Fine-tuning the orientation of cell polarization by a GTPase activating protein in
*Saccharomyces cerevisiae*

DISSERTATION

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Abstract

Orienting the precise axis of cell polarity and the plane of asymmetric cell division are essential for various cellular processes, including embryonic development and stem cell generation (Neumüller and Knoblich, 2009). Cells of the budding yeast *Saccharomyces cerevisiae* establish the axis of cell polarity directed by cell-type specific spatial cues. The small GTPase Cdc42, a highly conserved member of the Rho family, regulates the organization of the actin cytoskeleton and secretory apparatus in order to direct cell polarization toward the presumptive bud site. The coordination of GTPase cascades, including the Rsr1 GTPase and Cdc42 GTPase module, largely governs the patterns of yeast budding in all cell types.

Although several studies have provided significant insights into the mechanisms of polarity-organizing systems lacking spatial cues, the link between spatial cues and polarity-establishment components remains to be fully understood. Interestingly, Cdc42-GAP Rga1 has an exclusive role in positioning a correct polarity axis that is not shared by any other Cdc42-GAPs (Chen et al., 1996; Stevenson et al., 1995; Smith et al., 2002; Tong et al., 2007). In this study, the regulation of Cdc42 polarization by spatial cues and a Cdc42-GAP Rga1 was thoroughly explored.

Remarkably, two distinct phases of Cdc42 polarization were found in the G1
phase, dependent on Bud3 and Cdc24, respectively (Kang et al., 2014). Interestingly, haploid daughter cells exhibit temporal changes of the position of the Cdc42-GTP peak surrounding the axial landmark during early G1, whereas the dynamic Cdc42-GTP cluster is not observed in cells with depleted Rga1 activity. A stable polarization site is determined in mid G1, which is concurrent with the beginning of the T2 phase of G1 (Di Talia et al., 2007) in both wild-type and rsr1Δ cells. The robust Cdc42 polarization in early G1 is dependent on Bud3 GEF activity and the Rsr1 GTPase. Remarkably, the dynamic changes of Rga1 localization to the bud-neck in addition to Rga1 localization at the adjacent site to the bud-neck during M to the next G1 phase are crucial for selection of a proper bud site in haploid cells.

Moreover, Rga1 is necessary for directing Cdc42-GTP polarization to the pole opposite to the division site in diploid a/α daughter cells. Rga1 activity that inhibits Cdc42 activation at the cytokinesis site is critical for choosing a single bud site despite the presence of both pole markers in diploid daughter cells. Surprisingly, a high level of Cdc42-GTP persistently polarized to the distal tip of daughter cells in more than 50% of rga1Δ diploid daughter cells during M and to the next G1 phase, implicating the important role of Rga1 in Cdc42-GTP polarization during an apical to isotropic switch.

These results provide the mechanisms of cue-dependent Cdc42 polarization and the unique role of Cdc42-GAP Rga1 in determining the proper axis of cell polarization in yeast. As the intracellular players and pathways involved in orienting the cell polarity are remarkably conserved, this study will help to identify the detailed mechanisms of polarity establishment in higher eukaryotes.
Dedication

This document is dedicated to my family.
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I am deeply grateful to my academic advisor Dr. Hay-Oak Park for her critical training throughout my graduate studies. This work would not have been possible without her outstanding supervision and patience.

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Major Field: Molecular, Cellular and Developmental Biology
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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular Fluorescence Complementation</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast microscopy</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine-nucleotide-dissociation inhibitor;</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>GDP-GTP exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>iMYTH</td>
<td>Integrated Membrane Yeast Two-Hybrid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PBD</td>
<td>p21-binding domain</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic complete</td>
</tr>
<tr>
<td>SD</td>
<td>synthetic dextrose</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ts</td>
<td>temperature sensitive</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast extract, peptone, dextrose</td>
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CHAPTER 1: Introduction

1.1. General patterns of cell polarization

Cell polarization is a fundamental biological process that governs a variety of cellular mechanisms in many cell types. Cells polarize or divide asymmetrically through the unequal distribution of intracellular molecules, and this process occurs in a wide range of organisms, from single-celled organisms to multicellular invertebrates and vertebrates (reviewed in Nelson, 2003) (Figure. 1.1). The establishment of an appropriate axis of cell polarization is crucial in order to mediate proper cellular function, differentiation and morphogenesis. For example, epithelial cells polarize in the direction of the apical-basolateral axis, which separates distinct membrane compartments both functionally and structurally (Drubin and Nelson, 1996; Nelson, 2003) (Figure. 1.1A). In addition, the establishment of the anterior-posterior axis in the *Caenorhabditis elegans* zygote is critical for its development (Figure. 1.1B). Cell polarity also determines the shape of a particular cell, and each shape is coupled with a specific cellular function. For example, the distribution of the dendrites and axons of neuronal cells across tissues provides communication over long distances, allowing rapid delivery of signaling molecules (Witte and Bradke, 2008) (Figure. 1.1C). Further, Cell polarization in *Saccharomyces cerevisiae* is also crucial for generating a bud-mother axis, which
ultimately determines the site of cell division during yeast budding (Figure. 1.1D).

Importantly, although the final shapes or structures of cells may vary, the fundamental mechanisms used to deliver surface proteins or to organize the polarized cytoskeletal assemblies are highly conserved processes in all eukaryotic cells (Nelson, 2003).

1.2. Cdc42, the master regulator of cell polarity, and its regulators

The small Rho-family guanosine triphosphatase (GTPase) Cdc42 plays a critical role in polarity establishment. Cdc42 was identified in the budding yeast S.cerevisiae through the isolation of temperature-sensitive mutants that arrested as unbudded cells and disorganized actin cytoskeleton along with multiple nuclei (Adams et al., 1990; Johnson and Pringle, 1990). Cdc42 function is highly conserved, and remarkably, human Cdc42 can replace the function of Cdc42 in S. cerevisiae (Munemitsu et al., 1990). Cdc42 exhibits a high degree of structural and functional conservation among different species including humans (Johnson and Pringle 1990; Munemitsu et al., 1990; Shinjo et al., 1990; Chen et al., 1993; Luo et al. 1994; Miller and Johnson 1994; Bi and Park, 2012). In mammalian cells, Cdc42 regulates actin polarization and induces JNK (c-Jun N-terminal protein kinase)/SAPK (stress-activated protein kinase) cascades (Coso et al., 1995). Cdc42 is also involved in phagocytosis (Chen et al., 1996), chemotaxis (Jones et al., 1998) and cell proliferation (Pruitt and Der, 2001). In budding yeast, Cdc42 mediates the polarization of several actin-dependent events including bud emergence, mating-projection formation, and filamentous growth under starvation (Bi and Park, 2012). The
active form of Cdc42 interacts with numerous downstream effectors and its regulators in order to trigger the polarized assembly of actin and septin cytoskeletons to the presumptive bud site.

Like all other Rho GTPases, Cdc42 acts as a molecular switch that converts between a GDP-bound (inactive conformation) and a GTP-bound form (active conformation). Cdc42 GTPase cycling between a GTP-bound and a GDP-bound state is essential for its proper function as dominant active alleles of *CDC42* exhibit multiple buds (Ziman *et al*., 1991). Active Cdc42 regulates several biological processes by turning on the activities of numerous downstream effectors. Cdc42 is activated by Cdc24, a guanine nucleotide-exchange factor (GEF) for Cdc42 (Zheng *et al*., 1994; Tcheperegine *et al*. 2005). Cdc24 has long been known as the only GEF for Cdc42 in budding yeast (Zheng *et al*., 1994), but a recent study has shown that Cdc42 is activated by two GEFs, initially by Bud3 and then, subsequently by Cdc24 in the G1 phase (Kang *et al*., 2014; Discussed in Chapter 2). Cdc24 localizes to the site of polarized growth in late G1 (Nern and Arkowitz, 1999; Toenjes *et al*., 1999; Shimada *et al*., 2000) and is necessary for polarity establishment and maintenance (Hartwell, 1971b; Sloat and Pringle, 1978; Sloat *et al*., 1981; Adams *et al*., 1990).

The hydrolysis of Cdc42-GTP is catalyzed by GTPase-activating proteins (GAPs), Rga1, Rga2, Bem3 and Bem2 (Zheng *et al*., 1993, 1994; Stevenson *et al*., 1995; Marquitz *et al*., 2002; Smith *et al*., 2002). All GAPs are specific to Cdc42 except Bem2, which acts as a GAP for other Rho GTPases including Cdc42, Rho1 and potentially for Rho2 and Rho4 (Zheng *et al*., 1993; Chen *et al*., 1996; Marquitz *et al*., 2002; Gong *et al*., 2013).
Although each GAP is not essential for viability, the synthetic lethality of the \textit{rga1\textDelta} \textit{bem2\textDelta} double mutant (Chen \textit{et al}., 1996) shows the redundant function of GAPs for viability.

Three GAPs (Rga1, Rga2, and Bem3) regulate a septin ring assembly at the incipient bud site, presumably by boosting the GTP hydrolysis of Cdc42 for Cdc42 cycling between its GTP and GDP-bound states (Gladfelter \textit{et al}., 2002; Smith \textit{et al}., 2002; Caviston \textit{et al}., 2003). Septins are GTP-binding proteins that are highly conserved from yeast to humans (Bi and Park, 2012). In budding yeast, five septins (Cdc3, Cdc10, Cdc11, Cdc12 and Shs1/Sep7) are expressed and assembled into filaments and eventually form a ring at the mother-bud neck (Bi and Park, 2012). Overexpression of Rga1 rescues the septin defects and that defects are less effectively rescued by the overexpression of Bem3 or Rga2 (Gladfelter \textit{et al}., 2002; Caviston \textit{et al}., 2003). Although the \textit{in vitro} GAP activity of each Cdc42 GAP seems to be similar (Smith \textit{et al}., 2002), suppression levels of defective septin or \textit{cdc42} mutant phenotype by overexpression of each Cdc42 GAP are different (Caviston \textit{et al}., 2003). In addition, all GAPs have a unique role in different processes of cell polarization that are not shared by others. For example, Bem3 has a role in cell morphogenesis and Rga1 has a role in haploid invasive growth, suggesting that Cdc42 GAPs act through unique effectors for different cellular processes (Smith \textit{et al}., 2002).

Cdc42 GAPs exhibit different localization patterns during the cell cycle. All GAPs localize to the presumptive bud site in the G1 phase (Caviston \textit{et al}., 2003; Knaus \textit{et al}., 2007; Sopko \textit{et al}., 2007). The localization patterns of Rga2 and Bem3 are similar
to the patterns of Cdc42 polarization unlike Rga1, which initially localizes to the site of bud emergence and then spreads throughout the bud cortex as a bud grows and then concentrates to the bud neck during cytokinesis (Caviston et al., 2003; Tong et al., 2007). Bem2 also localizes similar to Cdc42 except that Bem2 localization to the mother-bud neck appears in the S phase (Caviston et al., 2003; Huh et al., 2003; Knaus et al., 2007). During cytokinesis, Rga1 localizes as double rings to the bud-neck region (Tong et al., 2007). Remarkably, Rga1 undergoes dynamic changes of its localization pattern during M to the next G1 phase (further discussion in Chapter 2). In addition to the bud-neck localization of Rga1, Rga1 also localizes to old bud site(s) overlapped with bud scars at the adjacent to the mother-bud neck (see Chapter 2 for details). Rga2 concentrates as a single patch closer to daughter side of the bud within two septin rings during cytokinesis (Tong et al., 2007). The localization of Bem3 or Rga1 at the bud-neck are dependent on septins, but not in other locations including at the presumptive bud site or at the bud tip (Caviston et al., 2003). Bem3 localizes to sites of polarized growth via vesicle trafficking, implicating that endocytosis and secretion is likely to be involved in the polarized distribution of Cdc42 regulators (Mukherjee et al., 2013).

It is important to note that Rga1 is uniquely required for prevention of Cdc42 activation (and hence budding) at the cytokinesis site (Tong et al., 2007). Cells lacking Rga1 form a new bud within the old division site by accumulating high amount of the Cdc42-GTP at the neck, resulting in the same-site budding phenotype (Tong et al., 2007). Deletion of RGA1 in haploid cells increases the bipolar budding pattern in haploid a and α cells (Chen et al., 1996, Smith et al., 2002), revealing its unique role in bud site selection. The additional roles of Cdc42 GAPs are open to future investigation regarding
its contribution to the establishment and maintenance of cell polarity.

Cdc42 is also regulated by Rdi1, the only known guanine nucleotide dissociation inhibitor (GDI), which extracts Cdc42 from the plasma membrane and internal membranes by binding to the COOH terminal prenyl group of Rho GTPases (Masuda et al. 1994; Eitzen et al. 2001; Richman et al. 2004; Tcheperegine et al. 2005; Tiedje et al. 2008; Johnson et al., 2009).

1.3. Cdc42 signaling - Effectors

Cdc42 GTPase cycle and its effectors are closely connected to the yeast cell polarity by regulating the cytoskeletal organization and secretion toward the axis of polarized growth (Bi and Park, 2012). Indeed, several key actin-required morphogenetic events are mediated by Cdc42 signaling pathways (Bi and Park, 2012). In mammalian cells, Cdc42 is also involved in regulating the intracellular trafficking, endocytosis and actin assembly (Johnson et al., 1999; Engqvist-Goldstein, et al., 2003).

Yeast cells contain three members of the Pak (p21-activated kinase) serine/threonine kinases family, including Ste20, Cla4, and Skm1, which have distinct roles in cellular processes including mitogen-activated protein kinase (MAPK) pathways, cytoskeletal remodeling and polarity establishment (Bokoch, 2003). The P21-activated kinases are activated by binding with GTP-bound Rac or Cdc42 via a Cdc42/Rac interactive binding motif (CRIB) domain, which results in the release of PAKs from their auto-inhibited states (Cvrckova et al., 1995; Zenke et al., 1999). The ste20Δ cla4Δ double mutants exhibit synthetic lethality, but not in each single mutant, suggesting that both
genes share an essential function for cell survival (Cvrckova et al., 1995). In yeast, active Ste20 regulates the MAP kinase signaling in the pheromone response pathway as well as the haploid invasive growth, and the osmosensing high-osmolarity glycerol (HOG) pathway (Eby et al., 1998; Holly, 1999; Leberer et al., 1992; Mosch, 1996; O’Rourke and Herskowitz, 1998; Raitt et al., 2000; Roberts et al., 1994). Cla4 forms a complex with a scaffold protein Bem1 together with Cdc24 and Cdc42-GTP, thus playing a crucial role in polarity establishment (Gulli et al., 2000; Bose et al., 2001). Septin ring assembly requires the direct phosphorylation of septins by Cla4 (Longtine et al., 2000; Versele and Thorner, 2004). Skm1 was discovered by sequence similarity to Cla4 and Ste20, and has a role in the down regulation of sterol-uptake (Martin et al., 1997; Lin et al., 2009).

Gic1 and Gic2 specifically bind to the active form of Cdc42 via CRIB (Cdc42/Rac interactive binding) domain, which are required for actin and septin cytoskeletal organization in vivo (Brown et al., 1997, Chen et al., 1997, Iwase et al., 2006). Cells lacking both GIC genes exhibit defective actin and microtubule organization similar to cells lacking Cdc42 function (Adams et al., 1990). Deletion of GIC1, GIC2 together with the formin BNII cause cell lethality at all temperatures (Tcheperegine et al., 2005). Gic1/2 proteins play an important role in the initiation of budding together with Rsr1 as rsr1Δ gic1Δ gic2Δ mutants failed to initiate a new budding event and formed unbudded, large, and multinucleated cells (Kawasaki et al., 2003).

Two formins, Bni1p and Bnr1p, in Saccharomyces cerevisiae, are required for cell polarization and cytokinesis (Bi and Park, 2012). Both proteins are downstream targets of both Cdc42 and Rho1 (Kohno et al., 1996; Evangelista et al., 1997; Imamura et
Bni1 localizes to the site of polarized growth and Bnr1 localizes to the mother side of the bud neck from bud emergence to the onset of cytokinesis (Pruyne et al., 2004; Buttery et al., 2007). Thus, Bni1 and Bnr1 nucleate actin filaments at different locations: one directed toward the bud tip and the other surrounds the mother-bud neck, respectively (Pruyne et al., 2002; Sagot et al., 2002a,b; Moseley et al., 2004; Pruyn et al., 2004; Buttery et al., 2007). Bni1 nucleates a linear actin filament to promote bud growth or to a ring filament at the bud neck later in the cell cycle to promote cytokinesis (Pruyne et al., 2004). \(bni1\Delta\ bnr1\Delta\) double mutant cells exhibit synthetic lethality, reflecting several functions shared by these two proteins (Kamei et al., 1998; Vallen et al., 2000).

1.4. Spatial control of Cdc42

Cells respond to external spatial cues such as cell-cell contacts, chemoattractants, or morphogen gradients. For example, G protein-coupled receptors at the membrane recognize chemoattractants to trigger signaling pathways in both neutrophils and dictyostelium (Chung et al., 2001). Yeast cells react to a chemical signal, called pheromone, through the accumulation of receptors, leading to the activation of Cdc42 GTPase signaling pathways (Nern and Arkowitz, 1999)

In the budding yeast \textit{Saccharomyces cerevisiae}, selection of a bud site is dependent on the intrinsic mechanisms programed by a series of \textit{BUD} genes (Bi and Park, 2012). During vegetative growth, yeast cells possess an ability to choose a specific growth site depending on their cell types (not by ploidy). In haploid \(a\) and \(\alpha\) cells, both mother and daughter cells select a new bud site adjacent to the previously-used division site, which is called an axial budding pattern. In diploid \(a/\alpha\) cells, mother cells can bud
either at the adjacent or at the opposite to the previous division site, but daughter cells exclusively bud at the site opposite to the previous division site, which is referred to as a bipolar budding pattern (Freifelder, 1960; Hicks et al., 1977; Chant, 1991; Chant, 1995; Chant and Pringle, 1995; Figure 1.3A). The distinct patterns of budding display different patterns of bud scars, marking the previous division sites in mother cells (see Figure 1.3B).

Choosing a correct bud site is crucial for determining an axis of cell polarization and thus, the selection of a division plane that is perpendicular to the polarization axis. In haploid a and α cells, the division site is marked by transient spatial landmarks. In diploid a/α cells, both poles of the cell are defined by persistent cortical landmarks (Chant and Pringle, 1995). Both axial and bipolar landmark proteins recruit the Rsr1 (Ras-like) GTPase module through its interaction with the Rsr1-Guanine nucleotide exchange factor (GEF) Bud5 to the site of cell polarization (Kang et al., 2001). Bud5 localizes to the division site as well as to the presumptive bud site and its localization is dependent on landmark proteins (Kang et al., 2001). The active form of Rsr1 binds to Cdc24, which may result in its activation possibly through conformational changes of Cdc24 by releasing the PB1 domain from an intramolecular binding site (Zheng et al., 1995; Park et al., 1997; Park et al., 2002; Shimada et al., 2004).

Collectively, a model of morphogenetic hierarchy of the budding pathway has been developed based on genetic and physical interactions (see Figure 1.4). Cortical landmark proteins, which are specific to cell types (1st step) trigger the activation of general site-selection machinery (2nd step), thus leading to the activation of polarity
establishment machinery (3rd step). \textit{RSR1}, \textit{BUD5} and \textit{BUD2} are required for selection of a bud site, but are not essential genes for budding (Bender, 1993; Chant, 1991; Chant, 1995; Park \textit{et al.}, 1993).

In the axial budding pattern, the division site is marked by transient spatial landmarks; \textit{BUD3}, \textit{BUD4}, \textit{AXL1}, and \textit{AXL2/BUD10} (Chant and Pringle, 1995). These axial landmarks provide time-dependent spatial signals by dictating the subsequent bud site in order to direct a new bud site next to the immediately preceding division site (Chant and Pringle, 1995). In the absence of any axial landmarks, cells bud in a bipolar pattern although disruption of these genes does not affect the bipolar budding pattern in diploid a/a cells (Chant and Herskowitz, 1991; Fujita \textit{et al.}, 1994; Adames \textit{et al.}, 1995; Chant \textit{et al.}, 1995; Halme \textit{et al.}, 1996; Roemer \textit{et al.}, 1996; Sanders and Herskowitz, 1996). All deletion mutants of axial landmarks except \textit{axl2}Δ cells can be reverted to the axial budding cells when Rax1, a bipolar landmark, is depleted (Fujita \textit{et al.}, 2004; Kang \textit{et al.}, 2004a; Lord \textit{et al.}, 2002). It has been shown that Axl2 localization to the mother-bud neck is important for a proper bud-site selection while its localization to the presumptive bud site in late G1 is likely to be important for organizing the septin filaments (Gao \textit{et al.}, 2007). Axl2, a transmembrane protein, is likely to be delivered through the secretory pathway, unlike other cytoplasmic axial landmarks, which are likely to be delivered by diffusion or high affinity to cell wall components, and also possibly by other polarity molecules (Halme \textit{et al.}, 1996; Lord \textit{et al.}, 2000). In \textit{erv14}Δ mutants, which are defective in the ER-vesicle secretory cargo, Axl2 accumulates in the ER and haploid cells also exhibit a non-axial budding pattern (Powers and Barlowe,
Septins also play a critical role in determining the axial budding pattern as septin mutants including *cdc10-10* and *cdc11-6* ts mutants exhibit defective axial budding patterns (Flescher *et al*., 1993; Chant *et al*., 1995).

Ax11 is the only landmark protein that is specifically expressed in haploid cells (Fujita *et al*., 1994). Overexpression of Ax11 in a/α diploid cells (~70%) causes an axial budding pattern (Fujita *et al*., 1994). Both Ax11 and Ax12 localize as double rings at the mother-bud neck before cytokinesis and both mother and daughter cells inherit a single ring after cytokinesis, thus marking the previous division site (Halme *et al*., 1996; Roemer *et al*., 1996).

Bud3 and Bud4 form double rings at the mother-bud neck during and after the G2 phase and a single ring at the division site after cytokinesis, which subsequently disappears when a new septin ring forms in the next cell cycle (Chant *et al*., 1995; Sanders and Herskowitz, 1996). Localization of Bud3 and Bud4 is dependent on septin integrity as seen in poor localization of both proteins at the neck in *cdc10Δ* and *cdc11Δ* mutants (Chant *et al*., 1995; Sanders and Herskowitz, 1996; Frazier *et al*., 1998). Bud4 functions as a platform that links septins to the axial landmarks (Kang *et al*., 2012). Bud4 localizes to the mother-bud neck first by indirectly interacting with septins, presumably via the interaction with Bud3, and then recruits other axial landmarks (Kang *et al*., 2012; Sanders and Herskowitz, 1996). Bud3 and Bud4 are also important for septin ring integrity during/after cytokinesis (Kang *et al*., 2012). Although all axial landmarks are expressed in both cell types, Bud4 and Axl2 associate with Bud5 only in haploid cells.
and also in the presence of Axl1, which is only expressed in haploid cells (Kang et al., 2012). The axial landmarks directly communicate with the Rsr1 GTPase module via its GEF Bud5 and establish a polarization site to a predetermined site (Kang et al., 2001). In cells budding in a bipolar pattern, both poles of diploid a/α cells are marked by persistent cortical landmarks, Bud8, Bud9, Rax1 and Rax2, which are required for maintaining the bipolar budding pattern in diploids (Fujita et al., 1994; Zahner et al., 1996; Chen et al., 2000; Fujita et al., 2004; Kang et al., 2004a). Disruption of these genes affects the bipolar budding pattern, but not the axial budding pattern in haploid a and α cells. Cells lacking Bud8 predominantly bud at the proximal pole (proximal to the birth pole), whereas bud9Δ mutants mainly bud at the distal pole (the pole opposite to the birth pole) (Zahner et al., 1996). These unipolar budding patterns form clusters of bud scars near either pole of cells with non-specific patterns, unlike an axial budding pattern, which forms a long chain-like shape of bud scars (see Figure 1.3).

Both Bud8 and Bud9 are transmembrane proteins that exhibit sequence similarity between their short cytoplasmic compartments, which are bracketed by two membrane domains (Harkins et al., 2001, Kang et al., 2004). These cytoplasmic domains may be recognized by the same downstream components of the Rsr1 GTPase module, activating the common morphogenetic pathway of yeast budding (Figure 1.4; Harkins et al., 2001).

Bud8 localizes to the distal pole and Bud9 localizes to the proximal pole of daughter cells, representing the role of spatial marks at the distal and proximal pole, respectively (Chen et al., 2000, Harkins et al., 2001, Kang et al., 2004, Schenkman et al., 2002). Delivery of Bud8 is dependent on actin, and the delivery of Bud9 is dependent on
actin and septins (Harkins et al., 2001; Schenkman et al., 2002). Bud8 also fails to localize to the bud tip in the absence of Bni1, and Rax1 whose localization is dependent on Bni1 (Kang et al., 2004). Bud8 is delivered to the distal pole of a new born cell just before bud emergence and to the distal pole of daughter cells after division, although the BUD8 mRNA peak is high in late G2/M (Harkins et al., 2001). Bud9 is delivered to the bud site of the mother-bud neck just before cytokinesis, although the BUD9 mRNA peak occurs in late G1, revealing the delay of translation and delivery of proteins (Harkins et al., 2001).

Rax1 and Rax2 are integral membrane proteins that are involved in diploid bud site selection, which localize to the distal pole of daughter cells as well as to the division sites in both mother and daughter cells (Kang et al., 2004). Rax1 and Rax2 interact with each other and also with Bud8 and Bud9 in bipolar budding (Kang et al., 2004). Rax1 and Rax2 appears to be required for the efficient delivery of Bud8 and Bud9 to the proper sites, suggesting that these proteins function together to provide spatial information to the polarity machinery in diploids (Kang et al., 2004). Bud8, Bud9 and Rax2 appear to have a long N-terminal glycosylated domain at the extra cytoplasmic space, which may serve as a stable anchor to the cell wall and thus prevent further diffusion, allowing a focused localization of landmarks during apical growth (Kang et al., 2004).

It is likely that bipolar landmarks are established and maintained by endocytosis and polarized exocytosis, unlike axial landmarks (Tuo et al., 2013). Deletion mutants of polarisome components including Spa2, Bni1 and Bud6, exhibit defective bipolar budding patterns, suggesting the involvement of intracellular trafficking in the delivery of
bipolar markers in these mutants (Zahner et al., 1997; Snyder 1998; Bi et al., 2000).

1.5. Cell-cycle control of Cdc42 – Temporal regulation

Yeast cell polarization is tightly controlled by the cell-cycle dependent regulation, which is coordinated by the cyclin/CDK complexes. Cells enter to a new cell-cycle by G1-CDK activity (Pringle and Hartwell, 1981), which is called a ‘START’ checkpoint, initiating the transcription of a large set of genes required for G1/S transition (see Figure 1.5). In late G1, Cdc42 polarization is directed to the incipient bud site by Cdc24 activation, which exit from the nucleus by Far1 degradation upon its phosphorylation by Cln1/2-Cdc28 activity (Nern and Arkowitz, 2000). Cdk1 activity is also required for bud growth, presumably by phosphorylating components of the Cdc42 GTPase signaling module, suggesting its critical role in both the initiation and the maintenance of cell polarity (McCusker et al., 2007).

During G1 phase, two independent regulatory steps are separated by the nuclear residence of Whi5, a G1-transcriptional repressor. Whi5 enters to the nucleus late in mitosis and exits in the middle of G1 phase (Costanzo et al., 2004). Briefly, Whi5 binds and represses G1/S transcription factors, SBF (Swi4–Swi6) and MBF (Mbp1–Swi6), at the G1/S promoter elements and SBF/MBF is dissociated by the CDK-dependent phosphorylation (Cdc28/Cln3) of Whi5, allowing the transcription of the hundreds of genes responsible for G1/S transition (Costanzo et al., 2004; de Bruin et al., 2004; see Figure 1.6). The first step is a size-sensing module that depends on both Cln3 and the size of cells, whereas the second phase is a size-independent timing module that depends on Cln2 (Di Talia et al., 2007; see figure 1.7). Cln3 activity is most likely correlated with
cell size, such that the rate of Cln3 synthesis increases with an increased volume of cytoplasm (Rupes et al., 2002). The period between cytokinesis and Whi5 exit is called T1, and the period between Whi5 exit and budding is called the T2 phase (Di Talia et al., 2007).

Cdc42 regulators are involved in linking the cell polarization to the cell-cycle regulation. The spatial and temporal regulation of Cdc24, a GEF for Cdc42, is under the control of cyclin-CDK activity. In haploid cells, Cdc24 is sequestered in the nucleus via a physical interaction with the adaptor Far1 during late M to the early G1 phase (Shimada et al., 2000). Cdc24 is released from the nucleus to the presumptive bud site via the phosphorylation of Far1 by Cln2-Cdc28 kinase at the G1/S transition that subsequently results in the ubiquitin-dependent degradation of Far1 (Gulli et al., 2000; Shimada and Gulli, 2000; Moffat and Andrews, 2004; Henchoz et al., 1997; Shimada et al., 2000). The relocation of Cdc24 to the presumptive bud site is critical for the initiation of new bud emergence (O’Shea and Herskowitz, 2000). Interestingly, Far1 expression is dependent on cell types as FAR1 transcription is blocked in diploid cells (Chang and Herskowitz, 1990).

Cdc24 phosphorylation by Cdc28 has been confirmed in vitro (McCusker et al., 2007). However, mutational analysis of the predicted CDK1 phosphorylation sites (up to 35 sites) showed no obvious polarization defects in vivo (Gulli et al., 2000; Wai et al., 2009), suggesting that other ways of Cdc24 regulation are more important than Cdc24 phosphorylation, including the interaction of Rsr1 and Bem1 to Cdc24 or the oligomerization of Cdc24 (Park et al., 1997; Shimada et al., 2004; Butty et al., 2002;
Irazoqui et al., 2003; Mionnet et al., 2008).

The GAP activities of Rga2, Bem2, and Bem3 are inhibited by CDK-dependent phosphorylation, thus activating Cdc42 in a time-dependent manner during bud emergence (Knaus et al., 2007; Sopko et al., 2007). Rga2 phosphorylation by G1-CDK was demonstrated in both in vitro and in vivo (McCusker et al., 2007; Sopko et al., 2007) and defects in phosphorylation resulted in impaired polarized growth (Sopko et al., 2007). Thus, the proper activation of Cdc42 is mediated by CDK activity in G1 phase, which is tightly linked to the regulation of a Cdc42-GEF and GAPs.

Cdc42 polarizes in response to external or internal cues, but the clustering of Cdc42 can also arise spontaneously at a random site in the absence of preexisting cues, which is called a ‘symmetry-breaking’ (Wedlich-Soldner and Li, 2003; Irazoqui et al., 2003). The local accumulation of active Cdc42 (GTP-bound form) at the cell cortex is triggered by stochastic fluctuations of polarity factors initiated by a small random perturbation, which could be further amplified by positive feedback loops (Turing et al., 1952). Positive feedback loops promote more Cdc42 accumulation by recruiting more Cdc42 GTPases to sites where a small amount of active Cdc42 is already clustered (Johnson et al., 2011, Slaughter et al., 2009).

Two types of positive feedback loops are suggested to contribute to Cdc42 polarization in yeast. One is the Bem1-mediated amplification of active Cdc42 (Irazoqui et al., 2003, Kozubowski et al., 2008) to a polarization site, which is independent of actin cytoskeletons. The idea is that the scaffold protein Bem1 mediates the link between the P21-activated kinase Cla4, which is bound to preexisting GTP-Cdc42, and the Cdc42
GEF into one complex, thus facilitating Cdc24 activation presumably via hyperphosphorylation (Kozubowski et al., 2008). This Cdc24-Bem1-Cdc42 positive feedback mechanism is based on a Turing-type mechanism that explains the spontaneous emergence of cellular polarity by local positive feedback and global inhibition by Cdc42 GAPs (Turing, 1952; Goryachev and Pokhilko, 2008). However, a recent study (Smith et al., 2013) has challenged the critical role of Bem1 in the symmetry breaking model. In fact, the synthetic lethality of bem1Δ rsr1Δ double mutants, which is the basis of a ‘symmetry breaking’ mechanism by Bem1, was not seen in different strain backgrounds (Smith et al., 2013). The disruption of Cdc42-Bem1 interaction by a point mutation indicates that the symmetry-breaking process does not require Bem1 binding to the active Cdc42 (Smith et al., 2013).

Another positive feedback mechanism is based on the actin-mediated transport of vesicle-bound Cdc42-GTP (Wedlich-Soldner et al., 2003), particularly in the absence of Bem1 (Wedlich-Soldner et al., 2004). The delivery of Cdc42-bearing vesicles initiated by the active Cdc42 and enhanced by Cdc42-GTP stimulated actin polymerization generates a stable polarity axis via an actin-mediated positive feedback loop (Wedlich-Soldner et al., 2003). However, the relevance of actin cytoskeletons and secretory vesicles for yeast polarization are still controversial (Johnson et al., 2011, Kozubowski et al., 2008; Irazoqui et al., 2005, Savage et al., 2012, Yamamoto et al., 2010). An opposite role of actin in cell polarization has been supported by several studies, which explains the perturbation of polarity by endocytosis-mediated dispersal of Cdc42 (Irazoqui et al., 2005, Ozbudak et al., 2005, Layton et al., 2011). The role of actin was also emphasized in
delivering landmark proteins rather than Cdc42 itself, which may result in more activation of Rsr1 (Wu et al., 2013). It seems that the constant recycling of Cdc42 is needed to drive and maintain Cdc42 polarization by both positive feedback loops (Wedlich-Soldner et al., 2004, Slaughter et al., 2009). Cdc42 polarization also involves the coordination of polarized exocytosis and the corralling of endocytic events (Jose et al., 2013).

The core polarity regulators, including Cdc42, Bem1 and Cdc24, exhibit oscillatory behavior in its cluster, suggesting the presence of a delayed negative feedback loop (Novak and Tyson, 2008; Howell et al., 2012). A time-delayed negative feedback loop and a positive feedback loop promote Cdc42 oscillation (Das et al., 2012b). In principle, a negative feedback loop may operate by GTPase-mediated stimulation of GAP activity, or inhibition of GEF activity but its exact mechanism still requires further investigation (Wu and Lew, 2013). In fact, a recent study has shown that the Cdc42 GEF phosphorylation by Cdc42-stimulated effector kinases provides a negative feedback loop via the inhibition of GEF activity (Kuo et al., 2014). The presence of negative feedback loops may retain the limited Cdc42 levels, thus restraining the spread of a polarized domain on the membrane (Howell et al., 2009). Negative feedback loops may also facilitate the disappearance of the losing cluster during a winner-takes-all competition, thus affecting the dynamics of competition (Howell et al., 2009).
1.6. Aims of dissertation research

Many cell types must polarize at certain stages, and the establishment of a correct polarity axis is crucial for the cellular identity and function in most eukaryotic cells. Owing to a high degree of its polarized growth, cells of the budding yeast *Saccharomyces cerevisiae* offer an attractive system for studying cell polarity and asymmetric cell division. Importantly, yeast cells polarize to a pre-determined position oriented by the internal stimuli (i.e. upstream landmark cues). Although several key players involved in cell polarization processes were found, the link between the pre-programmed spatial information and the polarity machinery remains unclear.

The aim of this dissertation is to determine how the axis of cell polarization is established to a proper bud site by a Cdc42 GTPase activating protein in response to spatial cues. This study has shown the following.

The determination of a polarity axis to the proper bud site is largely dependent on the Cdc42-specific GAP Rga1 activity when spatial cues mark several potential area for the subsequent bud site in both cell types. In haploid a and α cells, the peak of the Cdc42-GTP cluster dynamically moves around the axial landmark before selecting a single polarity axis for an axial budding in mid G1. This dynamic changes of the axis of Cdc42-GTP polarization is dependent on Rga1. Moreover, two novel findings on Rga1 localization— next to the bud neck (of mother cells) and the dynamic changes at the bud neck — are necessary for selection of a proper bud site in haploid cells.

In diploid a/α cells, the preferential polarization of Cdc42-GTP to the distal pole
of daughter cells is dependent on a Cdc42-GAP Rga1 activity at the cytokinesis site as well as at the bipolar landmark, Bud8. Moreover, a large portion of $rga1\Delta$ cells (~50%) exhibit that the axis of Cdc42-GTP polarization remains to the distal tip of $rga1\Delta$ daughter cells during M to the next G1 phase, suggesting an additional role of Rga1 in spatiotemporal regulation of Cdc42, and also in polarized morphogenesis.

Collectively, this study revealed the mechanisms of cue-dependent Cdc42 polarization patterns in both cell types, and the unique role of a Cdc42 GTPase activating protein, Rga1, in orienting a proper polarity axis. Based on the high conservation among polarity regulators, understanding the fine-tuning of cell polarization will help to reveal the regulatory mechanisms of polarity establishment in all eukaryotic cells.
Figure 1.1. Diversity of polarized cells. (not to scale) This figure depicts a few example of polarized cells. Cell polarization is tightly correlated with cellular functions. (A) Epithelial cells require the segregation of apical and baso-lateral proteins and membrane domains separated by tight junctions (in red). (B) The asymmetric protein distribution (red/green gradient) in the cytoplasm results in two unequal daughter cells during the development of the *C. elegans* zygote. (C) Targeting of specific proteins to the axonal and dendritic membrane domain is critical for the development of neuronal cells, which is the most striking polarized organization of any cell types. (D) In budding yeast, the establishment of a bud-mother axis is crucial for its polarized growth, which ultimately determines the plane of cell division. Parts of the figure were adapted from Etienne-Manneville, 2004.
Figure 1.2. Regulation of the Cdc42 GTPase. Spatial or temporal signals activate Cdc24 (GEF), which converts Cdc42 to its active GTP-bound form. Active Cdc42 binds to several downstream targets to promote cellular function. Cdc42 GTPase activating proteins (Rga1, Rga2, Bem2, and Bem3), which are also regulated by internal and external cues, inactivate Cdc42 by stimulating its intrinsic GTPase activity. Rdi1 (Rho GDI) cycles Cdc42-GDP between the plasma membrane (PM) and the cytosol. Parts of the figure were adapted from Bi and Park, 2012.
Figure 1.3. Patterns of bud-site selection in *S. cerevisiae*.
(A) Axial and bipolar patterns of cell division. Red arrows depict the axes of cell polarization. (B) The patterns of bud scars on the surface of yeast mother cells. Bud scars are visualized by staining with the dye Calcofluor (as shown in blue). In the axial pattern, scars form a continuous chain. In the bipolar pattern, scars cluster around the birth scar (proximal pole) and the pole opposite to the birth end (distal pole). Figure was adapted from Bi and Park, 2012.
Figure 1.4. A morphogenetic hierarchy of yeast budding event. Signaling pathways underlying the axial and bipolar budding in haploid (a or α) and diploid (α/α) cells. Step 1 involves the selection of a bud site by axial or bipolar landmarks. Step 2 includes the Rsr1 GTPase module, which promotes the proper bud-site selection. Step 3 involves the Cdc42 GTPase module, which mediates the bud-site assembly. This step is essential for cell growth. Several protein-protein interactions are speculated based on genetic and localization data but physical interactions were shown in many cases (see details in chapter 1.4). Figure was adapted from Bi and Park, 2012.
Figure 1.5. Cell-cycle dependent regulation of cell polarity in yeast. Cdc42 becomes polarized in G1/S phase (called START). The site of polarized growth (in red) changes during the cell cycle. Black and white arrows indicate the direction of cell growth. These changes are controlled by the action of Cyclin/CDK complexes (see details in chapter 1.5) Figure was adapted from Yoshida and Pellman, 2008.
Figure 1.6. A simple model for START regulation by the cell-cycle machinery in *S. cerevisiae.*
The exclusion of Whi5 from the nucleus through Cln3-Cdc28 mediated phosphorylation activates SBF/MBF, thus leading to the expression of numerous genes including *CLN1/2* that are required for the G1/S transition (Start) in yeast. Figure was adapted from Skotheim et al., 2008.
Figure 1.7. Partitioning of the G1 phase by the nuclear exit of the transcriptional repressor Whi5.

T1 phase is the time between cytokinesis and Whi5 exit. T2 phase is the time between the exit of Whi5 from the nucleus and a new bud emergence. The duration of T1 phase is longer in daughter cells due to its smaller size compared to mother cells (Hartwell and Unger, 1977). Figure was adapted from Di Talia et al., 2007.
CHAPTER 2: Regulation of cue-dependent cell polarization by Rga1, a Cdc42 GTPase activating protein, and the Rsr1 GTPase in budding yeast.


2.1. Introduction

The proper selection of a polarity axis is essential for cell morphogenesis, proliferation and differentiation in most eukaryotic cells. Cells of the budding yeast S. cerevisiae polarize to a defined axis in its native physiological state, which serves as an outstanding organism to study the underlying mechanisms of cell polarity in response to intrinsic cues. In the budding yeast, cells exhibit two distinct types of polarized growth depending on cell types. Haploid a and α cells bud in an axial pattern in which both mother and daughter cells bud next to the previous division site. In contrast, diploid a/α mother cells can bud at the opposite pole or at the adjacent site to the previous division site, whereas diploid a/α daughter cells preferentially bud at the opposite pole to the previous division site (Bi and Park, 2012).
The axial pattern of budding requires transient cortical landmarks including Bud3, Bud4, Axl1 and Axl2 (Chant and Herskowitz, 1991; Chant et al., 1995; Chant and Pringle, 1995; Fujita et al., 1994; Halme et al., 1996; Lord et al., 2002; Roemer et al., 1996; Sanders and Herskowitz, 1996). In the bipolar budding pattern, cells require persistent cortical markers, including Bud8, Bud9, Rax1 and Rax2, directing a new axis of polarization toward the predetermined positions (Chen et al., 2000; Fujita et al., 2004; Harkins et al., 2001; Kang et al., 2004a; Zahner et al., 1996).

The common machinery for both cell types is the Rsr1 GTPase module, which is consisted of the Ras-like GTPase Bud1/Rsr1, its GTPase activating protein (GAP) Bud2, and its GDP-GTP exchange factor (GEF) Bud5 (Bender et al., 1989; Chant et al., 1991; Chant, 1991; Powers, 1991; Park, 1993; Bender, 1993). The Cdc42 GTPase, a highly conserved Rho family of GTPase, regulates specific signaling pathways involved in cell polarization (Etienne-Manneville, 2004). Both Rsr1 and landmark proteins interact closely with Cdc42, thus building up a proper axis of cell polarization (Bi and Park, 2012).

A considerable number of studies have shown that yeast cells possess a self-organizing system in polarity establishment even when pre-existing cues are eliminated, called a ‘symmetry breaking’ (Irazoqui et al., 2003, Wedlich-Soldner et al., 2004). However, these studies have neglected the importance of spatial cues in the process of polarity establishment.

Remarkably, recent studies by Dr. Pil Jung Kang show that Bud3 activates Cdc42 as a novel Cdc42 GEF and that this activity of Bud3 is critical for the axial budding pattern (Kang et al., 2014). Here I briefly summarize his findings that have led to my
studies on Cdc42 polarization in haploid cells. 1) Bud3 catalyzes GDP release from Cdc42 and Bud3 preferentially binds to the GDP-bound form of Cdc42 both in vitro and in vivo. 2) The bud3ΔCR2 mutant, which Dr. Kang generated by introducing deletions of two residues (aa 325-326) and a substitution mutation (D328Q) in the conserved region (CR2; aa 311-324) of the putative DH domain of Bud3, disrupts the axial landmark assembly and Bud3-GEF activity. 3) The bud3ΔCR2 mutant cells also budded in the bipolar pattern as bud3Δ cells. 4) Among several temperature-sensitive cdc42 mutants, Dr. Kang identified a specific cdc42ts mutant that buds in the bipolar pattern at the semi-permissive temperature, and found that the axial landmark does not assemble properly in this cdc42ts mutant. Using live-cell imaging, I provided evidence that Cdc42 is indeed activated in two steps during the G1 phase, first by Bud3 and then by Cdc24. I also provide additional insight into the regulation of Cdc42 polarization in haploid cells as described in this Chapter.

Several questions regarding spatial-cue directed cell polarization remain unanswered in the axial budding pattern. Importantly, all axial landmark proteins form uniform ring(s) at the division site. How is a single, new bud site selected even though the perimeter of the ring is large enough to accommodate multiple sites? And, how are old bud sites excluded from being used again? Although it has been shown that Rga1, a GAP for Cdc42, creates a zone of inhibition for Cdc42 activation at the cytokinesis site and thus blocks re-budding at the same site (Tong et al., 2007), it is unclear how other earlier bud site(s) that are adjacent to the immediately preceding division site is prevented from being used again (see Figure 2.1B).
Using high resolution time-lapse microscopy, I found that daughter cells of haploid cells exhibit the transient movement of the Cdc42-GTP cluster near the axial landmarks until the axis of cell polarity is stabilized in mid G1. The dynamic behavior of the active Cdc42 cluster and selection of a single bud site is largely dependent on a Cdc42-GAP Rga1 and the Rsr1 GTPase. Interestingly, dynamic distribution of Rga1 at the neck and Rga1 localization at the adjacent to the bud neck are crucial for proper bud-site selection in haploid cells. Thus, this chapter provides two distinct regulatory mechanisms of Cdc42 polarization that is necessary for specifying a single polarity axis in the proper orientation during the G1 phase.

2.2 Materials and methods

**Strains, plasmids and genetic methods**

Standard methods of yeast genetics, DNA manipulation, and growth conditions were used (Guthrie and Fink, 1991), unless indicated otherwise.

Gic2-PBD-tdTomato, GFP-RGA1, Bud3-Rga1GAP (aa 700-1007)-GFP, CDC3-mCherry, CDC3-GFP, Whi5-GFP were expressed from the chromosomal loci except Myo1-mCherry (pRS314-Myo1-mCherry, CEN, TRP1, provided by E. Bi, University of Pennsylvania, Philadelphia, PA). To construct a strain expressing Gic2-PBD fused to the tdTomato at its C terminus, a plasmid encoding GIC2-PBD (aa 1-208 of Gic2) tagged with the 1.5 copies of tdTomato (YIp211-GIC2-PBD-RFP, integrative, URA3) was linearized with ApaI and integrated at the *ura3* locus as previously described (Tong *et al.*, 2007). To express Rga1 fused to the GFP at its N terminus, a DNA fragment carrying GFP-Rga1 from the HindIII-digested plasmid pRS315-GFP-RGA1 (Caviston *et al.*, 2007)
was transformed to replace *rga1Δ::URA3*-KanMX6 and the correct targeting was confirmed by GFP positive signals as previously described (Tong *et al*., 2007). To construct a strain expressing Bud3-Rga1GAP (aa 700-1007) fused to the GFP at its C terminus, a plasmid encoding the Bud3-C terminal fragment (aa 1477-1636) without a stop codon fused to the Rga1GAP domain (aa 700-1007) including yEGFP3-TCYC1 (pRS306-BUD3-RGA1-C1-GFP) was linearized with MluI and integrated at the BUD3 locus of the strain YEF2324 (*rga1Δ*) cells (Tong *et al*., 2007). To express Cdc3 fused to the mCherry or GFP at its N-terminus, a plasmid YIplac128-CDC3-mCherry (integrative, LEU2) or YIplac128-CDC3-GFP (integrative, LEU2) was digested with BglII and integrated at the CDC3 locus on the chromosome as previously described (Tong *et al*., 2007). To construct RSR1 deletion, *rsr1Δ::URA3* cassette from the EcoRI and BamHI digested pPB181 plasmid (Bender and Pringle, 1989) was used to delete the chromosomal *RSR1* gene, yielding HPY753(*rsr1Δ::URA3*). HPY753 was used to construct HPY2653 and HPY2669. To construct a plasmid expressing Gic2- PBD<sup>W23A</sup> fused to the tdTomato at its C terminus, the site-directed mutagenesis was performed to introduce W23A mutation in the PBD domain (aa 1-208), yielding a plasmid YIplac211-PBD<sup>W23A</sup>-RFP (integrative, URA3) [which lacks the residual Gic2 activity due to the W23A mutation] as previously described (Okada *et al*., 2013). To express Gic2-PBD<sup>W23A</sup>-tdTomato at the *leu2* locus, a DNA fragment carrying PBD<sup>W23A</sup>-tdTomato from the SacI-digested YIplac211-PBD<sup>W23A</sup>-RFP plasmid (integrative, URA3) was cloned to the YIplac128, yielding the YIplac128-PBD<sup>W23A</sup>-RFP (integrative, LEU2) and then linearized with PpuMI for integration at the *leu2* locus as previously described (Kang *et al*., 2014). To express Whi5 fused to the GFP at its C terminus, a DNA fragment carrying
GFP-TRP1 or GFP-KanMX6 was amplified by PCR using pFA6a-GFP(S65T)-TRP1 or pFA6a-GFP(S65T)-KanMX6 (Longtine et al., 1998) as a template and primers oWHI51 (5'-CGAAACGGAGCCCCGATACCAGACTGGGAGACGTCTCGGATCCC CGGTTAATTAA-3') and oWHI52(5'-CTAACCTCGAGATGCGAGAAACTCG TACTACCACA GAATTCGAGCTCGTTTAAC-3'). The resulting PCR product was transformed into strains by one-step-replacement method (Rothstein, 1991). Correct targeting was confirmed by colony PCR using primers oWHI53 (5'-CGGTGTCGAAA CACTGATGC-3') and oWHI54 (5'-GCAGAGTACGCACTAGTGAGC-3'). See the complete genotype of each strain in Table 2.1.

**Microscopy and data analysis**

Cells were grown in synthetic medium with dextrose, unless indicated otherwise, overnight and freshly subcultured for 3~ 4 hrs in the same medium. Cells were then harvested and mounted on a slab containing the same medium and 2% agarose. The slab was put on a stage directly (at 22°C) or in a temperature-control chamber set to 30°C or 37°C, as indicated.

For two-color time-lapse imaging, images were captured every min at 22°C or every two min at 30°C using a spinning-disk confocal microscope (Ultra-VIEW VoX CSU-X1 system; PerkinElmer) equipped with a 100×, 1.4 NA Plan Apochromat objective lens (Nikon), 440-, 488-, 515-, and 561-nm solid-state lasers (Modular Laser System 2.0; PerkinElmer), and a backthinned electron-multiplying charge-coupled device camera (ImagEM C9100-13; Hamamatsu Photonics) on an inverted microscope (Ti-E; Nikon).
Time-lapse imaging of the *cdc24-4* mutant was performed similarly, except the following: cells were grown overnight at room temperature, diluted to pre-warmed media (37°C), and shaken for 2 hrs at 37°C prior to imaging every 2 min on a slab placed in a temperature-control chamber at 37°C. Maximum intensity projections were generated using UltraView Vox software to generate figures and movies.

To quantify fluorescence intensity of PBD-RFP clusters, average projections were generated from 3 Z-sections (spaced at 0.3 μm) and then a threshold method was used after background subtraction using ImageJ software (NIH), as previously described (Okada *et al.*, 2013). The sum of all pixels in either mother or daughter cells was analyzed separately from the onset of cytokinesis until bud emergence (in mother cells), and intensity at each time point was normalized to the intensity at the onset of cytokinesis (t = 0). Because of cell-to-cell variations of the G1 length as previously reported (Di Talia *et al.*, 2007), the representative normalized intensity of individual cells were shown instead of averaging them at each time point (see Figure 2.2).

To test whether cells that had depleted Cdc24 activity could polarize Cdc42-GTP, *cdc24*<sup>ts</sup> cells that carry either a multicopy BUD3 plasmid, a multicopy *bud3ΔACR2* plasmid, or a vector control were grown at room temperature overnight and then by shifted to 37°C for 3 hrs before imaging using the confocal microscope as described above. To count cells with polarized Cdc42-GTP, unbudded cells that had a PBD-RFP cluster were identified by a threshold method using ImageJ (NIH) software. The percentage of cells with polarized Cdc42-GTP cap was shown below each images (n=130 ~ 200) in Figure 2.6. To quantify fluorescence intensity of PBD-RFP cluster in individual polarized cells, fluorescence thresholds were set to capture either a PBD-RFP cluster or
the whole cell, and summed fluorescence intensity was measured using ImageJ software from summed images after background subtraction (using the average whole cell intensity of an untagged strain). Summed fluorescence intensity of a PBD-RFP cluster was divided by the summed intensity in the whole cell, multiplied by 100, to calculate the fraction of the polarized PBD-RFP, and plotted for each individual polarized cell (Figure 2.6).

For two-color time-lapse imaging in Figure 2.7-13, images were captured (up to 9 stacks at 0.3 µm Z steps) every min at 22°C or every 2 min at 30°C. Although there were some cell-to-cell variations, cell growth at 22°C was typically slower than at 30°C, and thus helped to observe dynamics of Cdc42 more in detail in daughter cells in the G1 phase. However, I used images captured at 30°C for the analysis of daughter cells from cytokinesis to late G1 in the next cell cycle to avoid any variations of potentially sick cells due to phototoxicity caused by repeated capturing for longer time. I confirmed little difference in the budding pattern at both temperatures. Kymographs and heatmaps were generated from maximum intensity projection images of Z-stacks using the multiple kymograph plugin and heatmap histogram plugin for ImageJ.

To obtain end-on views of the bud neck or division site, freshly grown cells were mixed with the same warm medium containing melted agarose (0.6%). The agarose mix containing diluted cells was spread over the poly-lysine coated glass bottom dish (MatTek) before imaging. To visualize bud scars with PBD-RFP and Bud3-Rga1GAP-GFP (together with Whi5-GFP) by three-color time-lapse imaging, cells were stained with Calcofluor white (final concentration, 0.5µg/ml) for 10 min before mounting on a
slab.

For quantification of Cdc42-GTP level around the septin ring, a single best focused Z-section (selected based on Cdc3-GFP) was used to measure the intensity of a single spatial position covering the area of 8 pixels over time using ImageJ software (National Institutes of Health). Three spots were selected around the Cdc3-GFP ring; the central position of the septing ring, and two spots at both sides of the septin ring. Representative images were generated by maximum intensity projections of Z-stacks using UltraView Vox software. Kymographs were generated using the National Institute of Health ImageJ (http://rsb.info.nih.gov/ij/) and the Multiple Kymograph plugin (http://www.embl-heidelberg.de/eamnet/html/kymograph.html).

For quantification of Cdc42-GTP level from individual cells at T1 and T2 phase, fluorescence thresholds were set to capture PBD-RFP clusters in the whole cell (Figure 2.10) or in the bud neck region (Figure 2.16), and fluorescence intensity was measured using ImageJ software from the summed images of five selected Z sections (spaced at 0.3 μm) after background subtraction as described previously (Kang et al., 2014).

Summed fluorescence intensity of PBD-RFP clusters at each timepoint was normalized to the timepoint at the onset of cytokinesis (t=0) and the highest PBD-RFP peak values of the individual cells in T1 and T2 phase were plotted for WT and mutant cells (n=20). Statistical differences between two sets of data were determined by a two-tailed Student’s t test using Excel (Microsoft).
Analysis of bud sites

To analyze the position of bud scars and birth scars, cells were stained with Calcofluor and WGA-FITC (wheat germ agglutinin conjugated to fluorescein isothiocyanate), as previously described (Frydlová et al., 2009). Calcofluor stains chitin-rich structures such as bud scars and the base of buds, while WGA-FITC stains both bud scars and birth scars. Briefly, cells were grown overnight (typically 16~20 hrs) at 30°C in YPD (yeast extract, peptone, dextrose), washed with 0.1M phosphate-buffered Saline (PBS) buffer (pH7.3), and then double-stained with Calcofluor white (final concentration, 0.5µg/ml) and WGA-FITC (final concentration, 100µg/ml). Cells were then washed with PBS three times before examining under the microscope. The spinning-disk confocal microscope (Ultra-VIEW VoX CSU-X1 system; PerkinElmer), described above, was used to capture 15 stacks (0.3 µm Z-steps), and the maximum intensity projections were generated to make Figure 2.14A. Images shown in Figure 2.14B. were captured at 24°C using a microscope (E800; Nikon) fitted with a 100×, 1.3 NA oil Plan Fluor objective lens (Nikon), a charge-coupled device camera (ORCA-ER; Hamamatsu Photonics), and FITC/GFP and DAPI filters (Chroma Technology Corp.), and using SlideBook software (Intelligent Imaging Innovations). Maximum intensity projections were generated from 15 stacks (0.3 µm Z-steps) to make Figure 2.14.

2.3. Results

2.3.1. Cdc42-GTP polarization in early G1 is dependent on Bud3.

To monitor Cdc42 activation in vivo, I performed time-lapse imaging of haploid
wild-type and *bud3ΔCR2* cells, which contain several mutations in the putative DH domain of Bud3 (see introduction 2.1. for details), using a reporter for Cdc42-GTP, which is ‘PBD-RFP’ (the p21-binding domain of Gic2 fused to tdTomato) (Okada *et al.*, 2013). This reporter binds Cdc42-GTP specifically. These strains also expressed Cdc3-GFP as a marker for cytokinesis. I imaged these cells every minute from M to the next G1.

Little Cdc42-GTP accumulated at the onset of cytokinesis (when the Cdc3 ring splits: t = 0, Figure 2.2), presumably due to the suppression of Cdc42 activity by Cdc5/Polo kinase during mitotic exit as previously identified (Atkins *et al.*, 2013; Okada *et al.*, 2013). Surprisingly, Cdc42-GTP started to accumulate within 5 min after the onset of cytokinesis at 22°C (100%, n = 10; Figure 2.2A). Since Myo1 ring (Type II myosin heavy chain) contraction took about 6 ~ 8 min under similar conditions, the result indicates that Cdc42 activation begins in telophase or in early G1 phase.

When *bud3ΔCR2* cells were imaged under similar conditions as WT cells, little Cdc42-GTP was observed in early G1, although robust Cdc42-GTP polarization was observed at later time points (100%, n = 14; Figure 2.2B). This late Cdc42-GTP peak, which appeared at either pole of the *bud3ΔCR2* cells, was approximately concurrent with appearance of the new septin ‘clouds’ in the next G1 phase.

Strikingly, quantification of PBD-RFP clusters in individual mother and daughter cells revealed two waves of Cdc42 activity during G1, although two waves are less clearly separated in mother cells. In WT mother cells, Cdc42-GTP accumulated soon after the onset of cytokinesis, which was immediately followed by the second ‘wave’ of
Cdc42-GTP, despite some fluctuation in its level. In contrast, the first Cdc42-GTP wave was missing in the bud3ΔCR2 mother cells (Figure 2.2C). The first Cdc42-GTP peak was also evident in WT daughter cells, but more time elapsed between the early and the late Cdc42-GTP waves, likely because of their longer G1 phase (Figure 2.2C). Both Cdc42 waves were evident within 25–30 min after the onset of cytokinesis in WT daughter cells at 30°C (n = 10; Figure 2.3 Ac). In the bud3ΔCR2 daughter cells, only the second Cdc42 activation was observed at 30°C within 30 min after the onset of cytokinesis (n = 18; Figure 2.3 Ac). Despite cell-to-cell variations in timing of these two Cdc42 peaks, which are likely due to the G1 variability among individual cells (Di Talia et al., 2007), the average time interval between the onset of cytokinesis and the appearance of new septin was about the same in WT and bud3ΔCR2 cells (see Figs 2.2; 2.3A). The absence of the first Cdc42-GTP wave in bud3ΔCR2 cells is thus likely due to the lack of Bud3 GEF activity rather than different length of the G1 phase.

2.3.2. Stepwise activation of Cdc42 is correlated with the temporal partitioning of the G1.

Since Cdc42 is activated by two sequential steps during G1 phase, this activation pattern is likely to be related to the temporal G1 regulation. The G1 phase is partitioned into two functionally distinct steps by nuclear exit of the transcriptional repressor Whi5: the first step depends on the G1 cyclin Cln3 and the second step depends on the late G1 cyclin Cln1/Cln2 (Di Talia et al., 2007; Skotheim et al., 2008). Once Whi5 exits from the nucleus, SBF (Swi4–Swi6) and MBF (Mbp1–Swi6) activate transcription of hundreds of
genes, including Cln1 and Cln2. Consistent with the predicted idea, two Cdc42-GTP waves were temporally separated by the exit of Whi5 from the nucleus in daughter cells (Figure 2.4). This separation of Cdc42-GTP waves was not detectable in mother cells at 30°C, likely due to immediate Cdc24 activation in mother cells, which have obtained a critical cell size and thus traverse a minimal G1 phase (i.e. short T1 phase; the period between cytokinesis and Whi5 exit). Cdc24 is sequestered within the nucleus in haploid cells during G1 (Nern and Arkowitz, 2000; Shimada et al., 2000; Toenjes et al., 1999), and its relocation to the presumptive bud site depends on activation of the late G1 CDK (Gulli et al., 2000; Moffat and Andrews, 2004). The second Cdc42-GTP wave is thus likely due to Cdc42 activation by Cdc24. Consistent with this idea, when I imaged cdc24ts cells expressing PBD-RFP after temperature upshift to 37°C, the first Cdc42-GTP peak appeared transiently in early G1 but the second Cdc42-GTP peak was not observed (100%; n = 9) (Fig 2.5).

To confirm whether Bud3 indeed activates Cdc42 in vivo, I monitored Cdc42-GTP levels in cdc24ts mutants using PBD-RFP as a reporter for Cdc42-GTP in static images (Okada et al., 2013). When cdc24ts cells expressing PBD-RFP from the chromosome and carrying a multicopy BUD3 or bud3ACR2 plasmid or a vector control were shifted to 37°C for 3 hrs, most cells were arrested as unbudded cells and the level of Cdc42-GTP was significantly reduced as previously reported (Atkins et al., 2013). Cdc42-GTP clusters were observed in a larger number of the cells overexpressing Bud3 (39%, n = 128) than in cells with a vector (6.6%, n = 212) or in cells overexpressing Bud3ΔCR2 (1.5%, n = 202) (Figure 2.6). The level of Bud3 and Bud3ΔCR2 proteins were
equally expressed (Data not shown; Data provided by Dr. Pil Jung Kang). Quantification
of PBD-RFP intensity indicated that the Cdc42-GTP levels were not statistically different
regardless of whether the clusters were developed in cells carrying the BUD3 plasmid or
the control (Figure 2.6B). The polarized Cdc42-GTP in cells with the vector might be due
to the Bud3 activity encoded by the chromosomal BUD3 gene or residual Cdc24 activity
remaining after the temperature upshift. In contrast, the level of Cdc42-GTP was
significantly lower in those few polarized cells with the bud3ΔCR2 plasmid (Figure
2.6B), suggesting that bud3ΔCR2 might compete with the endogenous Bud3 for Cdc42
association. Together, these results indicate that Bud3 activates Cdc42 in early G1.

2.3.3. Cdc42-GTP polarization in WT haploid cells is dynamic in early G1.

Using live cell imaging, I confirmed that Cdc42 is activated by Bud3 in vivo,
emphasizing a crucial link between the axial landmark and polarity establishment
machineries (Kang et al., 2014). My subsequent studies focused on Cdc42 polarization
during axial budding. One of key questions in understanding the bud-site selection in
haploid cells is that how and when a single site is selected during axial budding (see 2.1.
introduction). To gain insight into the detailed mechanisms of axial bud-site selection, I
focused on the stabilization of Cdc42 polarization axis at a single spatial position as an
indicative of bud-site selection in the cell cycle. Thus, I monitored the location of Cdc42-
GTP polarization using cells expressing PBD-RFP as a reporter for Cdc42-GTP (Tong et
al., 2007) and Cdc3-GFP as a marker for timing of cytokinesis and also for marking the
bud neck every min at 22°C in WT haploid cells (Figure. 2.7A).
Surprisingly, I found different patterns of Cdc42 polarization between mother and daughter cells. In WT mother cells, the highest Cdc42-GTP level at a single site was established quickly within 5 min after the onset of cytokinesis (t = 0), despite some fluctuation of the level, and this position did not change significantly until the new septin was visible (86%, n = 14). Kymographs generated either along the cell periphery of mother cells or at the mother side of the bud neck showed development of robust Cdc42-GTP peak at a site adjacent to the septin ring (Figure. 2.7B, a & b), as expected for cells budding in the axial pattern. Interestingly, haploid daughter cells exhibit the dynamic motion of the Cdc42-GTP peak only around the septin ring (i.e. axial landmarks) in early G1 (100%, n = 14; Figure. 2.7Bc) and becomes stabilized to a single presumptive bud position 10-12 min prior to bud emergence as previously reported (Okada et al., 2013).

2.3.4. The dynamic movement of the Cdc42-GTP peak in daughter cells is likely to occur during T1.

Next, I wondered when the axis of Cdc42-GTP polarization becomes stabilized at a spatial position during G1 and whether the dynamic behavior of Cdc42-GTP peak is correlated with the temporal G1 regulation. Thus, I monitored Cdc42-GTP in cells expressing the transcriptional repressor Whi5-GFP, whose exit from the nucleus partitions the G1 phase into two temporal steps — T1 and T2 (Di Talia et al., 2007). Indeed, time-lapse imaging of PBD-RFP and Whi5-GFP indicated that the axis of Cdc42-GTP polarization remained spatially stable after Whi5 exits from the nucleus (i.e., during T2) in daughter cells (Figure 2.8) whereas a couple of Cdc42-GTP clusters were occasionally observed around the bud neck during T1 (see Figure 2.8 at t = 6 & 20). The
axis of cell polarization is thus likely to be stabilized approximately at the beginning of T2. These results also account for little changes of the position of the Cdc42-GTP cluster observed in mother cells, which have very short T1. Thus, two different dynamic phases of Cdc42-GTP polarization are tightly controlled over cell cycle progression during G1 phase.

2.3.5. The axis of cell polarization is determined in mid G1 in rsr1Δ cells.

A previous study reported that cells deleted for RSR1 exhibit spatially unstable axis of cell polarization before selecting a single and a random site during the G1 phase (Ozbudak et al., 2005). It remained unclear whether the axis of Cdc42-GTP polarization continuously changes even in T2 phase of rsr1Δ cells unlike in WT cells. Thus I monitored the position of Cdc42-GTP clusters in rsr1Δ cells every min at 22°C by time-lapse imaging (Figure. 2.9A). I did not observe strong Cdc42 polarization in early G1 in rsr1Δ cells (see 2.3.6 below), while robust Cdc42-GTP polarization was evident in mid-late G1, albeit at a random site relative to the septin ring. Surprisingly, once the strong Cdc42-GTP cluster developed, the axis of Cdc42 polarization did not change significantly, and new septin ‘clouds’ appeared at the same site where Cdc42-GTP cluster developed (88%; n = 33). These results thus suggest that even in cells lacking a spatial information (i.e., cells budding in a random pattern), the axis of cell polarization is determined around mid G1. The rsr1Δ cells occasionally abandoned robust Cdc42-GTP cluster and then developed a new one at a distant site (12%; n = 33). However, such ‘relocation’ was seen only in those rsr1Δ cells that were slower in undergoing bud
emergence, presumably due to the unstable axis of Cdc42-GTP polarization during the G1 phase.

2.3.6. Robust Cdc42-GTP polarization in early G1 is dependent on Rsr1

Less Cdc42-GTP signals in rsr1Δ cells in early G1 suggested that RSR1 is necessary for Cdc42 polarization in T1. Indeed, when I imaged rsr1Δ cells expressing PBD-RFP and Whi5-GFP at 30°C, I observed weak, sporadic Cdc42-GTP elevations in rsr1Δ daughter cells during T1 (when Whi5 remains within the nucleus), while robust Cdc42-GTP cluster developed during T2 (Figure. 2.9 B). Quantification of Cdc42-GTP cluster in the whole cells (each daughter or mother cell) over time confirmed that the first Cdc42-GTP wave during T1 was significantly compromised in rsr1Δ daughter cells, despite activation of Cdc42 by Bud3 (Kang et al., 2014), but not in T2 phase (Figure. 2.9B; Figure. 2.10). These results thus suggest that Rsr1 is necessary not only for selection of a bud site but also for Cdc42-GTP polarization in early G1, likely as a part of the Cdc42 activation circuit triggered by Bud3 (Kang et al., 2014).

2.3.7. Rga1 is likely to be involved in dynamic Cdc42-GTP polarization.

What mechanism(s) operate to mediate the movement and/or fluctuation of the Cdc42-GTP peak? Because Rga1 is the only Cdc42 GAP that specifically affects proper bud-site selection (Chen et al., 1996; Lo et al., 2013; Stevenson et al., 1995; Tong et al., 2007), I monitored the Cdc42-GTP polarization dynamics of rga1Δ cells that express PBD-RFP and Cdc3-GFP by time-lapse microscopy. I then quantified the Cdc42-GTP levels in three regions nearby the septin ring in daughter cells. While the Cdc42-GTP
level was often higher on either side of the septin ring for the first few min after the onset of cytokinesis in rga1Δ daughter cells (79%, n = 19), the Cdc42-GTP cluster eventually developed at the central region of the division site in daughter cells (100%, n = 19) and most mother cells (95%, n = 19) of an rga1Δ mutant (Figure. 2.11A), consistent with a previous report (Tong et al., 2007). These results thus suggest that Rga1 is likely to be involved in the movement of the Cdc42-GTP peak in the WT daughter cells.

Interestingly, I often found that rga1Δ cells exhibited high Cdc42-GTP accumulation at the tip of a bud at the onset of cytokinesis (t = 0; Figure. 2.11B; see discussion) unlike WT cells. When I compared the overall level of Cdc42-GTP cluster at the bud neck region (normalized to the same at t = 0), I found that Cdc42-GTP level was much highly elevated in rga1Δ cells compared to WT cells, despite the presence of other Cdc42 GAPs (Figure 2.11B). When I compared overall level of Cdc42-GTP cluster at the bud neck region (normalized to the same at t = 0), it was about 4 ~ 5 fold higher in rga1Δ cells than the average values of Cdc42-GTP peaks of WT cells in T1 and T2 phase (90%; n = 10; see Figure 2.11Bb). Two distinct Cdc42-GTP waves in the G1 phase were not evident in rga1Δ cells, likely because of such high elevation of Cdc42-GTP level.

2.3.8. Dynamic changes in Rga1 distribution during cytokinesis and to the next G1 phase.

Dr. Wing-Cheong Lo (Mathematical Biosciences Institute, OSU) tried to explain different dynamics of Cdc42-GTP polarization in haploid mother and daughter cells using mathematical modeling by incorporating the presence of fixed Cdc42 GAP ring at
the bud-neck during cytokinesis as previously reported (Caviston et al., 2003; Tong et al., 2007) but computational modeling always predicted that Cdc42-GTP polarizes within the bud-neck, contrary to the in vivo observation (Data not shown).

Thus I wondered whether Rga1 indeed localizes as a homogeneous ring at the bud neck and then to the subsequent division site during M to the next G1 phase. Although Rga1 localization to the bud neck is important for preventing re-budding within the immediately preceding division site (Tong et al., 2007), it remains unclear how any other adjacent, previous bud site(s) are prevented from being used again. To address these questions, I performed time-lapse imaging of GFP-Rga1 together with either Cdc3-mCherry or Myo1-mCherry. Surprisingly, I found two unique features of Rga1 localization patterns that had not been previously appreciated. First, Rga1 localized to a site (occasionally even to a few sites) next to the bud neck in mother cells (77% of large budded cells prior to the onset of cytokinesis, n = 17; see an arrow at t = -4, Figure. 2.12A), and the new septin ring always appeared at a position on the other side of the bud neck (but still adjacent to the division site) (see an arrow at t = 52, Figure. 2.12A: see more discussion below). Rga1 localization adjacent to the neck also disappears around the time when a new septin appears (see t = 52, Figure 2.12A). Second, the GFP-Rga1 intensity at the bud neck decreased during cytokinesis and then started to increase at the division site again in G1 (100%; n = 17; Figure. 2.12A). Line scan analysis of time-lapse images of GFP-Rga1 with Myo1-mCherry showed the asymmetric distribution of Rga1 between mother and daughter cells during cytokinesis and the G1 phase (Figure. 2.13).

To examine these changes of Rga1 localization more clearly, I took end-on views of large budded or newly divided cells that had the rings of GFP-Rga1 and Myo1-
mCherry (or Cdc3-mCherry) in the center of cells (as depicted in Figure. 2.1). GFP-Rga1, which appeared as a homogeneous ring at the bud neck, underwent drastic changes during cytokinesis: the ring became fragmented as the Myo1 ring started to contract, and then amorphous GFP-Rga1 signal appeared at the division site soon after the completion of Myo1 ring contraction, forming a ring again (100%, n = 5; Figure. 2.12Ba). The fragmented GFP-Rga1 often appeared within the Cdc3-mCherry ring in newly born daughter cells (in which the septin ring is bigger than in the newly divided mother cells or at the mother-bud neck) (100%, n = 12; Figure. 2.12Bb). This fragmented disassembly of Rga1 ring is also likely to occur in mother cells. However, it was difficult to observe in mother cells likely because of the shorter G1 phase and also due to the presence of another Rga1 ring on the daughter side. Taken together, these results thus suggest that the homogeneous Rga1 ring disassembles during cytokinesis and early G1.

2.3.9. Time-dependent Rga1 distribution is required for proper bud-site selection

The next question is whether these new Rga1 localization patterns in addition to the bud neck localization are necessary for selection of a proper bud site. First, I wondered whether Rga1 also localizes to the old division site(s) next to the bud neck, thus preventing re-budding from the site(s) in mother cells. To test the idea, I stained cells expressing GFP-Rga1 with Calcofluor, which stains chitin-rich bud scars (thus all previous bud sites) and the base of a bud. Remarkably, GFP-Rga1 localized to the site(s) overlapping the bud scar(s) or within the birth scar (i.e., the division site in daughter cells, which is low in chitin content) (100%; n = 45) (marked with an arrow and an arrowhead in Figure. 2.14A), supporting the hypothesis.
In order to address the functional significance of the time-dependent changes of Rga1 localization patterns, I tested whether limiting Rga1 localization only to the bud neck as a homogeneous ring could affect the bud-site selection by expressing the Rga1 GAP domain fused to Bud3 (‘Bud3-Rga1GAP’). Cells expressing Bud3-Rga1GAP-GFP exhibited the same localization pattern as Bud3: i.e., it localized to the bud neck as a homogenous double ring during M – G1 but no other site(s) next to the bud neck, as previously reported (Tong et al., 2007). The level of Cdc42-GTP at the bud neck was significantly reduced in rga1Δ cells expressing Bud3-Rga1GAP fusion compare to rga1Δ cells, confirming that Bud3-Rga1GAP fusion acts as a GAP that inactivates Cdc42 similar to WT cells (Figure 2.16).

Remarkably, expression of Bud3-Rga1GAP failed to rescue ‘the budding-within-the-old-division-site phenotype’ of an rga1Δ mutant: when these cells were doubly stained with Calcofluor and WGA-FITC, almost all daughter cells budded within the birth scar, which was stained with WGA-FITC (Figure 2.14B). Even in mother cells that had more than one bud scars, most bud scars were observed within the birth scar, similar to rga1Δ cells (Figure 2.14B). When I counted only those cells that had at least more than two bud scars, a significant number of rga1Δ cells exhibited the bipolar pattern (62%, n = 50), as previously reported (Chen et al., 1996; Stevenson et al., 1995).

Since budding events within a bud scar were sometimes difficult to distinguish clearly from static images (see Figure 2.14Bb*), I also examined Cdc42-GTP polarization in the rga1Δ cells expressing Bud3-Rga1GAP-GFP by time-lapse imaging after Calcofluor staining (n = 22). This analysis revealed an interesting difference
between mother and daughter cells: Cdc42-GTP cluster polarized within the Bud3 ring of all daughter cells (100%). In mother cells, Cdc42-GTP became polarized either within the immediately preceding division site (32%) or to a site next to the bud neck (68%) but most of these sites overlapped with a bud scar (93%) (marked with an arrow; Figure 2.15), indicating that these sites were old division sites. These analyses indicate that Bud3-Rga1 GAP is not functional, although the defect was less severe in mother cells. Taken together, these results suggest that this transient Rga1 localization — next to the bud neck (of mother cells) and the dynamic changes at the bud neck — contributes to selection of a proper bud site.

2.4. Discussion

Despite a large number of studies of bud-site selection and polarity establishment in budding yeast, how spatial cues are linked to the determination of the axis of cell polarity is not fully understood. A recent study by Dr. Pil Jung Kang indicates that stepwise activation of Cdc42 in G1 is necessary for proper assembly of the axial landmark and establishment of a proper bud site in haploid budding yeast (Kang et al., 2014). By live-cell imaging, I showed that the early Cdc42 activation is indeed dependent on Bud3, and the late Cdc42 activation relies on Cdc24 in vivo. Two Cdc42-GTP waves are separated by two temporally uncorrelated phases during G1 in haploid cells. A number of questions still remain in understanding the mechanisms of axial budding pattern: Is there a positive feedback circuit involved in robust Cdc42 polarization in early G1? How does the patterns of the Cdc42-GTP polarization different between early G1 and late G1? When and how does the axis of cell polarization determined in response to
the axial landmark? The findings reported here provide part of the answers.

Time-lapse analyses of Cdc42-GTP clusters uncover two distinct Cdc42 polarization dynamics in mother and daughter cells during cytokinesis and early G1. While the Cdc42-GTP cluster moves around the division site in daughter cells during T1, the location of the Cdc42-GTP cluster becomes stabilized in T2 in both mother and daughter cells, suggesting that the axis of cell polarization is determined in mid G1.

I also showed that the Rsr1 GTPase is necessary for robust Cdc42 polarization in early G1. This data supports the previous study, indicating the crucial role of Rsr1 in selection of a proper bud site and also for the efficient Cdc42 polarization (Kozminski et al., 2003). Surprisingly, the Cdc42-GTP cluster, once formed in mid G1, exhibited relatively little changes of its position in rsr1Δ cells, which bud in the random pattern. These observations suggest that Cdc24 arrives at the already selected bud site and then contributes to further Cdc42 activation. In contrast, Cdc42 activation by Bud3 is necessary for establishment of a proper bud site (Kang et al., 2014).

In addition, the axis of Cdc42 polarization dynamically changes its position in early G1 when Cdc24 is sequestered in the nucleus in haploids (Toenjes et al., 1999; Nern and Arkowitz, 2000; Shimada et al., 2000), thus Cdc24 is unlikely to be involved in the negative feedback loop that may be required for dynamic behavior of Cdc42-GTP polarization, contrary to a previous report (Kuo et al., 2014).

Another significant finding from this study is time-dependent changes of Rga1 distribution around the bud neck and the division site. These localization patterns of Rga1 are necessary for selection of an axial bud site. In contrast to the previous report (Tong et
al., 2007), computational modeling by Dr. Wing-Cheong Lo and experimental data indicate that when Rga1 GAP activity is limited only to the division site, similar to the septin ring, cells fail to choose a bud site adjacent to the previous bud site. These findings thus contrast with a previous report, concluding that the bud-neck localization of Rga1 is critical for its role in preventing re-budding at the previous division bud site (Tong et al., 2007). I suspect that it might have been difficult to uncover the defect of bud position of cells expressing Bud3-Rga1GAP in the previous study, which relied on the number of bud scars without visualizing the position of birth scars. Because a birth scar is a malleable structure unlike a bud scar, and it thus changes its size and fades significantly after multiple cell divisions (Powell et al., 2003), it might also have been missed in some mother cells. In this study, separation of budding patterns between mother and daughter cells have shown evident defects in the first bud position of daughter cells relative to a birth scar in cells expressing Bud3-Rga1GAP fusion protein. The time-lapse imaging analysis also visualizes the patterns of Cdc42-GTP polarization from a single cells, thus providing the precise conclusion of Bud3-Rga1GAP mutant phenotype. The defect of the axial budding pattern in cells expressing Bud3-Rga1GAP is unlikely due to a lack of Cdc42 GAP activity. In fact, this fusion seems to have even a higher GAP activity than WT Rga1, based on quantification of Cdc42-GTP (see Figure 2.16), suggesting the significance of the transient localization pattern of Rga1 in the axial budding pattern.

Interestingly, cells lacking Rga1 exhibit strong accumulation of Cdc42-GTP at the bud tip even at the onset of cytokinesis (t=0), suggesting that Rga1 is also important for depolarization of Cdc42-GTP from the bud tip to the neck during cytokinesis, similar to the case in rga1Δ diploid daughter cells (see Figure 2.11B; Figure 3.4B). The localization
of Rga1 at the bud tip has not been clearly seen in this study and also in previous reports (Caviston et al., 2003; Tong et al., 2007), thus it is likely that Rga1 may play an indirect role in Cdc42 inactivation at the bud tip during G2 to M transition (see Chapter 4 for further discussion).

How is then a single bud site chosen during axial budding? The live-cell imaging suggests that a bud site is determined within the potential area restricted by the axial landmark and the Rsr1 GTPase. Further restriction of this potential area is imposed by the inhibition of Cdc42 activity by Rga1, but the final selection of a bud site could be a random event within the potential area due to noise in the level of the landmark and Rga1 inhibition. The spatio-temporal regulation of Cdc42 activity during two distinctive phases of G1 is likely to be critical in linking the polarity machinery to the cell cycle progression and also to the proper establishment of cell polarity in haploid budding yeast. These mechanisms may implicate in general principles of cell polarization in other higher eukaryotic systems.
Figure 2. 1. A schematic diagram of the axial budding. 
A scheme of the axial budding pattern: a side view (A) and end-on view (B). The red rings depict the axial landmark and septin rings together (the new septin rings are shown in black); blue patches depict the Cdc42-GTP cluster; and the purple and green rings in (A) depict the cell division site in mother (‘bud scar’) and daughter cells (‘birth scar’), respectively. Solid and dotted red rings in (B) depict the division sites from the immediately preceding division and earlier division, respectively.
Figure 2. 2. The early Cdc42 activation is dependent on Bud3.
Time-lapse imaging of PBD-RFP and Cdc3-GFP in WT (A) and bud3ΔCR2 (B) cells at 22°C. Arrowheads mark first appearance of new septins in mother cells. Numbers indicate time (in minutes) from the onset of cytokinesis (t = 0). (C) Quantification of PBD-RFP in mother and daughter cells shown in A and B. PBD-RFP intensity is normalized to intensity at t = 0.
Figure 2. 3. Quantification of PBD-RFP and localization of PBD-RFP and Cdc3-GFP. (A) PBD-RFP intensity is compared in mother cells (a) and daughter cells (b) of WT and bud3ΔCR2 at 22°C (including those shown in Figure 2.1 C) and daughter cells (c) of WT and bud3ΔCR2 at 30°C. PBD-RFP intensity was normalized to intensity at the onset of cytokinesis (t = 0). Each colored arrowhead marks the time point when new septin clouds were first visible (or estimated) in each mother cell at 22°C (a) or in each daughter cell at 30°C (c) for each corresponding colored line. (B) Time-lapse imaging of PBD-RFP and Cdc3-GFP in WT cells at 30°C. Arrowheads mark the first appearance of a new septin in a daughter cell. Numbers indicate time (in minutes) from the onset of cytokinesis (t = 0). Quantification of PBD-RFP intensity in the daughter cell is shown in A (c, purple line on the top).
Figure 2. Two Cdc42-GTP waves are partitioned by the temporal G1 regulation. Time-lapse imaging of PBD-RFP and Whi5-GFP in a haploid WT cell at 30°C. (bottom) PBD-RFP intensity at each time point is normalized to intensity at t = 0. Arrows mark time points when Whi5-GFP completely exited from the nucleus. Bars, 3 μm.
Figure 2. 5. Cdc42 is activated by Bud3 in early G1 independently of Cdc24. Time-lapse imaging of PBD-RFP and Cdc3-GFP in cdc24-4 cells every 2 min at 37°C, and selected images are shown. Numbers (white) indicate the time (minutes) relative to the onset of cytokinesis (t = 0); numbers (black) below images indicate the time after shifting to 37°C. Bars, 3 μm. (Right) PBD-RFP intensity is normalized as in Figure 2.2.
Figure 2. 6. Bud3 activates Cdc42 in vivo.
(A) PBD-RFP polarization in cdc24<sup>ts</sup> cells carrying each multicopy plasmid after shifting to 37°C for 3 h. Percentages of cells with Cdc42-GTP clusters are shown.
(B) Quantification of PBD-RFP clusters in individual polarized cells is shown as a percentage of the whole-cell intensity with means (horizontal lines) ± SEM (error bars). * P = 0.86; ** P < 10<sup>-3</sup>.
Figure 2.7. Cdc42 polarization in WT haploid cells is dynamic in early G1.  
(A) Time-lapse analysis of Cdc42-GTP (PBD-RFP) dynamics with respect to Cdc3-GFP in WT haploid cells at 22°C. Numbers indicate time (in min) from the onset of cytokinesis (i.e., t = 0 when the Cdc3 ring splits).  
(B) Kymographs are shown below: along the mother cell perimeter (a); and for the region marked with a yellow rectangle in the mother cell (b) and the daughter cell (c). Empty and filled rectangles next to kymographs mark the positions of old and new septin rings, respectively. Heat maps (b and c) represent the Cdc42-GTP level of the kymographs shown on the left.
Figure 2.8. Time-lapse analysis of Cdc42-GTP and Whi5-GFP in WT haploid cells at 30°C.

A heat map represents the Cdc42-GTP level in the daughter cell (in the marked region) from t = 20 (2 min before T2 began) to t = 30 min (about 4 min before bud emergence). Note: The daughter cell was slightly pushed due to bud emergence in the mother cell. A dotted-line rectangle next to the heat map marks the position of the division site (estimated from the bud neck). Bars, 3 µm.
Figure 2. Cdc42-GTP polarization in early G1 is dependent on Rsr1.

(A) Time-lapse analysis of Cdc42-GTP dynamics with Cdc3-GFP in rsr1Δ cells at 22°C. Numbers indicate time (in min) from the onset of cytokinesis (t = 0). (right) A kymograph is shown along the perimeter of the mother cell. Empty and filled rectangles next to kymographs mark the positions of old and new septin rings, respectively.

(B) Time-lapse analysis of Cdc42-GTP clusters in rsr1Δ cells at 30°C. Arrows in the merged images mark sporadic appearance of Cdc42-GTP cluster in the daughter cell. (right) Quantification of PBD-RFP in five representative rsr1Δ daughter cells are shown at each time point (colored lines), normalized to intensity at t = 0. A WT daughter cell control is shown with a dotted black line. Each colored arrowhead and a black arrow mark the time point when Whi5 exits from the nucleus (or estimated) in each rsr1Δ (marked with each corresponding color) and WT, respectively. Bars, 3 µm.
Figure 2. Quantification of PBD-RFP clusters in WT and rsr1Δ daughter cells in T1 and T2 phases. PBD-RFP intensity from the whole cell is normalized to intensity at t=0 (the onset of cytokinesis) and the normalized values were plotted. Each dot represents the highest level of Cdc42-GTP peak from individual cells during the T1 and T2 phase with means (horizontal lines) ± SEM (error bars). *, P = 0.46; **, P < 10^{-10}
Figure 2. 11. Rga1 is likely to be involved in dynamic Cdc42-GTP polarization.

(A) Time-lapse analysis of Cdc42-GTP dynamics with respect to Cdc3-GFP in rga1Δ cells at 22°C. Numbers indicate time (in min) from the onset of cytokinesis (t = 0). PBD-RFP intensity in each selected region (marked on the merged images) in daughter cells of rga1Δ and WT (images shown in Figure 2.3) was normalized to intensity at the same region at t = 0. (B) Time-lapse analysis of Cdc42-GTP clusters in rga1Δ cells (with Cdc3-GFP) at 30°C. PBD-RFP intensity in the bud neck region of WT and rga1Δ daughter cells was normalized to the same at the onset of cytokinesis (t = 0), and three representative plots of rga1Δ cells were compared to WT. Bars, 3 µm.
Figure 2. 12. Time-dependent Rga1 localization during cytokinesis and to the next G1 phase.
(A) Time-lapse images of GFP-Rga1 and Cdc3-mCherry in WT cells at 22°C. Arrows at t = -4 and t = 52 mark GFP-Rga1 localized to a site next to the bud neck and a new septin ring, respectively. Numbers indicate time (in min) relative to the onset of cytokinesis (t = 0). Bar, 3 µm. (B) (a) End-on views of GFP-Rga1 and Myo1-mCherry localized to the bud neck and the division site during cytokinesis and G1 at 22°C. Numbers indicate time (in min) from the first image (a few min before the Myo1 ring started to contract). (b) End-on views of GFP-Rga1 and Cdc3-mCherry localized to the bud neck (upper panel) and to the division site of a daughter cell after cytokinesis (lower panel). Bars, 1 µm.
Figure 2. 13. Asymmetric localization of Rga1 at the mother-bud neck is likely to be important for proper bud-site selection.
Time-lapse images of GFP-Rga1 and Myo1-mCherry from cytokinesis to next G1 at 22°C. Arrowheads mark GFP-Rga1 localized to the side of the bud neck before and after the Myo1 ring contraction; and an arrow marks the new Myo1-mCherry. Line-scan graphs show fluorescence intensities (au) of GFP-Rga1 and Myo1–mCherry along the bud-mother axis at the selected time points.
Figure 2. 14. Two unique localization patterns of Rga1 are crucial for proper bud-site selection.

(A) Localization of GFP-Rga1 to the previous division site (in addition to the bud neck), which overlaps with (a) a bud scar (marked with an arrow), which is chitin-rich, thus stained with Calcofluor, or (b) a birth scar (marked with an arrowhead), which is chitin-less. Bar, 3 μm.

(B) Patterns of bud site(s) position (%) in mother (M) and daughter (D) cells of haploid WT, rga1Δ, and rga1Δ expressing BUD3-Rga1(aa 700-1007)-GFP. A representative image of each pattern in mother and daughter cells is shown: (a) bud scars adjacent to the birth scar, as in the axial budding pattern; (b) bud scars or a bud within the birth scar; (b*) bud scars (or a bud) within a bud scar (as well as within the birth scar); and (c) bud scars (or a bud) within the birth scar and at the opposite pole. In daughter cells, position of a bud relative to birth scar is scored similarly. Arrows indicate birth scars. Bar, 3 μm.
Figure 2. 15. The patterns of Cdc42-GTP polarization in rga1Δ cells expressing Bud3-Rga1GAP fusion.

Time-lapse analysis of an rga1Δ cell expressing (a) PBD-RFP, (b) Bud3-Rga1GAP-GFP and Whi5-GFP after (c) Calcoflour staining at 22°C. Merged images are shown below: PBD-RFP (red) with Calcoflour (cyan) (a + c); or with Bud3-Rga1GAP-GFP and Whi5-GFP (which was used as a marker for cell cycle progression) (green) (a + b). A Cdc42-GTP cluster (marked with an arrowhead) developed at an old bud site (stained with Calcofluor; marked with an arrow) in the mother cell; while a Cdc42-GTP cluster (marked with *) developed within the Bud3 ring in the daughter cell. Numbers indicate time (in min) from the onset of cytokinesis (t = 0) estimated by the appearance of Whi5 to the nucleus (t = -2 or -4). Bars, 3 µm.
Figure 2.16. Quantification of PBD-RFP clusters in WT, *rga1Δ*, Bud3-Rga1GAP daughter cells in T1 and T2 phase.
PBD-RFP intensity from the daughter side of the neck region is normalized to intensity at t=0 (the onset of cytokinesis) and the normalized values were plotted. Each dot represents the highest level of Cdc42-GTP peak from individual cells during the T1 and T2 phase with means (horizontal lines) ± SEM (error bars). *P = 0.08; **P < 10^{-8}
Table 2.1. Yeast strains used in this study

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*a Strains marked with * are congenic to HPY210.

b Strain marked with # is derived from Y147 (Bender and Pringle, 1989).
CHAPTER 3: Polarization of diploid daughter cells directed by spatial cues and GTP hydrolysis of Cdc42 in budding yeast.


3.1. Introduction

During vegetative growth, Cells of budding yeast Saccharomyces cerevisiae exhibit cell-type specific patterns of budding, which ultimately determines the axis of polarized growth. Haploid a and α cells choose a new bud site immediately next to the previous division site in both mother and daughter cells, a process called the axial budding pattern. In contrast, a/α diploid cells bud in a bipolar pattern in which daughter cells predominantly bud at the pole distal to the previous division site (distal pole) and mother cells can choose a new bud site near the proximal pole (birth pole) or the distal pole to the birth pole (see Fig 3.1A) (Chant and Herskowitz, 1991). The selection of a bud site is directed by spatial cues, which are specific to cell types. The Rsr1 GTPase module, which is consist of the Rsr1 GTPase, its GDP-GTP exchange factor (GEF) Bud5, and its GTPase activating protein (GAP) Bud2, mediates the communication between spatial cues and the polarity-establishment machinery, including the Cdc42 GTPase
module. Cdc42 thus polarizes to the predetermined cortical site to initiate new bud growth (Bi and Park, 2012).

Bud8 and Bud9 are structurally related transmembrane proteins, which are essential for the bipolar budding pattern of diploid a/α cells (Harkins et al., 2001, Kang et al., 2004). Bud8 localizes to the distal pole of newly born daughter cells, whereas Bud9 localizes to the daughter side of mother-bud neck (the proximal pole of a daughter cell) just before cytokinesis (Harkins et al., 2001). Cells of bud8Δ/bud8Δ and bud9Δ/bud9Δ exclusively bud at the proximal and distal poles, respectively (Harkins et al., 2001). The localization patterns and deletion phenotypes are consistent with the idea that Bud8 and Bud9 are putative distal and proximal pole markers, respectively. Rax1 and Rax2 also serve as stable cortical markers in the bipolar budding pattern (Bi and Park, 2012). Rax1 and Rax2 localize to the distal tip of daughter cells and to the division sites, which persistently present throughout multiple generations (Chen et al., 2000, Fujita et al., 2004, Kang et al., 2004). Previous genome-wide screenings of non-essential genes have found a large number of proteins affecting the bipolar budding pattern (Zahner et al., 1996, Ni and Snyder. 2001, Sheu et al., 2000, Yang et al., 1997). These studies have shown a close correlation between the bipolar budding pattern and the cell cycle progression (Sheu et al., 2000, Schenkman et al., 2002). Despite the known localization patterns of putative bipolar landmarks and genome-wide mutant analysis, the mechanism by which the bipolar pattern is established remains largely unknown. One of the key questions is why daughter cells of a/α diploids choose mainly the distal pole for their first budding despite the presence of Bud8 and Bud9 marking each pole.
Cells of the budding yeast *Saccharomyces cerevisiae* can initiate cell polarization through spontaneous clustering of Cdc42 and its subsequent stabilization by feedback loops in the absence of spatial cues, a process called ‘symmetry breaking’ (Wedlich-Soldner *et al.*, 2003, Irazoqui *et al.*, 2003, Wedlich Soldner *et al.*, 2004, Kozubowski *et al.*, 2008). Two positive feedback mechanisms have been suggested, one is actin-mediated positive feedback and the other relies on Cdc42 signaling network, including the scaffold protein Bem1 and the Cdc42 GEF Cdc24 (Wedlich-Soldner *et al.*, 2003, Irazoqui *et al.*, 2003, Wedlich Soldner *et al.*, 2004, Goryachev and Pokhilko, 2008, Kozubowski *et al.*, 2008). Endocytosis and GDI (Guanosine nucleotide dissociation inhibitor)-mediated recycling of Cdc42 and a negative feedback loop confer robust initiation of polarization (Ozbudak *et al.*, 2005, Marco *et al.*, 2007, Slaughter *et al.*, 2009, Howell *et al.*, 2012). These studies provide a mechanistic insight into spontaneous polarization in the absence of spatial cues. However, some aspects of these mechanisms and their physiological relevance are strongly debated (Johnson *et al.*, 2011, Layton *et al.*, 2011, Arkowitz and Bassilana, 2011). Importantly, it had been unknown whether and how the spatial cues are recognized and amplified through these feedback mechanisms in the process of normal budding events. In this chapter, the mechanisms of polarity establishment in diploid daughter cells were investigated by live-cell imaging as well as by mathematical modeling simulated by Dr. Lo. This study reports that both spatial landmarks and GTP hydrolysis of Cdc42 by Rga1 are required for robust Cdc42-GTP polarization to the distal pole of diploid daughter cells.
3.2. Materials and Methods

**Strains, plasmids and genetic methods**

Standard methods of yeast genetics, DNA manipulation, and growth conditions were used (Ausubel et al., 1999; Guthrie and Fink, 1991) unless indicated otherwise. Plasmids YIp211-GIC2-PBD-1.5tdTomato and YIp128-CDC3-GFP (kindly provided by E. Bi, University of Pennsylvania) were used to construct strains expressing Gic2-PBD-RFP and Cdc3-GFP, respectively, as previously described (Tong et al., 2007). Plasmids pRS314-HO and YCp50-HO (from the Park lab collection), which carry the HO gene allowing the mating-type interconversion (Jensen et al., 1983), were used to generate a/α diploids. Yeast strains used in this study are listed in the Table 3.1.

**Determination of the budding pattern and localization of Bud8**

To determine budding patterns, cells were spotted on a YPD plate after a brief sonication and then the position of each bud was monitored under the dissecting microscope at 25°C. For time-lapse imaging by DIC microscopy, cells were grown similarly, spotted on a slab of YPD medium containing 1% agarose, and then imaged using a Nikon E800 microscope (Nikon, Tokyo, Japan) fitted with a 100X oil-immersion objective (NA =1.30) with a Hamamatsu ORCA-2 CCD camera (Hamamatsu Photonics, Bridgewater, NJ) and Slidebook software (Intelligent Imaging Innovations, Denver, CO) at 25°C. Localization of Bud8 was examined as previously described (Kang et al., 2004) using YEpGFP-BUD8F (Schenkman et al., 2002).
3D Time-lapse Microscopy

To visualize GFP- and RFP-fusion proteins, a slab of SC-Ura was prepared as above using exponentially growing cells in SC-Ura media. Images were captured at 23–24°C every 2 min using a spinning disk confocal microscope (UltraView ERS, Perkin Elmer Life and Analytical Sciences, Waltham, MA) equipped with a 1006/1.4 NA objective lens (Nikon, Melville, NY), a 488-nm solid state laser and 568-nm argon ion laser, and a cooled charge coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Maximum intensity projections of Z-sections (spaced at 0.4–0.5 µm) are generated using UltraView ERS software.

3.3. Results

3.3.1. The dynamics of Cdc42-GTP polarization is consistent with the preferential distal-pole budding of diploid daughter cells.

Daughter cells of a/α diploids predominantly bud at the distal pole during their first and second bud-site selection (Chant and Pringle, 1995, Zahner et al., 1996, Harkins et al., 2001) (Fig 3.1A). To examine this preferential distal-pole budding pattern in diploid daughter cells more closely, I monitored the localization of Cdc42-GTP every 2 min in wild-type diploid cells expressing Gic2-PBD-RFP (tdTomato fused to the p21-binding domain of Gic2) as a reporter for Cdc42-GTP (Tong et al., 2007) and GFP fused to Cdc3, a component of septins, as a marker for the timing and the site of cytokinesis. As expected, Gic2-PBD-RFP localized to the periphery of a growing bud until the end of the M phase, and then transiently comes to the bud-neck (which becomes the proximal pole
of daughter cells) during cytokinesis, and then to the distal pole of the daughter cells in the next G1 phase (100%, n= 15) (Fig 3.1B). Although Cdc42 polarizes to the mother-bud neck (Tong et al., 2007, Richman et al., 2002), weak and transient Gic2-PBD-RFP signal at the proximal pole might be probably due to rapid hydrolysis of Cdc42-GTP by its GAP(s), consistent with a previous finding in haploids (Tong et al., 2007). Importantly, the time-lapse imaging was able to capture daughter cells at an intermediate stage that exhibited Cdc42-GTP localization at both proximal and distal poles at 22°C (see a cell marked with an arrowhead in Fig 3.1B). The dynamics of Cdc42-GTP polarization is thus consistent with the distal-pole budding of diploid daughter cells.

It remained unclear why daughter cells of a/α diploids exhibit such dynamics of Cdc42-GTP polarization despite the presence of spatial cues marking at both poles. Dr. Wing-Cheong Lo (Mathematical Biosciences Institute) used a mathematical modeling to answer this time-evolved polarization of Cdc42-GTP in diploid daughter cells. Briefly, this model was built upon the assumptions that the distal and proximal poles compete for Cdc42 or its effectors and regulators that are also based on the Bem1-mediated positive feedback mechanism (Goryachev and Pokhilko, 2008, Kozubowski et al., 2008). Most importantly, the Cdc42 GAPs, which account for the weak Gic2-PBD-RFP at the division site, and the spatial cues at both poles are included in the model (Data not shown). The mathematical modeling by Dr. Wing-Cheong Lo indeed recapitulated the robust Cdc42 polarization to the distal-pole of a/α daughter cells despite the competition between two poles for recruiting the Bem1 complexes and Cdc42-GTP (Data not shown).
3.3.2. Deletion of *RGA1* affects the distal-pole budding in daughter cells of a/α diploids.

The computational modeling by Dr. Lo indicates that Cdc42-GTP hydrolysis rate at the division site contributes to robust distal-pole budding in a/α daughter cells. All known Cdc42 GAPs localize to the mother-bud neck at cytokinesis (Tong *et al.*, 2007, Caviston *et al.*, 2003, Knaus *et al.*, 2007, Sopko *et al.*, 2007). Next, I wondered which Cdc42 GAP(s) play a role in a bias for budding at the distal pole of daughter cells. Thus, I scored the position of the first bud in newly born daughter cells of diploid wild-type and mutants singly deleted for a Cdc42 GAP, including Rga1, Rga2, Bem2, or Bem3. As expected, daughter cells seldom budded at the proximal pole in wild-type cells (3.4±2.3%, n = 106). In contrast, a significant number of daughter cells in *rga1*Δ diploid strains budded at the proximal pole (29.3±1.9%, n = 144; see daughter cells marked with arrows in Fig 3.3A), which is statistically significant (p<10⁻⁵). Deletions of *RGA2* or *BEM3* did not exhibit the proximal-pole budding in daughter cells (0%, n = 56 and 53, respectively). While cells lacking *BEM2* resulted in a little increased proximal-pole budding (6.4±2.8%, n = 108), the difference between wild type and *bem2Δ* does not seem to be statistically significant (p = 0.22) (Fig 3.3A). It is less clear whether Bem2, which is known as a GAP for Rho1, also functions as a GAP for Cdc42 *in vivo* (Knaus *et al.*, 2007, Zheng *et al.*, 1993, Zheng *et al.*, 1994, Marquitz *et al.*, 2002). Taken together, these results suggest that among the Cdc42 GAPs, Rga1 is uniquely required for the preferential distal-pole budding of a/α daughter cells, and such role is not shared by
other GAPs. I thus focused on Rga1 in subsequent studies.

Because Rga1 is uniquely required for preventing budding at the division site (Tong et al., 2007), it is possible that the diploid rga1Δ daughter cells that failed to bud at the distal pole also budded at the division site. Unlike mother cells, which have bud scars (chitinous scar tissue, which is one component of polysaccharide, located at the division site), daughter cells have a much less discrete birth scar (which has little or no chitin) at the division site (Bacon et al., 1966). Daughter cell-specific chitinase Cts1 is secreted only in daughter cells, which dissolves the primary septum together with other cell wall-degrading enzymes, leaving daughter cells with chitin-less birth scar whose exact composition still remains unknown (Frydlova et al., 2009).

To examine the first bud position in daughter cells relative to birth scar more closely, I stained cells with Calcofluor, which stains bud scars as well as the base of a bud, and FITC-labeled wheat germ agglutinin (WGA-FITC; Lectin from wheat), which stains both bud scars and birth scars (Frydlova et al., 2009). As expected, almost all wild-type daughter cells formed a bud opposite to the birth scar (which is marked with an arrow in Fig 3.3). In contrast, all of the rga1Δ daughter cells that failed to bud at the distal pole indeed budded within the birth scar (n = 65; note: this number includes some mother cells of rga1Δ because those mother cells that repeatedly budded within the birth scar could not be easily distinguished from daughter cells). As expected, almost all bud8Δ daughter cells budded at the proximal pole, but the position of a bud in bud8Δ was adjacent to, rather than within, the birth scar (97.4%, n = 39) (Fig 3.3). A
small number of daughter cells of the diploid wild-type (3.5%, n = 56) and bem2Δ mutant (6.3%, n = 63) also budded at the proximal pole, but these buds hardly appeared within the birth scar (data not shown). Interestingly, almost of all rga1Δ bud8Δ cells also budded within the birth scar (99.2%, n = 137; this counting is also likely to include some mother cells due to deletion of RGA1, see above). Taken together, these observations suggest that reduced distal-pole budding in the diploid rga1Δ daughter cells might result from the increased Cdc42-GTP at the division site, thus budding at that site, consistent with a previous report (Tong et al., 2007).

3.3.3. Polarization of Cdc42-GTP in diploid daughter cells lacking RGA1.

Although some diploid rga1Δ daughters budded within the birth scar, the majority of them (~70%) still budded preferentially to the distal-pole of daughter cells. To gain insight into this cellular process, I monitored the localization of Cdc42-GTP (using Gic2-PBD-RFP as a reporter) in diploid rga1Δ cells every 2 min. Gic2-PBD-RFP localized to the periphery of a growing bud in an rga1Δ mutant as in wild type until cytokinesis. During cytokinesis and in the next G1 phase, however, three different patterns of Gic2-PBD-RFP localization were observed in rga1Δ daughter cells (n = 19 movies): 1) Gic2-PBD-RFP localized to the proximal pole and then to the distal pole, as seen in wild-type (15.8%; not shown); 2) Gic2-PBD-RFP remained at the proximal pole (26.3%; Fig 3.4Aa); and 3) Gic2-PBD-RFP continuously localized to the distal pole in a large percentage of daughter cells (57.9%; Fig 3.4Ab). Both the first and third patterns of Cdc42-GTP localization were expected to lead to the distal-pole budding in a/a
rga1Δ daughter cells (see summary in Fig 3.4B). The localization patterns of Gic2-PBD-RFP are thus consistent with the budding phenotype of the rga1Δ daughter cells (see Fig 3.4B).

While an increase of Cdc42-GTP at the proximal pole was expected given the lack of GAP-assisted Cdc42 hydrolysis at the division site in the rga1Δ mutant (Tong et al., 2007), it seemed counterintuitive that a significant percentage of rga1Δ daughter cells exhibited Cdc42-GTP polarization persistently at the distal pole. The one caveat is that the imaging was not fast enough to capture transient localization to the proximal pole in the third pattern (Fig 3.4B). Nonetheless, these observations indicate that the dynamics of Cdc42-GTP in rga1Δ cells is different from that in wild-type cells. Rga1 might thus have a unique role in Cdc42 polarization in diploid cells in addition to its role in clearing Cdc42-GTP at the division site (see below).

3.3.4. Bud8 is necessary for polarization of Cdc42-GTP to the distal pole in diploid rga1Δ daughter cells.

Since Bud8 acts as a distal pole marker important for normal bipolar budding pattern (Harkins et al., 2001), I wondered whether the persistent distal-pole localization of Cdc42-GTP in the rga1Δ daughter cells is dependent on Bud8. Alternatively, Cdc42-GTP might be polarized to the distal pole independently of Bud8 as seen in the distal-pole budding of the rsr1Δ mutant during haploid invasive growth (Cullen and Sprague, 2002).

To distinguish these possibilities, I examined the Gic2-PBD-RFP localization in
cells lacking both RGA1 and BUD8 by time-lapse microscopy. While Gic2-PBD-RFP still localized to the periphery of growing buds prior to cytokinesis in the rga1Δ bud8Δ cells, it always localized to the proximal pole during cytokinesis and remained at the proximal pole in the rga1Δ bud8Δ cells (100%, n = 8 movies) (Fig 3.5A). This finding indicates that Bud8 functions as a spatial cue for the enrichment of Cdc42-GTP at the distal pole of the rga1Δ daughter cells as in wild-type cells. Interestingly, Gic2-PBD-RFP localized to a site within the old Cdc3 ring (i.e., within the birth scar) in the rga1Δ bud8Δ daughter cells. In contrast, Gic2-PBD-RFP localized to the division site at cytokinesis but subsequently to a site adjacent to the old Cdc3 ring in bud8Δ cells (100%, n = 7 movies) (Fig 3.5 B). These Cdc42-GTP polarization patterns are thus consistent with the first bud positions in daughter cells of these mutants (see Fig 3.3).

Why is it that the persistent enrichment of Cdc42-GTP at the distal pole was observed only in rga1Δ daughter cells (see Fig 3.4B)? I wondered how Rga1 controls Cdc42-GTP polarization. At the early phase of the cell cycle, most growth is targeted to the tip of the bud in budding yeast. This ‘apical’ growth is switched to ‘isotropic’ growth in the G2 phase, during which growth is distributed diffusely within the bud, and then cells are repolarized at the site of cytokinesis (Howell and Lew, 2012).

It has been suggested that apical growth and repolarization during cytokinesis are critical for marking spatial cues at the distal and proximal poles, respectively, and thus subsequent positioning of the division plane in diploid cells (Sheu et al., 2000). Interestingly, the rga1Δ cells have elongated bud morphology (Smith et al., 2002, Chen et al., 1996, Stevenson et al., 1995, Watanabe et al., 2009), suggesting a delay in the
transition from apical growth to isotropic growth. Thus it is possible that the prolonged apical growth of the \textit{rga1Δ} mutant might result in more efficient delivery of the distal-pole marker such as Bud8 to the distal pole. To test the idea, I examined Bud8 localization.

A significant percentage of large-budded cells also showed Bud8-GFP localization at both the bud tip and the bud side of the mother-bud neck, although the latter was often weaker, as previously reported (Kang \textit{et al.}, 2004, Harkins \textit{et al.}, 2001, Taheri \textit{et al.}, 2000). Interestingly, more large-budded \textit{rga1Δ} cells exhibited Bud8-GFP localization to the bud tip (46.2\pm1.5\%, \textit{n} = 165) compared to wild type (34.5\pm0.1\%, \textit{n} = 174) (Fig 3.6 Histogram), and this difference is statistically significant (\textit{p} = 0.006). Bud8-GFP often appeared to be confined to the extreme bud tip in these \textit{rga1Δ} cells (Fig 3.6). A minor difference of Bud8 localization was also observed in unbudded cells of \textit{rga1Δ} compared to wild type (data not shown).

The mathematical modeling by Dr. Lo indicates that when the ratio of strength of landmark cue at the distal pole to that at the proximal pole increases beyond certain range, Cdc42-GTP localization to the proximal pole could be barely monitored (data not shown), which accounts for the persistent distal-pole localization of Cdc42-GTP observed in over 50\% of the \textit{rga1Δ} daughter cells (see Fig 3.4B). This data suggests that Cdc42-GTP localization depends on the initial level of Cdc42-GTP at the division site and the strength of landmark cues and thus predict that the relatively higher landmark at the distal pole or lower landmark at the proximal pole in the absence of Rga1 might result in the persistent Cdc42-GTP enrichment at the distal pole, which may result from natural variations in the efficiency of delivery of these cues to the poles.
Cells lacking Bud8 or Bud9 bud exclusively at the proximal and distal poles, respectively (Zahner et al., 1996). In the simulation, when the landmark cues are high only at either the proximal or distal pole, which mimics bud8Δ and bud9Δ diploids, the Cdc42-GTP polarizes to the proximal pole or distal pole, respectively (data not shown). Taken together, the computational modeling by Dr. Lo indicated different dynamics of Cdc42-GTP polarization when the levels of landmark and Cdc42-GTP were perturbed by noise in the model.

3.4. Discussion

By live-cell imaging and collaborative mathematical modeling experiments, these data suggest the idea that Bud8, a distal pole landmark protein, is more efficiently targeted to the bud tip (which becomes the distal pole of daughter cells) in rga1Δ cells, perhaps due to longer apical growth phase. However, it is less clear whether this different pattern of Bud8 localization uniquely accounts for persistent Cdc42-GTP polarization to the distal pole of rga1Δ cells. Indeed, I observed robust Cdc42-GTP polarization at the bud tip in large-budded cells of the bud8Δ rga1Δ mutant until cytokinesis (and even in bud8Δ cells, although Gic2-PBD-RFP appeared more broadly at the periphery of the buds in these cells) (see Figure 3.5), suggesting that this Cdc42-GTP polarization prior to cytokinesis is independent on Bud8. The level of PBD-RFP seems to be highly elevated at the bud tip of bud8Δ rga1Δ mutants compared to the level in bud8Δ mutants even prior to cytokinesis (Figure 3.5), emphasizing the unique role of Rga1 in Cdc42-GTP polarization during an apical growth. Rga1 might also affect the targeting of Bud9 to the
proximal pole or a component of the polarisome such as Spa2 or Ste20 to the bud tip (Park and Bi. 2007, Sheu et al., 2000), which might also affect Cdc42-GTP polarization prior to cytokinesis via unknown feedback mechanisms. Further investigation is necessary to understand the underlying mechanisms involved in polarized growth and selection of a bud site in diploids.

Further analysis will be needed to understand how Rga1 coordinates both bud morphogenesis and bipolar bud-site selection. While the exact mechanism remains unknown, a negative feedback loop involving Rga1 might be involved to buffer the level of Cdc42-GTP and thus to stop the polarity cluster from growing too large, as previously suggested (Howell et al., 2012).

In summary, this study provides evidence how diploid daughter cells change their polarity from the proximal pole (i.e., birth scar) to the distal pole (i.e., opposite to the birth scar). The live-cell imaging indicates that the distal-pole budding of diploid daughter cells is dependent on Bud8 at the bud tip and GTP hydrolysis of Cdc42 by Rga1 at the bud neck. By a collaboration of mathematical modeling that predicts the different dynamics of Cdc42-GTP polarization in vivo, this study provides that proper switching of the polarity axis from the proximal pole to the distal pole in diploid daughter cells requires robust Cdc42-GAP activity. While further investigation is necessary to fully understand the underlying mechanisms, this study also suggests that a Cdc42-GAP Rga1 affects the dynamics of Cdc42 polarization not only at the bud-neck but also at the distal tip of the bud, contributing to selection of a growth site in diploid daughter cells.
Figure 3. 1. Time-lapse microscopy of Cdc42-GTP polarization in wild-type \( a/\alpha \) diploids. (A) A schematic diagram of bipolar budding pattern. M and D stand for mother and daughter cells, respectively. Red arrows depict the axis of cell polarity. (B) Localization of Gic2-PBD-RFP and Cdc3-GFP in diploid wild-type cells (HPY2353). An arrowhead marks Gic2-PBD-RFP localized to the proximal pole in the daughter cell. Numbers indicate time (in min) from the first image. Size bar, 3 \( \mu \)m.
Figure 3. 2. Positions of the first bud in α/α daughter cells of wild-type and mutants deleted for each Cdc42 GAP.
Time-lapse DIC images of diploid cells of wild-type (YEF473) and rga1Δ (YEF1233). Arrows indicate budding events from daughter cells. Numbers indicate times (in min) from the first image. Size bars, 5 μm.
(Histogram) The position of the first bud of daughter cells was scored in wild type (HPY1680), rga1Δ (HPY2205), rga2Δ (HPY2246), bem2Δ (HPY2384), and bem3Δ (HPY2426). The mean percentage ±SD of each budding pattern is shown from three or four independent countings of wild type (n = 106), rga1Δ ( n = 144), rga2Δ ( n = 56), bem2Δ ( n = 108), and bem3Δ ( n = 53). Statistical significance was determined by Student’s t-test between proximal-pole budings in wild type and rga1Δ or bem2Δ (marked with asterisks): *p<10⁻⁵ (rga1Δ) and **p = 0.22 (bem2Δ).
Figure 3. 3. The position of the first bud relative to the birth scar in diploid daughter cells.
Cells were double stained with Calcoflour white and WGA-FITC (see material and method) from wild type (YEF473), \textit{rga1}\textsuperscript{Δ} (YEF1233), \textit{bud8}\textsuperscript{Δ} (YHH415), and \textit{rga1}\textsuperscript{Δ} \textit{bud8}\textsuperscript{Δ} (HPY2385). Arrows indicate birth scars. Size bar, 3 \textmu m.
Figure 3. 4. Localization of Gic2-PBD-RFP and Cdc3-GFP in rga1Δ cells. 
In rga1Δ cells (HPY2204), Gic2-PBD-RFP localized continuously to (a) the proximal pole or (b) the distal pole from cytokinesis to the next G1 phase. Arrows in (a) & (b) denote the Cdc3 ring splitting and an arrowhead in (a) denotes Gic2-PBD-RFP enriched at the division site (as well as the bud tip).
Numbers indicate times (in min) from the first image. Size bars, 3 μm. (B) Localization patterns of Gic2-PBD-RFP (red) prior to, during, and after cytokinesis (Cdc3-GFP in green) is summarized from time-lapse imagings of wild type (n = 15), rga1Δ (n = 19), bud8Δ (n = 7) and rga1Δ bud8Δ (n = 8). The proximal-pole localization pattern (marked with 2*) of rga1Δ or rga1Δ bud8Δ daughter cells is different from those seen in wild type and bud8Δ cells (see text for details).
Figure 3.5. Localization of Gic2-PBD-RFP and Cdc3-GFP in the diploids homozygous for $bud8^\Delta rga1^\Delta$ and $bud8^\Delta$.

Imaging was performed as in Figure 3.4 except in $bud8^\Delta$ (HPY2370) and $bud8^\Delta rga1^\Delta$ (HPY2371) cells. Arrows denote the Cdc3 ring splitting and arrowheads denote Gic2-PBD-RFP enriched at the proximal pole. Note: Gic2-PBD-RFP became enriched at a site adjacent to the Cdc3 ring in the $bud8^\Delta$ daughter cell, whereas it appeared within the Cdc3 ring in $bud8^\Delta rga1^\Delta$ daughter cell. Numbers indicate times (in min) from the first image. Size bars, 3 μm.
Figure 3.6. Localization of Bud8 in large-budded cells of the wild-type and rga1Δ carrying YEpGFP-BUD8F. Representative images are shown for each pattern (A-D) and the percentage (mean ± SD) of each pattern is shown from three independent experiments (n = 160–230). Student’s t-test was performed to compare the distal-pole localization in wild-type and rga1Δ (P = 0.006).
Table 3.1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotypea</th>
<th>Source/comments</th>
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| YEF473*        | a/α  
  *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 | (Bi and Pringle, 1996) |
| YEF1233*       | a/α  
  rga1Δ::HIS3/rga1Δ::HIS3 | (Tong et al., 2007)  |
| YHH415*        | a/α  
  bud8-Δ1::TRP1/ bud8-Δ1::TRP1 | (Harkins et al., 2001) |
| HPY2353*       | a/α  
  CDC3-GFP::LEU2/CDC3-GFP::LEU2 GIC2-PBD-RFP::URA3/GIC2-PBD-RFP::URA3 | This study |
| HPY2204*       | a/α  
  rga1Δ::HIS3/rga1Δ::HIS3 CDC3-GFP::LEU2/CDC3-GFP::LEU2 GIC2-PBD-RFP::URA3/GIC2-PBD-RFP::URA3 | This study |
| HPY2370*       | a/α  
  bud8-Δ1::TRP1/bud8-Δ1::TRP1 CDC3-GFP::LEU2/CDC3-GFP::LEU2 GIC2-PBD-RFP::URA3/GIC2-PBD-RFP::URA3 | This study |
| HPY2371*       | a/α  
  rga1Δ::HIS3/rga1Δ::HIS3 bud8-Δ1::TRP1/bud8-Δ1::TRP1 CDC3-GFP::LEU2/CDC3-GFP::LEU2 GIC2-PBD-RFP::URA3/GIC2-PBD-RFP::URA3 | This study |
| HPY2385*       | a/α  
  rga1Δ::HIS3/rga1Δ::HIS3 bud8-Δ1::TRP1/bud8-Δ1::TRP1 | This study |
| HPY1680@       | a/α  
  his3-Δ1/ his3-Δ1 leu2Δ0/ leu2Δ0 met15Δ0/ met15Δ0 ura3Δ0/ ura3Δ0 | Diploid of BY4741 (Open Biosystems) |
| HPY2205@       | a/α  
  rga1Δ::kanMX4/rga1Δ::kanMX4 | This study |
| HPY2246@       | a/α  
  rga2Δ::kanMX4/rga2Δ::kanMX4 | This study |
| HPY2384@       | a/α  
  bem2Δ::kanMX4/bem2Δ::kanMX4 | This study |
| HPY2426@       | a/α  
  bem3Δ::kanMX4/bem3Δ::kanMX4 | This study |

aStrains marked with * are isogenic to YEF473 and strains marked with @ are isogenic to HPY1680 except as indicated.
CHAPTER 4: Overall Discussion and Future Directions

Cell polarity and cellular asymmetry are fundamental to a wide range of cellular processes in nearly all types of cells. In budding yeast, the generation of cell polarity is critical for asymmetric cell division, providing an excellent model system to investigate the underlying mechanisms of cell polarity at a single-cell level. This work has focused on spatial cue-mediated cell polarization of the budding yeast. The position and the timing of polarity axis determination were largely dependent on a Cdc42-GAP Rga1 in addition to spatial cues in both a and α haploid and a/α diploid yeast cells. The delicate analysis of time-lapse imaging provides critical findings on transient localization patterns of polarity proteins including Cdc42 and Rga1, and the role of Rsr1 in Cdc42 polarization, which ultimately determine the proper bud-site selection in budding yeast. Thus, this study provides a deeper understanding of regulatory signaling networks during yeast budding.

As discussed in Chapter 2, haploid cells choose a single site within the potential area in response to the axial landmark and selection of a bud site is dependent on a GTPase activating protein (GAP) for Cdc42, Rga1, which inhibits the activation of Cdc42 at an old bud site adjacent to the division site as well as at the division site.
Particularly, dynamic changes of Rga1 distribution at the bud neck and at the site adjacent to the bud neck are novel findings from this study; this time-dependent changes of Rga1 is crucial for proper bud site selection. Overall, this study emphasizes the significance of spatiotemporal control of Cdc42 polarization by upstream regulators in axial bud site selection.

As discussed in Chapter 3, selection of a bud site to the pole distal to the division site is coordinated by the Rga1-GAP activity at the cytokinesis site and by the distal pole marker, Bud8, in diploid daughter cells. Surprisingly, a large number of rga1 Δ diploid daughter cells exhibit Cdc42-GTP continuously polarizing to the distal tip until a new bud emerges at that site. This observation suggests that Rga1 may play a distinct role in connecting between bud morphogenesis and selection of a polarization axis.

Both chapters provide a number of mechanistic insights on the determination of cell polarity axis during yeast budding. Several aspects of the underlying mechanisms of cell polarization still remain unanswered and can be addressed in the future research as discussed below.

1) Selection of a single bud site in response to spatial cues

Previous studies have uncovered several key players involved in bud-site selection and polarity establishment. However, much less is known about how spatial cues communicate with downstream signaling molecules in order to establish a unique polarity axis.

In cells budding in the axial pattern, a single bud site is selected within potential
area dictated by the axial landmark, which forms a symmetric ring at the bud neck (Kang et al., 2012; Kang et al., 2013). Therefore, the mechanisms of preventing a new bud from forming at a previously-used cytokinesis site (i.e., bud-neck) partially explain the decision-making process for axial budding.

As discussed in Chapter 2, it has been shown that Rga1 and Rsr1 cooperate with Cdc42, thus act as additional layers of regulation in polarity determination. The transient Rga1 localization at the mother bud-neck, and at the site adjacent to the bud neck are critical for proper bud-site selection. How does Rga1 then localize to the older division site? It remains unclear how Rga1 remains associated with the site adjacent to the bud neck. It is interesting to speculate that landmark proteins recruit Rga1 to prevent re-budding at the old bud site. Interestingly, Rax1 and Rax2 localize to all division sites in mother and daughter cells, which persistently remain throughout multiple cell divisions in diploid a/α cells (Kang et al., 2004). Although the role of Rax1 and Rax2 remained unclear in the axial budding pattern, their localization patterns lead to the speculation that Rax1 or Rax2 might be involved in localizing Rga1 to the old adjacent bud site. Future study can be directed to test whether Rga1 localization to an old bud site is affected by the absence of RAX1 or RAX2. As Rga1 does not have transmembrane or membrane-binding domains, it is possible that Rax1 or Rax2 may provide an anchor for Rga1 localization at the old bud site. Whether Rga1 directly binds to Rax1/2 remains to be further investigated. If so, it will be interesting to test whether Rga1 interaction with the landmark proteins facilitates the Rga1-GAP activity in order to prevent the reactivation of Cdc42 at an old bud site. Interestingly, a recent study has shown that Nba1 inhibits
Cdc42 activation at older bud sites in Gps1 and Rax1/2-dependent manner (Meitinger et al., 2014). It has been suggested that Nba1 inhibits the Cdc24 association with Rsr1, and Nba1 was found to be the only Cdc42 inhibitor at older bud sites (Meitinger et al., 2014). Does Rga1 localize to all old division sites similar to Nba1? I found that a majority of large-budded cells exhibit the Rga1 localization to an old bud site next to the bud neck (occasionally at two or three old bud sites next to the bud neck; unpublished) in freshly growing cells. It may be possible to visualize Rga1 localization to several old bud sites in cells that have budded multiple times. However, it remains unclear whether Rga1 transiently localizes to all older bud sites. Rga1 may play a major role in preventing re-budding from the most recent old bud site, which is directly adjacent to the division site in addition to the immediately preceding division site. In fact, it may not be necessary to inhibit Cdc42 reactivation in all earlier bud sites because Cdc42 is activated by Bud3 only near the immediately preceding division site.

I also found that the intensity of GFP-Rga1 to a site adjacent to the neck diminishes during cytokinesis, and disappears in the next G1 phase (see Chapter 2). Currently, it remains unclear when Rga1 arrives to the old division site. Since I have not carefully examined the Rga1 localization in other phases of the cell cycle, future studies can be directed to examine the detailed Rga1 localization patterns throughout the cell cycle by long-term time-lapse imaging.

Rga1 also affects bud-site selection in diploid a/α cells, albeit in a less severe phenotype compared to haploid a and α cells (Tong et al., 2007; Lo et al., 2013). It remains possible that Rga1 also localizes to all previous division sites in diploid mother
cells and possesses the same inhibitory function as in haploid cells. Further investigation will be necessary to test this idea.

This study indicates that dynamic change of the polarity axis becomes stabilized at a single spatial position in mid G1, which is correlated with the temporally regulated G1 phase (See chapter 2) in both wild-type and rsr1Δ cells. What other factors contribute to the stabilization of a polarity axis other than the cell cycle control? During symmetry breaking, polarity clusters compete for the limiting scaffold protein Bem1, thus a single cluster wins, leading to the cell polarization at a single site (Howell et al., 2012). In WT cells, I found that daughter cells developed more than one Cdc42-GTP cluster in early G1 (see Chapter 2), and that clusters become resolved to a single Cdc42-GTP cluster in mid G1. In this time window, Cdc24 remains within the nucleus in haploid cells during early G1 (Shimada et al., 2004), thus the Bem1-mediated positive feedback mechanism involving Cdc24 may not be responsible for dynamic behavior of the Cdc42-GTP cluster in early G1 (i.e., competition mechanisms found in cells breaking symmetry). Whether other molecular players involved in the symmetry breaking system also play the same role even in the presence of spatial cues remains to be challenged for future studies. The final selection of a bud site from any potential positions, excluding an old bud site by Rga1, may still depend on a random event, relying on the self-organization properties of cell polarization. Since the initiation of polarity cluster is confined to near the landmark proteins in WT cells, it may be possible that competition among nearby clusters may occur faster (i.e., more effectively) than the competition among distant clusters in cells undergoing a ‘symmetry breaking’. However, a recent study indicates that the presence of
Rsr1 slows the initial Cdc42 polarization and competition (Wu et al., 2013).

Establishment of a robust and unique polarization axis may depend on other mechanisms involving actin-dependent processes, such as vesicle trafficking or the regulation of Cdc42 dynamics in relation to the plasma membrane. Formation of a stable Cdc42 cluster is maintained by recycling of Cdc42 molecules and also by rapid Cdc42 GTPase cycle, preventing fast lateral diffusion on the membrane (Wedlich-Soldner et al., 2004; Freisinger et al., 2013). Cdc42 recycling pathways were also found in the slow-acting endocytic pathway and fast-acting GDI pathway (Slaughter et al., 2009). It remains unclear whether the rate of Cdc42 recycling or cycling of the Cdc42 is different between T1 and T2 phases of the G1, which may affect the dynamics of Cdc42 polarization. It is possible that stochastic noise coming from vesicle traffic may be also important for generating dynamic behaviors of Cdc42.

Although the process of cell polarization involves membrane trafficking, opinions on the role of actin in the formation of a stable axis are highly controversial (Yamamoto et al., 2010; Johnson et al., 2011; Kozubowski et al., 2008; Irazoqui et al., 2005; Savage et al., 2012). However, it seems that a stable polarization axis is dependent on actin (Wedlich-Soldner et al., 2003; Ozbudak et al., 2005), and lateral diffusion of Cdc42 might be balanced by the actin cable-mediated delivery (exocytosis) and actin patch-mediated dispersal (endocytosis) of Cdc42 to the polarization site (Irazoqui et al., 2005). For future study, it may be worthwhile to test whether actin is involved in the stable formation of Cdc42-GTP cluster in T2 phase of G1 (after mid G1) similar to the case when unstable Cdc42 polar cap was observed by inhibiting the actin-mediated transport in a ‘symmetry-breaking’ system (Wedlich-Soldner et al., 2004). However, disrupting
actin would cause many indirect consequences by interfering with the overall polarization processes, which needs to be considered in future research.

The role of Rho GDP-dissociation inhibitor (GDI) in the maintenance of cell polarity has been thoroughly studied (Das et al., 2012a; Freisinger et al., 2013; Smith et al., 2013). Rdi1, the only known GDI, is critical for actin-independent polarization of Cdc42 (Freisinger et al., 2013; Smith et al., 2013). In fact, a correct polarity establishment is likely to be orchestrated through coupling of multiple feedback loops involving actin and GDI (Freisinger et al., 2013). Whether and how actin and GDI are involved in the robustness of Cdc42 polarization during early and late G1 phase remains to be fully answered.

All other mechanisms discussed above were not included in the mathematical modeling by collaborators, further investigation would be necessary to fully understand the formation of a single and stable polarity axis during cell polarization.

This study indicates that robust Cdc42-GTP accumulation by Bud3-GEF activity in early G1 involves the Rsr1 GTPase cycle (see chapter 2 & K. E. Miller; unpublished). It would be interesting to see whether and how Rsr1 plays a different role during early and late G1 phase. The total level of Cdc42-GTP in rsr1Δ cells is reduced in early G1, but not in late G1 (see chapter 2), suggesting that the Rsr1 GTPase cycle may be mainly involved in robust Cdc42-GTP polarization in early G1, presumably via a Bud3-mediated positive feedback mechanism.

Do other Cdc42 GAPs affect the dynamics of Cdc42 polarization? The dynamics of Cdc42-GTP polarization can be examined in cells lacking RGA2 or BEM3. However,
it is more likely that Rga1 has a specific role in bud-site selection and other GAPs, including Rga2 and Bem3, may play a significant role at bud emergence (see Chapter 1.5; Sopko et al., 2007; Knaus et al., 2007).

Interestingly, Rga1 has 15 potential phosphorylation sites (Holt et al., 2009). However, the functional significance of all Rga1 phosphorylation sites remains unknown. It is unclear whether phosphorylation of Rga1 has an inhibitory role of its GAP function similar to Rga2 and Bem3 (Sopko et al., 2007; Knaus et al., 2007) or activates Rga1-GAP function. It is unclear whether the level of Rga1 phosphorylation is cell-cycle dependent. One phosphorylation site (S291) of Rga1 seems to be dependent on cyclin-dependent kinase Cdk1 (Holt et al., 2009). It is possible that Cdk1 may act as an upstream regulator of Rga1, providing the time-dependent regulation of Rga1.

A transient and delayed negative feedback loop may operate during the T1 phase of G1 (i.e., in early G1) based on the mathematical modeling performed by Dr. Lo. Currently, it is not completely understood what molecules are involved in a negative feedback loop. It is also unclear what mechanisms dampen a negative feedback loop prior to the stabilization of Cdc42 polarization axis. It is highly possible that a transient negative feedback may involve Rga1 as an inhibitory regulator of Cdc42 polarization. Additionally, Cdc24 is unlikely to be involved in this negative feedback mechanism during T1 phase, because Cdc24 is sequestered in the nucleus in early G1 in haploid cells (Nern and Arkowitz, 2000; Shimada et al., 2000; Toenjes et al., 1999).

It will be interesting to test whether the Cdc42-activated signal transducing kinases, PAKs (p21-activated kinase) are also involved in phosphorylating Rga1 and also activating its catalytic activity. Negative feedback regulation of Cdc42 might occur
through the GAP activity of Rga1, which is stimulated by Cdc42-activated effector kinases, thus linking the negative regulation of Cdc42 to its own activation as expected for a negative-feedback loop.

To examine the consequence of Rga1 phosphorylation in dynamic behavior of the Cdc42 cluster, cells expressing PBD-RFP as a reporter for Cdc42-GTP in phosphorylation site mutants of Rga1 can be examined and observed to see whether the dynamic behavior of Cdc42 clusters is abolished. It may be possible that phosphorylation site mutants of Rga1 also affect the dynamic localization of Rga1 itself to the bud-neck similar to the regulation of septin dynamics by their phosphorylation states (Dobbelaere et al., 2003).

Additionally, it would be interesting to test whether Bud3-mediated Cdc42 polarization in early G1 also plays a role in bud-site selection of a/α diploid cells. It is likely that the assembly of the axial landmark and its link to the Rsr1 GTPase module are defective because the expression of AXL1 is absent in diploid a/α cells (Kang et al., 2012). Therefore, robust Cdc42 activation involving the Rsr1 GTPase module in early G1 (see Chapter 2) may not be present in diploid cells. It is noteworthy that Cdc24 remains in the cytoplasm as the expression of FAR1 is absent in diploid cells (Chang and Herskowitz, 1990). It is unclear whether Cdc24 remains constantly active in the cytoplasm. Does bud3Δ cells exhibit the same patterns of Cdc42 activation as in a/α diploid cells? Testing whether/how Bud3-GEF activity is distinctively regulated in a/α diploid cells would be interesting.
2) The role of Rga1 in proper bud-site selection.

In chapter 2, this study revealed that Rga1 undergoes drastic changes of its localization to the bud neck during M to the next G1 phase, which is crucial for proper bud-site selection. What could be the mechanism(s) governing the dynamic distribution of Rga1 localization? Many cytoplasmic proteins are distributed by the regulation of endocytosis and vesicle trafficking during polarity establishment, but the exact function of each protein has been a subject of great research interest (Engqvist-Goldstein and Drubin, 2003; Conner and Schmid, 2003).

None of previous studies have thoroughly investigated the localization of Rga1 in the vesicles and its delivery through actin cables. It remains possible that the delivery of Rga1 is mediated by secretory pathways similar to another Cdc42 GAP Bem3, which is targeted to the site of polarized growth by endocytic and recycling pathways (Mukherjee et al., 2013). It has been shown that epsin endocytic adaptors directly interact with all three yeast GAPs for Cdc42: Rga1, Rga2 and Bem3 (Aguilar et al., 2006). Thus, further investigation will be necessary to test whether and how Cdc42 regulators are involved in cellular trafficking processes. Future experiments can be directed to identify endocytosis mutants that exhibit the defective Rga1 localization to the site adjacent to the bud neck, or the dynamic localization of Rga1 to the bud neck. To confirm whether actin is involved in this process, the inhibition of actin polymerization by latrunculin A, which inhibits nucleotide exchange in an actin monomer, or deletion of formin, Bni1, will answer the involvement of actin polymerization in Rga1 localization. It remains possible that endocytosis-mediated dispersal of Rga1 may operate in the process of dynamic Rga1 localization.
When does Rga1 arrive to old division sites during the cell cycle? It remains unclear whether Rga1 localization at the old bud site is inherited from previous cell cycle or recruited from cytoplasmic pools by diffusion. It will be interesting to test whether the old Rga1 molecules are recycled and used again to form another ring for marking the subsequent division site.

Rga1 undergoes dynamic assembly and disassembly of its structure (see chapter 2). FRAP experiments may explain whether the uniform Rga1 ring prior to cytokinesis is less mobile compare to the stage when the distribution of Rga1 to the neck region is dynamically maintained. Depending on the cell-cycle stage, it is possible that Rga1 may exhibit different diffusion rates on the membrane or the cytosol-membrane exchange rate. In order to ensure that any Cdc42-GTP molecules that diffuse away from the cluster are inactivated by GAPs (Kozubowski et al., 2008), it may be possible that endocytosis and exocytosis optimize Rga1 localization on the membrane.

In Chapter 3, distinct dynamics of Cdc42 polarization by Rga1 was found by time-lapse microscopy in diploid a/α daughter cells. Interestingly, over 50% of diploid daughter cells lacking Rga1 exhibit continuous Cdc42-GTP polarization to the distal pole, suggesting the unique role of Rga1 in regulating the patterns of Cdc42 polarization. This was an unexpected observation because Cdc42 polarization is normally redirected to the mother-bud neck to promote cytokinesis, and then to the distal pole in wild-type daughter cells during late G2 and until the next G1 phase (Bi and Park, 2012). As evidenced by the elongated bud morphology of rga1Δ diploid cells (Smith et al., 2002; Chen et al., 1996; Stevenson et al., 1995; Watanabe et al., 2009), one possible reason could be due to the
delayed transition from the G2 to M phase in diploid daughter cells, resulting in more targeting of distal pole markers to the distal tip (see chapter 3.4 for discussion) as the apical growth and repolarization to the bud neck are critical for delivering spatial cues in order to position the correct division planes in diploid cells (Sheu et al., 2000).

It remains possible that Rga1 directly affects cell-cycle progression (i.e., core cell-cycle machinery), and thus affects the morphology of diploid cells because it remains obscure whether the defective morphologic consequence of rga1Δ is directly due to a mis-regulated apical growth phase or a delayed switch from apical to isotropic growth.

It is possible to speculate that the delayed apical to isotropic growth switch in rga1Δ cells may be due to the failure of CDK-inhibitory kinase Swe1 degradation (Sia et al. 1996; Sia et al. 1998). The negative genetic interaction (synthetic lethality or synthetic sick interactions) between Rga1 and Swe1 has been suggested by high-throughput studies (Costanzo et al., 2010, Sharifpoor et al., 2012). Swe1p localizes to the daughter side of the mother-bud neck (Longtine et al., 2000) upon its exit from the nucleus, which is required for Swe1 degradation in G2/M (Keaton et al., 2008). As Rga1 localizes to the mother-bud neck (Caviston et al., 2003), it remains possible that Rga1 might be involved in communication with the Swe1p degradation pathway. However, this hypothesis needs to be thoroughly tested.

It has been shown that the apical-isotropic switch is regulated by the translocation of phospholipids by flippase complex Lem3p-Dnf1/2p (Saito et al., 2007). This study showed that phospholipid asymmetry at the bud tip is altered and Cdc42-GAP activity is low in the absence of functional phospholipid flippases that translocate lipids across the
membrane, thus resulting in slow dispersal of Cdc42 from the bud tip and delay of the apical-isotropic switch. Overexpression of Rga1-GAP rescued elongated bud morphology of lem3Δ mutants, leading to the idea that lipid flipping regulates GAP activity in vivo. However, the localization of Rga1 at the bud tip was not clearly seen in previous reports and in this study (Caviston et al., 2003; Tong et al., 2007; see Chapter 2), contrary to the unpublished data described in Saito et al., 2007. Although I often detected the GFP-Rga1 in one or two puncta near the cortex (unpublished data), Rga1 mainly localizes to the mother-bud neck in addition to the site adjacent to the bud neck in large-budded cells. We also observed the high elevation of Cdc42-GTP to the bud tip of rga1Δ diploid and haploid cells, it remains unclear whether Rga1 indeed functions at the tip to depolarize Cdc42-GTP during the apical-isotropic growth switch as previously suggested (Saito et al., 2007).

Cells of the budding yeast Saccharomyces cerevisiae never choose a new bud site overlapping with previous bud sites in axially budding, bipolar budding, or in randomly budding (mutant) cells (Chant and Pringle, 1995). In earlier study, it was postulated that the chitin-rich structure of bud scars might not be amenable for new bud growth (Pringle et al., 1989). It remained possible that continuous budding within the same site affects the thickness of yeast cell wall, and thus cells become less resistant to environmental stresses.

Even though the bud-scar cue impedes a new budding at the same site, how do cells recognize such cue prior to the establishment of a new polarity axis? There are several mechanisms governing the prevention of Cdc42 reactivation (thus budding) within the old division site, including Rga1-mediated inactivation of Cdc42 and Gps1-
mediated negative polarity cues (Tong et al., 2007; Meitinger et al., 2013; Meitinger et al., 2014). Interestingly, the daughter-cell specific gene DSE1 prevents re-budding at the birth scar (Frydlová et al., 2009), suggesting the involvement of CWI pathway activation, which may govern non-overlapping budding events. Identifying other cell wall components that are involved in preventing the reuse of old bud sites would be interesting to pursue further.

Another fundamental question is what is the significance of preventing re-budding from old division sites in yeast? In naturally occurring yeast strains, daughter cells are attached to their mothers even after cytokinesis (for laboratory purposes, yeast strains were manipulated to make cells detach easily in order to minimize clumping), thus it would be impossible to re-bud at the same site if daughter cells remain at the division site (Tong et al., 2007). rga1Δ cells are capable of forming a new bud within a bud scar, but it does not affect cell viability (Tong et al., 2007). gps1Δ cells that bud inside old division sites resulted in daughter cell death by making cells more vulnerable to the defects in secondary septum biogenesis (Meitinger et al., 2013). It has been suggested that re-budding at the same site narrows the diameter of mother-bud neck, which may impede nuclear segregation or partitioning of organelles between mother and daughter cells, resulting in a shorter replicative life span of mutants including rga1Δ cells compare to WT cells (Meitinger et al., 2014). Therefore, preventing rebudding from previous division sites appears to be beneficial for cell survival.
APPENDIX A: The Rho1 GTPase acts together with a vacuolar glutathione S-conjugate transporter to protect yeast cells from oxidative stress.


A.1. Introduction

All aerobic organisms are constantly exposed to reactive oxygen species (ROS), which are byproducts of normal cellular metabolism and also generated upon exposure to environmental stresses. The abnormal production of ROS perturbs the cellular redox system, which triggers several defense mechanisms in order to avoid any detrimental cellular damages and even cell death (Moradas-Ferreira et al., 1996). Although ROS can regulate several intracellular signaling pathways for intended biological processes, oxidative stress occurs when cells cannot efficiently neutralize or eliminate the abnormal production of ROS (Finkel, 2003).

Several antioxidant activities and oxidative-stress protection systems have now been elucidated in the budding yeast S. cerevisiae (Finkel, 2003). Global gene expression profiles have identified many genes whose transcripts or protein levels are up-regulated or repressed in response to oxidants in yeast (Morgan et al., 1997; Godon et al., 1998; Lee et al., 1999; Gasch et al., 2000). Multiple genes involved in ROS resistance have
been reported by the genome-wide mutational analysis (Thorpe et al., 2004). Although these studies have provided some insights into the regulatory responses and the oxidative stress response regulons, it is obscure how these gene products function together in various cellular contexts to protect cells from damages. Surprisingly, most genes required for resistance to oxidative stress are not induced in response to oxidative stress (Thorpe et al., 2004). Thus how cells respond to and recover from oxidative stress remains largely unknown.

Rho1, a member of Rho family of GTPases is essential for cell survival in yeast, which participates several different signaling events including MAP kinase (MAPK)-pathway in response to various stresses (Harrison et al. 2004; Kamada et al. 1995). Rho1 activates Pkc1, a yeast homolog of mammalian protein kinase C, which activates a MAP kinase (MAPK) cascade composed of the Bck1-Mkk1/2-Mpk1 MAP kinase cascade in response to cell wall stresses (Lee and Levin, 1992; Kamada et al., 1995; Harrison et al., 2004). Rho1 activates the catalytic subunits of 1, 3-β-glucan synthase (GS) (encoded by FKS1 and GSC2/FSK2), thus promoting cell wall biogenesis (Drgonova et al., 1996; Qadota et al., 1996; Bi and Park, 2012).

The isolation and characterization of several conditional lethal mutants (high-temperature–sensitive (ts) mutations) of RHO1 from a previous study elucidated the distinct functional domains of Rho1—one group of rho1<sup>ts</sup> mutants including rho1-2 and rho1-5 is defective in activation of Pkc1, while another group including rho1-3 is defective in activation of glucan synthase (Saka et al., 2001).

Cells lacking Rom2, the GTP/GDP exchange factor (GEF) for Rho GTPases, exhibits hypersensitivity to oxidants, suggesting a possible involvement of Rho1 or other
GTPases in the oxidative stress response (Park et al., 2005; Vilella et al., 2005). Rho1 exhibits a two-hybrid interaction with Skn7 (Alberts et al., 1998), which is a stress response transcription factor that regulates numerous osmotic or oxidative stress response genes (Krems et al., 1996; Morgan et al., 1997). However, it remains unclear whether Rho1 or the cell integrity MAPK cascade is activated by oxidative stress.

Interestingly, Tus1, another Rho1 GEF, associates with Ycf1 (yeast cadmium factor) by a membrane two-hybrid analysis and co-immunoprecipitation (Paumi et al., 2007). Ycf1 is a yeast ATP-binding cassette transporter (ABC), which acts as a vacuolar glutathione S-conjugate transporter involved in detoxifying metals such as cadmium and arsenite (Li et al., 1997). Tus1 modulates Ycf1 transporter activity via the Rho1 GTPase (Paumi et al., 2007). Several studies have shown that metals induce oxidative stress in many cell types (Ercal et al., 2001; Valko et al., 2005). For example, cadmium induces oxidative stress by increasing ROS indirectly in S. cerevisiae and neurons (Brennan and Schiestl, 1996; López et al., 2006; Cuypers et al., 2010).

Although these previous studies suggested a possible link between Rho1 and Ycf1, several important questions remain to be answered. It is unclear whether Ycf1 is an upstream regulator of Rho1 or a downstream effector of Rho1. It is uncertain whether Tus1 activates Rho1 on the vacuolar membrane where Ycf1 localization was found (Wemmie and Moye-Rowley, 1997; Mason and Michaelis, 2002). Rho1 localizes to the plasma membrane and to other sites including bud tips, the mother-bud neck, and endomembranes (McCaffrey et al., 1991; Drgonova et al., 1996; Qadota et al., 1996; Yoshida et al., 2009). Tus1 localizes to the presumptive bud site in unbudded cells and to the mother-bud neck during cytokinesis (Yoshida et al., 2006; Kono et al., 2008), but has
not been observed on the vacuolar membrane.

To examine a potential role of Rho1 under oxidative stress, I investigated the connection between Rho1 and Ycf1 \textit{in vivo}. Here we show that Rho1 is necessary for the resistance to oxidants and that Rho1 associates with Ycf1 in a GTP-dependent manner. This study thus suggests that Rho1 regulates Ycf1 and other downstream targets to reduce intracellular ROS levels in a part of oxidative stress defense mechanism.

A.2. Materials and Methods

**Plasmids and yeast strains**

Standard methods of yeast genetics and recombinant DNA manipulation were used (Guthrie and Fink, 1991; Ausubel \textit{et al.}, 1999). Yeast cells were grown under standard growth conditions at 30°C unless otherwise indicated. Yeast strains used in this study are listed in Table A.1. Plasmids used in this study are listed in Table A.2

**Plasmids and Yeast Strains for BiFC**

To construct a strain expressing YCF1 tagged with the N-terminal fragment of Venus (VN) at the C-terminus, a DNA fragment carrying VN-kanMX6 was amplified by PCR using pFA6a-VN-KanMX6 (Sung and Huh, 2007) as template and primers oYCF11 (5’-TTGTCTTTACAGATHGCGGTGAGCTGGTTGTTGTAATGAAAATCGGATCCGCCGTTTAATTAA-3’) and oYCF12 (5’-CTACGTACCAGATTGTGCGGGACAGGTTTTTATTAGTTTCACAGTGAATTCGAGCTCGTTTAAAC-3’). The resulting PCR product was transformed into NY2284 by one-step-replacement method (Rothstein, 1991), yielding HPY1710. Correct targeting was confirmed by colony
PCR using primers oYCF13 (5'-AGCCGAGTTTGACTCTCCGGGCCAG-3') and oYCF14 (5'-GCACCTGTTCTCCGGAGAAATGTTG-3').

To express Rho1 fused to the C-terminal fragment of YFP (YFP<sup>C</sup>) at its N terminus, first, the 252 bp NotI fragment of YFP<sup>C</sup> generated from pRS304-YFP<sup>C</sup>-RSR<sub>1</sub><sup>K16N</sup> (pHP1678) (Kang et al., 2010) was cloned into the NotI site of pRS426*-RHO1 (pHP1697), yielding pRS426*-YFP<sup>C</sup>-RHO1 (pHP1737). To express YFP<sup>C</sup>-Rho1 from a CEN plasmid, the 2.1 kb HindIII-Xhol fragment (carrying YFP<sup>C</sup>-RHO1 sequence) from pHP1737 was cloned into pRS316 digested with HindIII and Xhol, yielding pRS316-YFP<sup>C</sup>-RHO1 (pHP1765).

The RHO<sub>1</sub><sup>Q68L</sup> and the RHO<sub>1</sub><sup>T24N</sup> mutations were generated by PCR-based site-directed mutagenesis using pHP1737 as template and primer pairs oRHO19 (5'-GCGCTATGGGATACCGCTGGTCTAGAAGATTATGAT AGACTAAG-3') and oRHO110 (5'-CTTAGTCTATCATAATCTTCTAGACCAGCGTGATATCCATAGCGC-3'); and oRHO111 (5'-GGTGATGGTGCTTGTAAGAATCTTATTAATCGTCTTTTCAAGGGC-3') and oRHO112 (5'-GCCCTTGGAAAAGAC GATTAATA AACAGTTCTTACCACAGGCCACCATCACC-3'), yielding pRS426-YFP<sup>C</sup>-RHO1<sup>Q68L</sup> (pHP1744) and pRS426-YFP<sup>C</sup>-RHO1<sup>T24N</sup> (pHP1745), respectively. The correct mutations were confirmed by DNA sequencing. To express RHO1<sup>Q68L</sup> and RHO1<sup>T24N</sup> from CEN plasmids, pRS316-YFP<sup>C</sup>-RHO1<sup>Q68L</sup> (pHP1766) and pRS316-YFP<sup>C</sup>-RHO1<sup>T24N</sup> (pHP1768) were generated from pHP1744 and pHP1745, respectively, as described above.

To construct TUS1 deletion in the strain HPY1710, PCR was performed using pFA6a-TRP1 (Longtine et al. 1998) as template and primer pairs, oTUS15 (5'-
CGAATATAAACATTTAAAAACAAAAACTTATTGAGTGCAAGCAAGTTAACCG
GGATCCCGGGTTAATTAA-3') and oTUS16 (5'-TTATATTATTACAACGG
ATATTTACCATTAAAAAGTGCTATATCTTATAGAATTCTGCCTGC
ATTTAAAC-3'). The resulting PCR product was used to delete the chromosomal TUS1 gene in HPY1710 by one-step gene disruption (Rothstein, 1991), yielding HPY1737. Correct targeting was confirmed by colony PCR using primer pairs, oTUS17 (5'-CATACTGACT
CGTCGATAGGCGG-3') and oTRP11 (5'-GTTCACCTGTCCACCTGCTTCTG-3').

**Construction of ycf1 deletion mutants and the YCF1-GFP strain**

To construct YCF1 deletion in the NY2284 background, a DNA fragment (2.08 kb) carrying ycf1Δ::KanMX4 was amplified by colony PCR using HPY1904 (an ycf1Δ::KanMX4 strain obtained from Open Biosystems) and primers, oYCF15 (5'-
CTCCTGGTGTGATGCTTGGGCGGTG-3') and oYCF14 (5'-GCACCTGTCTTC
CGGAGAAATGTTG-3'). The resulting PCR product was used to delete the chromosomal YCF1 gene in NY2284 and NY2287 by one-step gene disruption (Rothstein, 1991), yielding HPY1738 and HPY1739, respectively. Colony PCR was performed using primers oYCF14 and oKanC (5'-CGAGTGATTGGATGCAG
AGCGTAATGGCTGG-3') to confirm the correct deletion, which generated a 0.8-kb DNA fragment. The phenotype of ycf1Δ was confirmed by checking growth on a plate containing 30 M CdCl2.

To construct a strain expressing Ycf1 fused to GFP at its C terminus, a DNA fragment encoding GFP-TRP1 was amplified by PCR using pFA6a-GFP(S65T)-TRP1
(Longtine et al., 1998) as template and primers oYCF11 and oYCF12. The resulting PCR product was transformed into NY2284 by one-step-replacement method, yielding HPY1955. Correct targeting was confirmed by colony PCR using primers oYCF13 and oTRP11.

**Plate assays**

To test the sensitivity to H$_2$O$_2$, cells were diluted to OD$_{600} = 0.8$ and then treated with 2 or 3 mM H$_2$O$_2$ for 200 min before plating on YPD or SC plates as indicated. The sensitivity to various concentrations of H$_2$O$_2$ was tested by halo assays. First, cells from a mid-logarithmic phase culture were diluted to OD$_{600} = 0.2$. To make a lawn of cells, 200 µl of the diluted culture was spread on YPD or SC plates as indicated. Sterilized filter disks (Whatman filter paper) were placed on the plate and then soaked with 5 µl of H$_2$O$_2$ (concentrations ranging from 0.1 to 4 M). The plates were then incubated at 30°C for 1–2 days to monitor zones of growth inhibition around the filter disks. The laboratory wild-type strains exhibited varying degrees of sensitivity to H$_2$O$_2$ depending on the background: BY4741 was more sensitive to H$_2$O$_2$ compared with other wild-type strains tested (Figure A.9 B and C), as previously reported (Higgins et al. 2002; Singh et al. 2008). The sensitivity of the ycf1Δ and rho1-5 mutants to cadmium was tested by making fivefold serial dilutions starting from OD$_{600} = 2$, followed by plating on SC containing 30 µM CdCl$_2$. The plates were then incubated at room temperature for 3–7 days.
Fluorescence microscopy and bimolecular fluorescence complementation

Image acquisition of GFP–Rho1 was carried out essentially as previously described (Kang et al., 2001) using a Nikon E800 microscope (Nikon, Tokyo, Japan) fitted with a 100× oil-immersion objective (N.A. = 1.30), a Uniblitz electronic shutter, a Prior Z-axis drive, and a Hamamatsu Orca ER cooled charge-coupled device. A series of optical sections was captured at 0.3-µm intervals using Slidebook software (Intelligent Imaging Innovations, Denver, CO) by exposing for 1 sec. Cells were treated with 1–2 mM H$_2$O$_2$ for 2–4 hr or mock treated, where indicated, before imaging.

Bimolecular fluorescence complementation (BiFC) assays were performed as previously described (Singh et al. 2008; Kang et al. 2010) with slight modifications. Rho1 was fused to the C-terminal fragment of YFP (YFP$^C$) at its N terminus and was expressed from a CEN or 2μ plasmid (where indicated). Ycf1 was fused to the N-terminal fragment of Venus (VN), a variant YFP (Nagai et al. 2002), at its C terminus, and was expressed from its chromosomal locus.

To monitor BiFC signals, a single optical section was captured using the YFP filter by exposing cells to UV for 8 sec. Imaging and image processing were performed under identical conditions for all BiFC assays. Where indicated, the vacuolar membrane was visualized by staining cells with FM4-64 for 30 min at room temperature as previously described (Vida and Emr, 1995). Localization pattern and pixel intensity of the bimolecular fluorescent complex and Ycf1–GFP were analyzed by counting at least 100 cells per experiment from three independent experiments. Image analysis and processing were performed with ImageJ software, and the data are presented as mean ±
SD. Statistical significance was determined using Student's t-test.

A.3. Results

A.3.1. Cells of the rho1<sup>ts</sup> mutants are hypersensitive to various oxidants.

To determine whether Rho1 affects the cellular response under oxidative stress, I examined sensitivity of the rho1<sup>ts</sup> mutants, rho1-2, rho1-3, and rho1-5, to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which can be easily converted to the highly reactive hydroxyl radical by metal ions or exposure to UV (Valko et al., 2005). These rho1 mutants were also sensitivity to H<sub>2</sub>O<sub>2</sub> to different extents, with rho1-2 and rho1-5 being particularly hypersensitive to H<sub>2</sub>O<sub>2</sub> (Figure A.1). The sensitivity of the rho1<sup>ts</sup> mutants to paraquat, diethyl maleate (DEM) was also examined (Data provided by Dr. Singh). Paraquat is an agent which generates superoxide (Cochemé and Murphy, 2008), and DEM depletes glutathione, which acts as a thiol-specific oxidant (Nguyễn-Nhu and Knoops, 2002).Thus both drugs increase intracellular ROS levels. When serial dilutions of these rho1 mutants were spotted on rich plates containing paraquat or DEM at 30°, rho1-3 and rho1-5 exhibit hypersensitivity to these oxidants compared to wild type, while rho1-2 were little sensitive to these drugs (Figure A.1). Taken together, these results suggest that Rho1 is necessary for the resistance to oxidants.

A.3.2. rho1<sup>ts</sup> mutants exhibit high ROS accumulation.

To test whether the hypersensitivity of the rho1<sup>ts</sup> mutants to oxidants resulted from its specific defect in maintaining cellular redox balance rather than general sickness, the intracellular ROS level was indirectly examined using DHR, which becomes fluorescent rhodamine 123 upon oxidation (Experiments performed by Dr. Singh; Herker
et al. 2004). When these cells were examined by flow cytometry after adding DHR, a high level of ROS was present in the rho1 mutants even when they were grown at the semipermissive temperature of 30° and a higher percentage of cells with increased fluorescence upon shift to 37°, but not in wild-type cells (data not shown; data provided by Dr. Singh). When the rho1-5 mutant was examined under the fluorescence microscope, high fluorescence was observed in the cytoplasm at 30° and in an even higher percentage of the cells after exposure to H₂O₂ (data not shown; data provided by Dr. Singh). These results thus show that the ROS removal from the cytoplasm of the rho1ts mutants are not efficient compare to wild-type cells.

A.3.3. Rho1 interacts with Ycf1 in vivo.

The rho1-2 and rho1-5 mutants fail to activate Pkc1 (Saka et al., 2001) and these mutants were hypersensitive to H₂O₂, thus it is interesting to examine whether Rho1 is the upstream regulator of the Pkc1–MAPK pathway under oxidative stress. However, very mild sensitivity of the pkc1Δ, bck1Δ or mpk1/sl12A mutant (the downstream components of Pkc1) to H₂O₂ compare to the sensitivity of rho1 mutants (Data not shown; Data provided by Archana Shenoy and Dr. Singh) suggest that the Pkc1–MAPK pathway may play a minor role in recovery from oxidative stress.

Thus Rho1 might regulate another downstream target in response to the oxidative stress. Because Tus1 interacts with Ycf1 (Paumi et al. 2007), it is unclear whether Ycf1 might be a downstream target of Rho1. It is possible that the mis-regulation of Ycf1 in the rho1ts mutants might lead to increased ROS accumulation in the cytoplasm. To test whether Rho1 interacts with Ycf1 in vivo, Dr. Snider at the University of Toronto
performed an integrated split-ubiquitin membrane yeast two-hybrid (iMYTH) analysis (Snider et al., 2010). This assay indicates that Rho1 specifically interacts with Ycf1, confirmed by growth and β-galactosidase expression on SC plates lacking Trp, Ade, and His but containing X-Gal (Data not shown).

Next, I performed a BiFC assay to monitor the Rho1–Ycf1 interaction in vivo. This assay identifies protein-protein interactions in live cells by monitoring the formation of a YFP fluorescent complex, which is visible when truncated YFP fragments (YFP\(^N\) and YFP\(^C\)) are close together by interaction of the two proteins fused to each truncated YFP fragment (Hu et al., 2002). YFP\(^C\)–Rho1 was expressed from a low-copy plasmid in a strain expressing Ycf1–VN on the chromosome. Ycf1–VN and YFP\(^C\)–Rho1 were partially functional based on the complementation of cadmium sensitivity of ycf1Δ and H\(_2\)O\(_2\) sensitivity of rho1–5, respectively (Figure A.2). When these fusion proteins were co-expressed, the majority of cells showed a strong YFP signal on the vacuolar membrane (Figure A.3a). Some of these cells also showed one or two fluorescent puncta at the sites where two vacuolar lobes overlapped (see Figure A.4B for quantitation). In contrast, no cells exhibited detectable fluorescence in a control strain that co-expressed Ycf1–VN and YFP\(^C\) only (without a Rho1 fusion) (Figure A.3b). These results thus indicate that Rho1 interacts directly or closely associates with Ycf1 in vivo.

### A.3.4. Association of Rho1 with Ycf1 is likely to depend on its GTP-bound state.

Next, in order to determine whether Rho1 interacts with Ycf1 in a GTP-dependent manner, I imaged cells expressing the GTP- and GDP-locked state of Rho1, YFP\(^C\)–Rho1\(^{Q68L}\) and YFP\(^C\)–Rho1\(^{T24N}\), respectively, (Nonaka et al. 1995) together with the
YCF1–VN strain. Cells co-expressing YFP<sup>C</sup>–Rho1<sup>Q68L</sup> and Ycf1–VN exhibited BiFC signals (Figures A.4A), although less percentage of cells exhibit signals compare to the Ycf1 interaction with wild-type Rho1 (see Discussion). In contrast, cells co-expressing YFP<sup>C</sup>–Rho1<sup>T24N</sup> and Ycf1–VN showed little fluorescence (Figures A.4A). The Rho1<sup>Q68L</sup>–Ycf1 BiFC signal was often observed on the vacuolar membrane and in several puncta on the vacuolar membrane (see Figure A.4B for quantitation). The YFP signals in these cells appeared less discrete than those observed in the cells co-expressing YFP<sup>C</sup>–Rho1 and Ycf1–VN. This is likely due to the vacuolar shape in cells expressing YFP<sup>C</sup>–Rho1<sup>Q68L</sup>, as visualized by differential interference contrast (DIC) microscopy. Staining with FM4-64 also revealed different morphology of the vacuolar membrane in these cells (Figure A.5). Despite these differences, these data thus suggest that Rho1–GTP specifically interacts with Ycf1.

It is possible that the formation of the Rho1–Ycf1 complex would depend on Tus1, which converts Rho1 to the GTP-bound state. To test this idea, I performed BiFC assays in a strain deleted for TUS1. When the interaction between YFP<sup>C</sup>–Rho1 and Ycf1–VN was examined in the <i>tus1Δ</i> mutant, fewer cells indeed show a detectable BiFC signal (compare Figure A.4A, c to Figure A.3a). However, a significant percentage of <i>tus1Δ</i> cells still showed the Rho1–Ycf1 bimolecular fluorescent complex (Figure A.4B). When fluorescence of these cells with positive BiFC signals was compared, the mean pixel intensity of the vacuolar membrane was about the same in wild-type and <i>tus1Δ</i> cells (Figure A.4C). Consistent with the BiFC results, iMYTH analysis in a <i>tus1Δ</i> reporter strain indicated that Rho1 interacts with Ycf1 specifically even in the absence of TUS1 (Data not shown; Data provided by Dr. Snider). It is thus likely that another GEF (such as
Rom1 or Rom2) compensates for the loss of Tus1 in tus1A cells. The Rho1Q68L–Ycf1 bimolecular fluorescent complex was also observed in tus1A cells (Figure A.4A, d and 4.4B), and the total fluorescence intensity in individual cells was not statistically different between wild type and the tus1A mutant.

A.3.5. The Rho1–Ycf1 interaction may increase upon exposure to H2O2.

Since Ycf1 formed a bimolecular fluorescent complex with the GTP-locked Rho1 but not with the GDP-locked Rho1, Rho1 might be activated upon exposure to oxidants and thus form more Rho1–Ycf1 bimolecular fluorescent complex. To test the idea, I performed BiFC assays in cells co-expressing Ycf1–VN and YFPC–Rho1 after treatment with H2O2. While the BiFC signals appeared on the vacuolar membrane similar to those in untreated cells, more cells showed several puncta with stronger fluorescence on the vacuolar membrane (Figure A.6A, a). This is particularly evident in cells expressing YFPC–Rho1 from a multicopy plasmid after exposure to H2O2 (Figure A.6B, b and 4.6D). Quantification of fluorescence intensity of these cells indeed indicated that the pixel intensity of individual cells and in each punctum increased from 9.57 ± 5.4 to 15.13 ± 9.9 (in arbitrary units, a.u.; \(P = 0.0002\)) and from 0.64 ± 0.1 to 0.94 ± 0.5 (\(P = 0.002\)), respectively, after H2O2 treatment (Figure A.6C). Despite the cell-to-cell variation, these differences are statistically significant. Cells expressing Ycf1–VN and YFP\(^C\) fragment only (without the Rho1 fusion), however, did not show such signal after H2O2 treatment (Figure A.6A, b), suggesting that these dots represent the Rho1–Ycf1 bimolecular fluorescent complex rather than any other endogenous proteins that became fluorescent after H2O2 treatment. The fluorescence signal was occasionally observed in the vacuolar
lumen in some cells upon exposure to H2O2, which might result from mis-targeting of the bimolecular fluorescent complex under oxidative stress. Taken together, these results suggest that the interaction between Rho1 and Ycf1 increased after H2O2 exposure (see Discussion).

A.3.6. Localization of GFP–Rho1 remains similar, while the Ycf1–GFP level is elevated after exposure to H2O2.

Next question is to address whether localization of the Rho1–Ycf1 bimolecular fluorescent complex indeed indicates the sites at which these two proteins interact with each other in vivo and how localization of Rho1 and Ycf1 is affected upon exposure to H2O2. I thus examined localization of Rho1 before and after exposure to H2O2 using a strain, which expressed GFP–Rho1 under its native promoter from the chromosome. Expression of GFP–Rho1 in rho1ts mutants restored the resistance to H2O2, although less efficiently than wild type (compare Figure A.2 to Figure A.1), indicating that GFP–Rho1 was partially functional. GFP–Rho1 localized to the plasma membrane and to the sites of polarized growth as well as to the vacuolar membrane as expected (Figure A.7). This localization pattern of GFP–Rho1 remained similar after exposure to H2O2, although diffuse signals were also occasionally seen in the vacuolar lumen in some cells (Figure Figure A.7b). Thus, Rho1 is likely to interact with Ycf1 on the vacuolar membrane where the two proteins colocalize, and localization of GFP–Rho1 is mostly unaffected by H2O2.

We next examined localization of Ycf1–GFP, which was expressed from the YCF1 chromosomal locus. While Ycf1–GFP localized to the vacuolar membrane similarly before and after exposure to H2O2 (Figure A.8A), the mean pixel intensity of the
vacuolar membrane increased from 59.2 ± 18.8 to 71.6 ± 30.1 (in a.u.) after H₂O₂ treatment (Figure A.8B). This increase is statistically significant (P = 0.03), albeit rather heterogeneous among individual cells, suggesting that the Ycf1 level is elevated under oxidative stress.

**A.3.7. rho1Δ mutants are hypersensitive to cadmium, while an ycf1Δ mutant exhibits slight sensitivity to H₂O₂.**

On the basis of these findings described above, together with previous observations (Paumi *et al.*, 2007), we hypothesized that Rho1 activates Ycf1. If this was the case, we would expect a *rho1Δ* mutant to be hypersensitive to cadmium and an *ycf1Δ* mutant to be sensitive to H₂O₂. To test these predictions, I examined the sensitivity of the *rho1-2, rho1-3,* and *rho1-5* mutants to cadmium. Indeed, these *rho1* mutants were sensitive to CdCl₂ to different extents (Figure A.9A), and the pattern of the differential sensitivity was similar to those seen for paraquat and DEM (Figure A.1).

Next, I examined the H₂O₂ sensitivity of an *ycf1Δ* mutant in two strain backgrounds. An *ycf1Δ* mutant exhibited similar sensitivity to H₂O₂ compared to each isogenic wild-type strain (Figure A.9, B and C) and the mutants lacking other vacuolar ABC transporters, *ybt1Δ* and *bpt1Δ* (Figure A.9B). At relatively higher H₂O₂ concentrations, however, *ycf1Δ* was slightly more sensitive to H₂O₂ than wild type (Figure A.10). In addition, when the *rho1-5* and *ycf1Δ* mutations were combined, the double mutant was slightly more sensitive to H₂O₂ than *rho1-5* (Figure A.9C). Taken together, these observations thus suggest that Ycf1 contributes to resistance to both metals and oxidants, although loss of *YCF1* alone does not result in hypersensitivity to
H$_2$O$_2$. These results also indicate that other targets of Rho1 as well as Ycf1 are likely to
modulate cytoplasmic ROS level, since rho$^{1ts}$ was much more sensitive to H$_2$O$_2$ than
ycf1$^{\Delta}$ (see Discussion).

A.4. Discussion

Rho1 activates the “cell integrity” MAPK pathway in response to various stresses
(Levin, 2005), but it has been unclear whether Rho1 or any other component of the
MAPK pathway is also involved in the oxidative stress response. Although the Rho1
GEF, Tus1, interacts with Ycf1 (Paumi et al., 2007), it remained unclear whether Ycf1
functions upstream or as a target of Rho1. The studies reported here now clarify some of
these outstanding issues and uncover complex cellular response to oxidative stress.

Temperature-sensitive rho1 mutants were hypersensitive to oxidants and
exhibited an elevated level of ROS accumulation in the cytoplasm. A membrane two-
hybrid analysis and a fluorescence-based complementation assay demonstrate that Rho1
interacts with Ycf1 in vivo, likely dependent on its GTP-bound state (see below).
Together with the previous finding that Ycf1 activity depends on Rho1 (Paumi et al.,
2007), these findings thus suggest that Rho1 activates Ycf1 to regulate the redox balance
in the cell. Neither the ycf1$^{\Delta}$ nor the pkc1$^{ts}$ mutants, however, exhibited such
hypersensitivity to H$_2$O$_2$, suggesting that Rho1 regulates the oxidative stress response
probably through multiple downstream targets. We observed high cell-to-cell variation in
cellular response to oxidative stress, including the levels of Ycf1, the Rho1–Ycf1
bimolecular fluorescent complex, and ROS accumulation upon exposure to H$_2$O$_2$. This is
likely due to a different age and physiological state of individual cells. In fact, cellular
age in eukaryotes is a particularly well-known determinant of heterogeneous resistance to oxidative burden (Avery, 2006).

Cells expressing the GTP-locked Rho1\textsuperscript{Q68L} showed a positive BiFC signal, whereas cells expressing the GDP-locked Rho1\textsuperscript{T24N} did not, suggesting that Rho1–GTP interacts with Ycf1. It is thus likely that Ycf1 is a downstream target of Rho1. The localization pattern of the Rho1\textsuperscript{Q68L}–Ycf1 bimolecular fluorescent complex appeared different from that of Rho1, reflecting the different vacuole morphology in cells expressing Rho1\textsuperscript{Q68L} (Figure A.5). Indeed, Rho1 is also involved in vacuole membrane fusion (Eitzen et al., 2001; Logan et al., 2010). It might also correspond to the intrinsic difference between the GTP-locked Rho1 and the GTP-bound Rho1, which can cycle back to the GDP-bound state, with respect to their association with Ycf1. Although fewer cells exhibited BiFC signals with Rho1\textsuperscript{Q68L} than with the wild type, this is likely due to the sickness of cells expressing Rho1\textsuperscript{Q68L} (Nonaka et al. 1995), which might have caused loss of the YFP\textsuperscript{C}–Rho1\textsuperscript{Q68L} plasmid in some cells. Since Tus1 also interacts with Ycf1 (Paumi et al. 2007), Tus1 may facilitate the interaction between Rho1 and Ycf1 on the vacuolar membrane as well as the GDP–GTP exchange on Rho1. We were, however, unable to observe convincing Tus1 localization to the vacuolar membrane before or after exposure to H\textsubscript{2}O\textsubscript{2}, likely due to transient localization or a very weak signal of Tus1–GFP. Rho1 still interacted with Ycf1 in \textit{tus1}\textDelta cells, albeit less efficiently, suggesting that another Rho1 GEF substitutes Tus1 function in a \textit{tus1}\textDelta mutant.

The Rho1–Ycf1 bimolecular fluorescent complex was observed on the vacuolar membrane and occasionally as one or two dots on the vacuolar membrane. Although the exact nature of these puncta remains unclear, both patterns of the BiFC signals were
dependent on Rho1 and Ycf1. Interestingly, the number of these puncta on the vacuolar membrane and their pixel intensity increased after exposure to H$_2$O$_2$, suggesting an increased interaction between Rho1 and Ycf1 upon exposure to H$_2$O$_2$. This might be due to the activation of Rho1 as well as elevation of the Ycf1 protein level upon exposure to H$_2$O$_2$ (Figure A.8), consistent with the fact that Yap1 regulates the expression of YCF1 (Sharma et al., 2002). It is also possible that these puncta reflect the coalescence of the Rho1–Ycf1 bimolecular fluorescent complexes after exposure to H$_2$O$_2$. Ycf1–GFP also appeared as one or two dots on the vacuolar membrane, which are thought to be multivesicular bodies (MVBs) (C. M. Paumi, unpublished observation), in addition to the vacuolar membrane, but these puncta did not particularly increase upon exposure to H$_2$O$_2$ (Figure A.8).

While the interaction between Rho1 and Ycf1 is clear from this study, Ycf1 is unlikely to be the sole Rho1 effector involved in the oxidative stress response. Cells lacking YCF1 exhibited little (or slight) hypersensitivity to hydrogen peroxide depending on H$_2$O$_2$ concentration. This could be due to the functional redundancy of other vacuolar membrane-residing transporters such as Ybt1 and Bpt1. However, none of the double or triple mutants of the vacuolar transporters was as sensitive as the rho1$^{ts}$ mutants to H$_2$O$_2$ (M.-E. Lee, C. M. Paumi, and H.-O. Park, unpublished observation). Despite the lack of clear sensitivity of ycf1Δ to oxidants, a couple of observations suggest that the Rho1–Ycf1 interaction is significant to confer resistance to both metals and oxidants. The differential sensitivity of the rho1$^{ts}$ mutants to paraquat and DEM is correlated with their sensitivity to cadmium (Figures A.1 and A.9A), which is well established as an inducer of oxidative stress in various cell types including yeast (Brennan and Schiestl, 1996). An
ycf1 deletion confers an increased sensitivity of a rho1ts mutant to H2O2 (Figure A.9C). The unique response of each rho1ts mutant to various oxidants also suggests that the hypersensitivity to oxidants is unlikely due to the general sickness of the rho1 mutants. This observation is consistent with the idea that different oxidants may trigger cellular responses by distinct mechanisms, as previously suggested (Thorpe et al., 2004).

Hydrogen peroxide is an uncharged species (unlike superoxide, O2−) that penetrates membranes freely (Imlay, 2008). While other oxidants such as diamide may affect the cell wall, H2O2 seems to affect the intracellular function (Vilella et al., 2005). It was found that the rho1-2 and rho1-5 mutants, which are specifically defective in Pkc1 activation (Saka et al., 2001), were particularly hypersensitive to H2O2, but their sensitivities to other oxidants were opposite. Thus their hypersensitivity to H2O2 could be due in part to the defect in Pkc1 activation, but the role of the Pkc1–MAPK pathway in response to other oxidants seems less clear.

The bck1Δ and mpk1Δ/slt2Δ mutants as examined here were mildly sensitive to H2O2. This is consistent with a previous report (Staleva et al., 2004), but differs from another study, which indicated that the bck1Δ and mpk1Δ mutants were not sensitive to H2O2 and diamide (Vilella et al., 2005). None of the pkc1 mutants that we tested exhibited such severe sensitivity to H2O2, unlike the report by Vilella et al., (2005). This discrepancy might be due to the different PKC1 alleles and the strain background. It is thus not clear whether the Pkc1–MAPK cascade plays a role under oxidative stress. The bifunctional transcription factor Skn7 might also be involved in the Rho1-mediated oxidative stress response (Alberts et al., 1998). Further investigation will be required to fully understand the mechanism by which Rho1 regulates the oxidative stress response.
In this study, we found that Rho1 is necessary for survival under oxidative stress. In contrast, Rho5 is necessary for cell death under excessive oxidative stress (Singh et al., 2008). Thus, despite the similar structure of these Rho GTPases, Rho1 and Rho5 seem to play opposite roles under oxidative stress. Cells may use an alternative program to promote either survival or death depending on the level of stress or cellular damage. It remains uncertain how cell fate is determined under different levels of oxidative stress. Although the details of the mechanism remain unknown, these findings suggest that Rho1 may regulate Ycf1 to get rid of heavy metals or other xenobiotics from the cytoplasm, and thus help yeast cells recover from oxidative stress. Because both Rho1 and Ycf1 belong to highly conserved families of proteins, Rho GTPases might also be involved in regulation of an ABC transporter in mammals, which brings us one step closer to unraveling the conserved mechanism of the GTPases-mediated stress defense in eukaryotes.
Figure A. 1. The rho1ts mutants are hypersensitive to oxidants. Five-fold serial dilutions (from left to right) of wild-type (NY2284), rho1-2 (NY2285), rho1-3 (NY2286), and rho1-5 (NY2287) cells were grown at 30°C for 2–4 days on YPD after treating with 3 mM H2O2 for 200 min or mock treatment. These mutants were also tested on YPD containing 400 mg/ml of paraquat or 1 mM DEM (Data provided by Dr. Singh).
Figure A. 2. Partial complementation of Ycf1 and Rho1 fusion proteins.

(A) YCF1-VN partially complemented the hypersensitivity of an ycf1Δ mutant to cadmium. Serial dilutions of WT (NY2284), YCF1-VN (HPY1710) and ycf1Δ (HPY1738) were plated in SC plate without or with 30µM CdCl$_2$, and then incubated for 4 days at room temperature.

(B) YFP$^C$-RHO1 expressed in a low copy plasmid partially complemented the hypersensitivity of rho1-5 to H$_2$O$_2$.

(C) GFP-Rho1 partially complemented the hypersensitivity of the rho1ts mutants to oxidants. Serial dilution of WT (HPY1574), rho1-2 (HPY1730), rho1-3 (HPY1731), and rho1-5 (HPY1732) mutants expressing GFP-Rho1 from the chromosome were treated with 3mM H$_2$O$_2$ for 200min, and plated on YPD and incubated for 3 days at 30°C.
Figure A. 3. The *in vivo* interaction between Rho1 and Ycf1.

BiFC assays were performed in wild-type cells (HPY1710), which express Ycf1–VN from its chromosomal locus and carry pRS316–YFP<sup>C</sup>–RHO1 (a) or YCp50–YFP<sup>C</sup> (b). Cells were grown in SC-Ura at 30°C. Images were captured with the for 8-sec exposures under YFP filter. Fluorescent images (YFP), DIC images (DIC), and fluorescent images overlaid with the DIC images (overlay) are shown for the representative cells. Bars, 5 µm.

See Figure B.4B for quantitation of the localization pattern of the Rho1–Ycf1 bimolecular fluorescent complex.
Figure A. 4. Rho1 interaction with Ycf1 is dependent on the GTP-bound state of Rho1 in vivo.
(A) BiFC assays were performed in the YCF1–VN strain (HPY1710), which carries (a) pRS316–YFP<sup>C</sup>–RHO1<sup>Q68L</sup> or (b) pRS316–YFP<sup>C</sup>–RHO1<sup>T24N</sup>, and the YCF1–VN tus1<sup>Δ</sup> strain (HPY1737), which carries (c) pRS316–YFP<sup>C</sup>–RHO1 or (d) pRS316–YFP<sup>C</sup>–RHO1<sup>Q68L</sup>. Cells were grown in SC-Ura at 30°C. Images were captured, processed, and presented as in Figure B.3. Bar, 5 μm.
(B) Localization pattern of the Rho1–Ycf1 bimolecular fluorescent complex: the vacuolar membrane (vm) only; the vm and a punctum on the vm; the vm and a few puncta on the vm; and others, which are mixed patterns with diffuse signal often enriched in the vacuole. Localization pattern of bimolecular fluorescent complex was quantitated from three independent experiments (n = 300–400), and mean (%) ± SD are shown.
(C, left) Mean pixel intensity of the vacuolar membrane of each individual cell was plotted and quantified using ImageJ software: WT (HPY1710 with pRS316–YFP<sup>C</sup>–RHO1), 18.7±5.0 (in arbitrary units, a.u.) and tus1<sup>Δ</sup> (HPY1737 with pRS316–YFP<sup>C</sup>–RHO1), 19.5±6.3 (in a.u.) (P = 0.6). (Right) Fluorescence intensity of each individual cell was analyzed: WT (HPY1710 with pRS316–YFP<sup>C</sup>–RHO1<sup>Q68L</sup>), 16.9±10.9 (in a.u.) and tus1<sup>Δ</sup> (HPY1737 with pRS316–YFP<sup>C</sup>–RHO1<sup>Q68L</sup>), 13.5±9.4 (in a.u.) (P = 0.1).
Figure A. 5. Localization of Rho1-Ycf1 and Rho1^{Q68L}-Ycf1 bimolecular YFP complex in cells stained with FM4-64.
BiFC complex was formed in WT (HPY1710), which co-expressed Ycf1-VN from the chromosome and (a) YFP<sup>C</sup>-Rho1 or (b) YFP<sup>C</sup>-Rho1^{Q68L} from a plasmid. Cells were grown in SC-URA at 30°C. Images were captured with the YFP filter for 8 sec exposure and TRITC filter for 200msec. Size bar 5µm.
The bimolecular fluorescent complex formation from Rho1 interaction with Ycf1 after exposure to H$_2$O$_2$.

(A) BiFC assays were performed in the YCF1–VN strain (HPY1710), carrying (a) pRS316–YFP$^C$–RHO1 or (b) YCp–YFP$^C$ (pHP1730). Cells were grown in SC-Ura at 30°C and incubated with 2 mM H$_2$O$_2$ for 2 hrs before imaging. Images were captured, analyzed, and presented as in Figure B.4. Bar, 5 μm.

(B) BiFC assays were performed in HPY1710, carrying pRS426–YFP$^C$–RHO1. Cells were grown in SC-Ura at 30°C and incubated with 2 mM H$_2$O$_2$ for 4 hr (+H$_2$O$_2$) or mock treated (no oxidant) before imaging.

(C, left) Fluorescence intensity of individual cells of HPY1710 with pRS426–YFP$^C$–RHO1 was plotted and quantified using ImageJ software: pixel intensity in untreated cells, 9.57 ± 5.4 (in a.u.) and in cells treated with 2 mM H$_2$O$_2$ for 4 hrs, 15.1 ± 9.9 (in a.u.) (P = 0.0002).

(C, right) Fluorescence intensity of each punctum in HPY1710 with pRS426–YFP$^C$–RHO1 was analyzed similarly: pixel intensity in untreated cells, 0.64 ± 0.1 (in a.u.) and in cells treated with 2 mM H$_2$O$_2$ for 4 hr, 0.94 ± 0.5 (in a.u.) (P = 0.002).

(D) Localization pattern of the Rho1–Ycf1 bimolecular fluorescent complex was analyzed as in Figure B.4B from strains HPY1710 with pRS316–YFP$^C$–RHO1, YCp–YFP$^C$, or pRS426–YFP$^C$–RHO1 after treatment with H$_2$O$_2$ for 4 hrs and HPY1710 with pRS426–YFP$^C$–RHO1 after mock treatment. Data are from three independent experiments (n = 300–400), and mean (%) ± SD are shown.
Figure A.7. Localization of GFP–Rho1 before and after H$_2$O$_2$ treatment. GFP-Rho1 expressed from the chromosome was examined in wild-type cells (HPY1574), grown in SC-Ura at 30°, (A) before and (B) after exposure to 2 mM H$_2$O$_2$ for 2 hrs. A series of Z sections was captured with the GFP filter and a single, representative Z section is shown. Bar, 5 μm. (experiments done in collaboration with Dr. Komudi Singh).
Figure A.8. Ycf1 localization after exposure to H$_2$O$_2$.
(A) Localization of Ycf1–GFP was examined in the YCF1–GFP strain (HPY1955), grown in SC-Trp at 30°C, before and after exposure to 2 mM H$_2$O$_2$ for 2 hrs. A series of Z sections was captured with the GFP filter and a single, representative Z section is shown.
(B) Fluorescence intensity of the vacuolar membrane was plotted and quantified using ImageJ software: pixel intensity in untreated cells, 59.2 ±18.8 (in a.u.); and in cells treated with H$_2$O$_2$, 71.6 ± 30.1 (in a.u.) (P = 0.03).
Figure A. 9. rho1Δ mutants are hypersensitive to cadmium while ycf1Δ mutant are slightly sensitive to H2O2.

(A) Fivefold serial dilutions (from left to right, starting from OD600 = 2) of wild-type (NY2284), ycf1Δ (HPY1738), rho1-5 (NY2287), rho1-2 (NY2285), and rho1-3 (NY2286) strains, all of which are in the isogenic background, were grown on SC or SC plate containing 30 mM CdCl2 at room temperature for 4 days (-) or 7 days (+30 mM CdCl2).

(B) Fivefold serial dilutions (from left to right, starting from OD600= 1) of wild type (BY4741) and isogenic deletion mutants of vacuolar transporters (ycf1Δ, ybt1Δ, and bpt1Δ) were treated with 2 mM H2O2 or mock treated, spotted on SC plates, and incubated at 30 °C for 2 days (experiments done in collaboration with Dr. Komudi Singh).

(C) Fivefold serial dilutions (from left to right, starting from OD600=1) of wild type (NY2284), ycf1Δ (HPY1738), rho1-5 (NY2287), and rho1-5 ycf1Δ (HPY1739) were treated as described above.
Figure A.10. Sensitivity of the vacuolar transporter mutants to H$_2$O$_2$.
H$_2$O$_2$ sensitivity of wild type (BY4741) and deletion mutants of vacuolar transports ($ycf1\Delta$, $ybt1\Delta$ and $bpt1\Delta$) tested by halo assays on SC plates with filter papers soaked with H$_2$O$_2$ solution as indicated. The plates were incubated at 30°C for 2 days.
(Experiments done in collaboration with Dr. Komudi Singh).
Table A.1. Yeast strains used in this study

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<th>Strain</th>
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a Strains marked with * are isogenic to NY2284, except as indicated; strains marked with @ are isogenic to BY4741, except as indicated.

*Strain constructed by Dr. Komudi Singh

^Strains constructed by Dr. Chan-Hun Jung
Table A.2. Plasmids used in this study

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#Plasmid constructed by Dr. Komudi Singh
APPENDIX B: Bimolecular Fluorescence Complementation (BiFC) Validation of yeast ABC transporter interactome


B.1. Introduction

ATP-binding cassette (ABC) transporters are multidomain integral membrane proteins that are highly conserved from yeast to human (Jones and George, 2004). ABC transporters typically utilize the energy of ATP hydrolysis to translocate a diverse range of molecules such as ions and xenobiotics across cellular membranes (Jones et al., 2009). These transporters are crucial for maintaining cellular homeostasis via several processes such as the export of drugs or metabolites, and nutrient uptake (Jones and George, 2004). Defects in ABC transporter genes are implicated in various inherited human diseases (reviewed in Dean and Annilo et al., 2005; Gottesman et al., 2001). Overexpression of ABC transporters also show a role in the drug resistance of cancer cells or pathogenic microorganisms (Cannon et al., 2009; Szakács et al., 2006). Budding yeast encodes 22 membrane proteins, which are homologous to ABC proteins found in human that are
linked to human disease (Paumi et al., 2009; Kovalchuk and Driessen, 2010). The study
of ABC transporters in yeast is therefore of huge clinical interests. However, despite
considerable research to date, their function and regulation remain still unclear.

One of powerful approaches to study protein function is by generating protein
interaction maps (interactomes), which provide a broad perspective of cellular processes
at the functional and regulatory mechanisms within the cell (Snider et al., 2013). Dr. Igor
Stagljar (University of Toronto, Toronto, CA) sets out to map the interactome of the
members of ABC superfamily using membrane yeast two-hybrid (MYTH) technology in
the budding yeast S. cerevisiae. This system is based on the split-ubiquitin assay, which
allows us to confirm the protein-protein interactions between insoluble proteins. Briefly,
two proteins of interests are fused to either a C-terminal ubiquitin (Cub), which is also
fused to a transcription factor (TF) or an N-terminal ubiquitin (Nub). Once a stable
ubiquitin complex forms by bait-prey interaction, this ubiquitin molecule is subjected to
the target of ubiquitin specific proteases, which cut off the transcription factor that moves
to the nucleus to turn on the reporter genes (Snider et al., 2010). Unlike the conventional
two-hybrid assay that requires the localization of fusion proteins within the nucleus, this
assay identifies interactions of full-length membrane proteins in their natural cellular
environment (Stagljar et al., 1998; Snider et al., 2010). As a part of their project, I
performed the Bimolecular Fluorescence Complementation (BiFC) experiment to
validate the positive interactions identified from MYTH results. Among 22 ABC
transporters, all 19 ABC transporters except the three inner mitochondrial membrane
proteins encoded by the budding yeast genome were mapped for their interactions with
their putative interactors. Particularly, BiFC assay helped to suggest the involvement of
ABC transporters in zinc homeostasis and osmotic stress response pathway. To sum up, several novel interactors of ABC transporters confirmed by a BiFC assay in this study will provide new insights into the cellular role and regulation of clinically relevant membrane transporters, which improved our current understanding of these proteins.

B.2. Materials and methods

**Plasmids and strains**

Standard methods of yeast genetics and recombinant DNA manipulation were used (Guthrie and Fink, 1991; Ausubel *et al.*, 1999). Yeast cells were grown under standard growth conditions at 30°C unless otherwise indicated. Plasmids pFA6a-VN-KanMX6 and pFA6a-VC-KanMX6 (Sung and Huh, 2007) were used to construct strains expressing VN or VC fusion proteins, respectively (Snider *et al.*, 2013). See the complete genotype of each strain in Table B.1.

**BiFC analysis**

BiFC analyses were carried out essentially as previously described (Sung and Huh, 2007). Briefly, selected ABC transporter genes were endogenously tagged in strain BY4742 at either their 5’ or 3’ end with sequence encoding the C-terminal (‘VC’) fragment of YFP Venus (Sung and Huh, 2007), while interactor genes were endogenously tagged in strain BY4741 at their 5’ or 3’ end with sequence encoding the N-terminal (‘VN’) fragment of YFP Venus. BY4741 and BY4742 strains were mated and resultant diploids examined for YFP signal by fluorescence microscopy. Image acquisition and processing were performed using Slidebook software (3i), as previously
described (Lee et al., 2011).

For strains with relatively weak BiFC signals, which is likely due to low expression level of particular proteins or longer distance between two proteins, I repeated imaging using a spinning disk confocal microscope (UltraView VoX; PerkinElmer Life and Analytical Sciences, Waltham, MA) equipped with a 100 × oil NA 1.4 Plan-Apo Nikon objective lens, the EM CCD ImagEM (Back-Thinned Electron Multiplier CCD Camera), and a solid state 514-nm diode laser. A single optical section was captured for 1 s exposure using 20% laser power (sensitivity 171) for a BiFC assay. To determine the localization of VC-PDR10/VN-ZRC1 interaction, the BiFC signal was captured in the presence of markers directed to the plasma membrane (pRS415-PMET-CFP-Cdc42) and endoplasmic reticulum (Sec63-mCherry). Image capture and processing were performed using the Volocity software package (PerkinElmer) and ImageJ software.

**Localization of BiFC complex at peroxisome**

To confirm BiFC results for Pxa1 and Pxa2 baits, the media containing an oleic acid (Sigma-Aldrich) was used to induce greater numbers of peroxisomes. Briefly, cells were grown in 2% raffinose containing SC-TRP media and then, freshly grown cells with the same media were pelleted and re-suspended into fresh media containing 0.1% raffinose, 0.15% oleic acid, 0.2% tween-40 detergent and then, grown for 16-18 hrs prior to observing on the microscope. To determine the localization of BiFC interaction at peroxisomes, the BiFC signal was captured in the presence of markers directed to the peroxisome (pRS415-Pts1-DsRed). Image capture and processing were performed using Slidebook software (3i), and ImageJ software as previously described (Lee et al., 2011).
Images were deconvolved, which improves the signal-to-noise ratio, using Slidebook software (3i).

B.3. Results

B.3.1. BiFC analysis of selected protein interactions identified by MYTH

The final data set from the group of Dr. Igor Stagljar (University of Toronto, Toronto, CA) revealed 285 unique interactions across 209 proteins (see details in Snider et al., 2013). As a secondary validation of MYTH results, I performed the Bimolecular Fluorescence Complementation (BiFC) assay as an orthogonal test to confirm the parts of interactions (Hu et al., 2002; Sung and Huh, 2007). This approach is based on the complementation between two nonfluorescent fragments of the yellow fluorescent protein (YFP), which gives YFP signals when proteins fused to each YFP fragment is brought together by bait-prey interactions. For BiFC screening, proteins of interests are endogenously tagged at their N or C termini using the C-terminal half of Venus (VC), a variant YFP (Nagai et al. 2002), in yeast strain BY4742 (for ABC transporters) or the N-terminal half of Venus (VN) in the opposite mating type of yeast strain BY4741 (for interactors). Corresponding strains were mated, and I examined resultant diploids by fluorescence microscopy to identify cells producing a YFP signal, indicating interaction of the constructs (Figure B.1). Of the 79 interactions tested, 44 (55.6%) produced a positive BiFC signal (See Figs. B.2A-F). The 35 interaction pairs that could not be confirmed by BiFC, 20 were confirmed by using co-immunoprecipitation (Co-IP) analysis by Dr. Stagljar’s group (Snider et al., 2013).

The results of these two assays resulted in an overall confirmation rate of 81.0%
(Figure B.4). The failure to confirm a subset of the tested interactions may have resulted from altered expression and steric issues, as MYTH allows detection of both prey fragments and full-length preys, whereas the BiFC and Co-IP approaches use full-length preys exclusively. Overall, orthogonal testing confirms a notably high percentage of MYTH interactions, providing excellent support for the quality of the ABC interactome.

**B.3.2. Revealing the subcellular localization of BiFC complex and its implication**

A bimolecular fluorescence complementation (BiFC) assay allows us to determine the locations of protein interactions in living cells (Kerppola et al., 2006). In BiFC analysis, most interactions were determined whether there is any positive interaction. Where these bimolecular fluorescent complexes exist remain inconclusive, although most interactions are likely to be on the vacuolar or plasma membrane.

However, a subset of BiFC interactions were thoroughly examined for their intracellular localization as the location of protein-protein interactions of two proteins is of fundamental importance to their proper function in cells. One of notable observation from the screening is the association of several ABC transporters with two major yeast zinc transport proteins, Zrc1p (a vacuolar zinc uptake transporter responsible for zinc storage and detoxification) and Zrt1p (a plasma membrane localized, high-affinity zinc uptake transporter induced under conditions of zinc limitation (Zhao et al., 1996; MacDiarmid et al., 2002). I successfully validated the interaction between Pdr10p and Zrc1p using BiFC assay, observing a punctate signal located mainly around the cellular periphery (Figure B.5). This signal co-localized with CFP-tagged Cdc42p (which localizes to plasma, vacuolar and nuclear membranes; Figure B.5a), whereas signal
produced by mCherry-tagged Sec63p (which localizes to the endoplasmic reticulum), was more discretely separated (Supplementary Figure B.5b), concluding that the signal appears in the plasma membrane rather than the cortical endoplasmic reticulum, although I also occasionally observed a weak BiFC signal on the vacuolar membrane. These results suggest that the Pdr10p-Zrc1p interaction occurs on the plasma membrane, with a small subpopulation of (potentially irreversible) BiFC complex traveling to the vacuole, although the possibility of complex forming in the vacuole and moving to the plasma membrane cannot be definitively ruled out. Determining the exact nature and functional significance of this interaction will require further investigation.

For testing BiFC complex formation with Pxa1 and Pxa2 (Subunits of peroxisomal ABC transport complex), cells were grown in an oleic acid containing media (see Materials and methods for details), which induces the increased peroxisome levels in cells, because the peroxisome-related BiFC interactions were not detected in the initial screening, presumably due to the low peroxisome levels. A significant part of protein interactions were validated by BiFC assay (Figure B.3). In peroxisome inducing conditions, I often found cells with high vacuolar lumen signals, and such background signals were observed with non-peroxisome proteins in cells grown in the peroxisome-inducing media (Data not shown). The similar vacuolar luminal signal was detected by a previous study, suggesting that these are background signals coming from media (van der Zand A et al., 2012).

By BiFC screening, I found that Sho1 interacts with Fps1, but not with Bpt1 (negative control) (Figure B.6). Strains containing the VC tagged Sho1p (at either terminus) and VN tagged Fps1p showed intense fluorescence at the plasma membrane,
while a strain containing an unrelated protein, Bpt1, in combination with tagged Fps1p showed no fluorescence (Figure B.6). Sho1 is an intergral membrane protein which plays a critical role in the High Osmolarity Glycerol (HOG) MAPK pathway in yeast (O’Rourke and Herskowitz, 2004). Under high osmotic stress, Sho1 is activated and interacted with other HOG pathway proteins to execute cell signaling events to prevent the damages and remain viable (O’Rourke and Herskowitz, 2004). Fps1 is a glycerol transporter, which has been implicated in the HOG response (Tamás et al. 1999). The confirmation and characterization of the physical interaction and functional relationship between Sho1p and Fps1p provide a significant contribution in understanding of the Sho1p-mediated HOG pathway in yeast. Other Sho1 interactomes were confirmed by BiFC assay and the manuscript is under preparation by Dr. Igor Stagljar’s group. The findings identified in this study will greatly increase the current understanding of the regulation of MAPK pathways in general.

B.4. Discussion

Among 30 ABC proteins found in S. cerevisiae, 22 are membrane-bound transporters, many of which are homologous to disease-linked human ABC proteins, making S. cerevisiae an ideal model organism for studying ABC transporter regulation and function (Paumi et al., 2009; Kovalchuk et al., 2010). Considering the ubiquitous nature of ABC transporters and their major role in human disease, obtaining a better understanding of their regulation and function is of great clinical importance.

The MYTH technology allowed us to successfully map the protein interactome of all 19 of the nonmitochondrial ABC transporters in the budding yeast S. cerevisiae. A
large number of functionally diverse interactors in cellular network suggest that ABC transporters have a more general involvement in cellular processes than previously suspected. Many interactors of unknown function found in this study together with the screenings performed by Dr. Stagljar’s group as well as the interactions previously reported in the BioGRID database (Stark et al., 2011; see Snider et al., 2013 for details), demonstrate that there is still much we do not understand about this protein class. Examination of the identifiable functional groups, however, does suggest substantial involvement in transport and metabolic processes. Additionally, over 50% of the interactions involve a prey with an identifiable human ortholog, and approximately 40% of these are associated with human disease, suggesting that further study of the ABC transporter interactome should provide information relevant to human disease states.

One interesting observation in the outcome of interactome is the tendency of full-size ABC transporters to interact with one another. Although some of these interactions have been previously reported, the iMYTH screening detected a number of previously unreported physical associations. Though the functional importance of these interactions has not been well studied, it is likely that they may have a regulatory role.

Regarding the BiFC confirmation data, the caveat of BiFC assays is that it takes time for fluorophore maturation, and reversibility of a bimolecular fluorescent complex is another potential issue depending on fusion proteins. Thus, the location where BiFC signals are observed thus may not necessarily reflect the site of interaction of the two fusion proteins. (Kang et al., 2010). However, BiFC screening data still provides informative cellular localization pattern. For example, given that Zrc1 is a vacuolar membrane zinc transporter and that Pdr10-GFP was located in discrete puncta in the
plasma membrane (Rockwell et al., 2009), thus it is reasonable to conclude that VC-Pdr10 and Vn-Zrc1 interacts on the vacuolar and plasma membranes. It is tempting to speculate that ABC transporters may have a general role as functional regulators of membrane proteins in addition to their known transport roles. Further study of the interactome should help determine whether such regulation is a common occurrence and what regulatory mechanism is used.

The investigation of the integrated ABC transporter interactome has provided a number of new insights into the cellular role of this important class of transport proteins. With the large number of other interactions still unexplored, further examination of the network promises to greatly increase the understanding of ABC transporter function and regulation. Considering the high percentage of interactors with human orthologs, much of the information gained should also be directly applicable to higher organisms. This lays the foundation for subsequent identification of potential therapeutic targets in human diseases associated with ABC transporter dysfunction. Analysis of the yeast ATP-binding cassette transporter interactome provides significant insights on the regulation and function of a protein family that determines tolerance to physiological conditions and externally imposed stress relevant to human health.
Figure B. 1. A schematic diagram of BiFC assay. Haploid cells expressing either VN or VC (variant of yellow fluorescent protein) that are fused to putative interactors (X or Y) on the chromosome were mated and resultant diploids were examined to detect the formation of a bimolecular fluorescent complex by protein-protein association (X and Y).
Figure B. 2. BiFC validation of selected interactions identified in iMYTH screening (A-F).
Left panels – YFP channel. Right panels – DIC channel. Scale bar is 5 μm. Images marked with a ‘*’ were obtained via confocal microscopy as described in the Methods.
Figure B. 2. Continued
Figure B. 2. Continued

(C) YFP    DIC
PDR5-VC + PDR5-VN
VC-YOL075C + VN-PDR5

PDR12-VC + VN-PDR5
VC-PDR11 + VN-PDR10 *

SINQ2-VC + PDR5-VN
VC-PDR10 + VN-PDR12
VC-PDR11 + VN-PDR12
VC-PDR11 + VN-PDR18

Scale bar

Continued
Figure B.2. Continued

(D)

**YOR1-VC**
- VN-YCK1 [*+*]
- VN-RAS2 [*+*]
- VN-YPL199C [*+*]
- VN-YCK2 [*+*]

**VN-PDR5 [*+*]**

**PDR12-VC**
- VN-YBR056W [*+*]
- VN-GTT1 [*+*]

**VN-YOR215C [*+*]**

**YOR1-VC**
- VN-YDL173W [*+*]
- VN-GYL1 [*-*]
- VN-YGL082W [*-*]

**VN-PTR2 [*-*]**

**PDR15-VC**
- VN-YER097W [*-*]

Scale bar
Figure B. 2. Continued

(E) VN-PDR11 VN-PDR5 VN-YGL081W
PDR11-VN VN-CCC1 VN-CVC
VN-OSW5 VN-AVT3 VN-SOP4
OSW5-VN VN-SSM4 VN-SRM4
VN-SSM4 VN-ZRC1
VN-ZRC1

Continued
Figure B.2. Continued
Figure B. 3. BiFC validation of selected interactions with peroxisome components. Left panels – YFP channel. Right panels – DIC channel. Arrows (in white) in PXA1-VC point to positive punctate signals corresponding to interaction at peroxisomes. (right) Wild-type diploids without VN or VC fusion proteins were examined under the same condition and no visible signal was detected. Scale bar is 5 μm.
Figure B. 4. Summary of the tested interactions. The number of interactions tested by orthogonal assay that were positively confirmed by BiFC or Co-IP versus the number of interactions that could not be confirmed (Snider et al., 2013).
Figure B. 5. Localization of PDR10-ZRC1 BiFC complex together with intracellular markers.
Co-localization of the bimolecular fluorescent complex of VC-PDR10 and VN-ZRC1 (YFP) with (a) the plasma membrane marker CFP-Cdc42 (2 views) and (b) the endoplasmic reticulum marker Sec63-mCherry. For YFP+CFP and YFP+RFP overlay images, YFP signal is shown in Green, while CFP and RFP signals are shown in Red.
Figure B. 6. Confirmation of the Sho1p-Fps1p interaction by Bimolecular Fluorescence Complementation (BiFC) assay. Fps1p, Sho1p, and the unrelated protein Bpt1p were tagged endogenously with the N-terminal fragment of YFP (VN) or the C-terminal fragment of YFP (VC) as indicated.
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*a Strains are isogenic to HPY1680 except as indicated. BiFC strains are named differently from other lab glycerol stocks.

*b Each haploid strain carrying either VC or VN was constructed by Dr. Igor Stagljar’s group. These haploid strains were used to generate a/α diploids in this study (see method for details).
References


Components required for cytokinesis are important for bud site selection in yeast. J Cell Biol. 122:373-86.


Sagot, I., Klee, S. K., Pellman, D. (2002). Yeast formins regulate cell polarity by


