Regulation of cell polarity by the cell cycle in *Saccharomyces cerevisiae*

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Abstract

Cell polarity in *Saccharomyces cerevisiae* is essential for bud formation, which is regulated by the cell cycle. How this regulation occurs is poorly understood. The master regulator of polarity is a Rho-GTPase called Cdc42, which accumulates at a region on the plasma membrane and recruits its downstream effectors and the cell’s cytoskeleton, leading to bud emergence. Previous work suggested that at a time in G1 called Start, the G1 CDK kinase promotes Cdc42 polarization. Recent findings have shown the opposite: Cdc42 is able to polarize prior to Start in daughter cells. Nevertheless, bud growth does not begin until after Start, which leads to the question: what exactly is this kinase regulating? One possibility is that G1 CDK regulates effectors of Cdc42. A partial survey of effectors showed that some were only able to polarize after the kinase activity increased. The aim of this study was to continue surveying effectors of Cdc42, focusing on Gic1 and Gic2. Confocal microscopy was used to obtain movies of yeast cells, which were analyzed using a customized MATLAB program. Gic1 polarization did not occur prior to Start, but Gic2 could polarize pre-Start in daughter cells. Future investigation into the structural difference between Gic1 and Gic2 in combination with that of other effectors may suggest potential ways that G1 CDK regulates effector localization.
Introduction

Cell shape is essential for cell functionality

From a single zygote, the human body grows into 37 trillion cells. Rough estimates have put the different cell types in the body at around 200, which seems appropriate given the amount of diversity and specialization in the organs and tissues (Bianconi et al., 2013). Each of these cell types has a defined shape, especially designed to guarantee harmonious functionality of the human body. The mechanism by which these cell types perform their functions requires a specific shape, usually defined during embryogenesis.

The sciatic nerve, for example, is the largest and thickest nerve in the human body. It emerges from the spinal cord and eventually splits and runs down to both legs all the way down to the big toe of each foot. This unique bundle of cells measures a meter to ensure its main function, which is to provide a channel for communication between the lower limbs and the central nervous system (Rea, 2015). Another example of cells with a completely different shape are those in the gastrointestinal tract, which have microscopic cellular membrane protrusions called microvilli (Lange, 2011). The core of these structures is mostly composed by a filamentous-actin cytoskeleton, which is responsible for providing structure to the elongated form exhibited by the microvilli. The large surface area of these membrane protrusions allows for faster nutrient absorption and increases in the number of digestive enzymes on the cell surface, making digestion as effective as possible.

While neuronal cells and cells of the gastrointestinal tract have their shapes determined early in development, other cell types in the human body constantly change shape, depending on the environment to which they are exposed. Platelets, for instance, are a cell type responsible for
coagulation, whose cytoskeleton mainly consists of actin, tubulin, and spectrin (Shin et al., 2017). A blood vessel sends out a signal when it gets damaged, and platelets in the blood stream will respond by transforming into their active form and accumulating in the area. In order to make contact with the broken blood vessel and help with coagulation, platelets then change shape, growing long projections that resemble the legs of a spider or the tentacles of an octopus (Shin et al., 2017).

The role of the cytoskeleton and Rho-GTPases in cell shape modification

The sciatic nerve, microvilli, and platelets are very different from one another and perform various functions, but they all have something in common that allows the development of their shape: the cytoskeleton. The cytoskeleton is an intricate network of protein filaments that supports the large cytoplasm and is especially important in animal cells that lack a cell wall. It is a dynamic structure that responds to its environment, allowing cells to be internally reorganized and restructured and to move from one location to another. Major roles of the cytoskeleton also include spatially organizing the organelles in a cell, segregating chromosomes, and pinching apart daughter cells at cell division (Alberts, 2014).

A key element of the cytoskeleton in maintaining and changing cell shape is actin, which polymerizes to form actin filaments. Actin filaments are thin, flexible fibers, approximately 7 nm in diameter and up to several micrometers in length, whose organization is regulated by different proteins according to the cell’s needs. These actin-binding proteins are essential for controlling the assembly, disassembly and stabilization of actin filaments within a cell (dos Remedios et al., 2003).

Regulation of actin allows cells to move, control their shape, divide, and secrete at a specific location. A number of signal transduction pathways have been identified in which cells
respond to extracellular signals to regulate the dynamics of actin inside the cell (Carpenter, 2000). These pathways often involve a group of closely related monomeric proteins called Rho GTPases that are localized to the plasma membrane. In platelets, for example, the interaction of the Rho GTPases RhoA, Rac1, and Cdc42 with the cytoskeleton is responsible for the formation of filopodia and lamellipodia, actin-rich plasma-membrane protrusions (Aslan and McCarty, 2013). These structures increase the platelet surface area, stabilizing platelet aggregates and thus promoting localized coagulation (Aslan and McCarty, 2013).

Rho-GTPases themselves are also regulated and behave as molecular switches. When bound to GTP, Rho-GTPases are active and when bound to GDP they are inactive. There are two classes of molecules that regulate the activation and inactivation of Rho-GTPases: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). In general, GEFs turn on the GTPases by catalyzing the exchange of G-protein bound GDP to GTP and GAPs turn off the GTPases by inducing the intrinsic GTPase activity, promoting GTP hydrolysis, and thus inactivation (Hodge and Ridley, 2016). GEFs and GAPs respond to extracellular signals or local cues and are localized to certain regions of the plasma membrane. Finally, there are proteins named guanine nucleotide dissociation inhibitors (GDI) that promote the release of Rho GTPases from the plasma membrane to the cytoplasm (Hart et al., 1992).
Three members of the Rho family have been studied in detail: Cdc42, Rac1, and RhoA. The first, Cdc42, was defined by conditional lethal mutations in yeast. Investigations of mutants that allowed progression through the cell cycle but not bud formation lead to the discovery of Cdc42 and its GEF, Cdc24 (Adams et al., 1990; Hartwell et al., 1970). Saccharomyces cerevisiae or baker’s yeast is a highly genetically tractable organism with a fast rate of proliferation and genes highly homologous to those of humans. Even though Cdc42 was first discovered in yeast, it is also present in many eukaryotic cells, and therefore studying it could lead to the understanding of its function in other organisms, such as mammals. For these reasons, the budding yeast is a very convenient organism and was therefore the model system chosen in this study.

There is, however, a substantial difference between animal cells and yeast cells that changes the way actin functions to determine and define the shape of these two cells: the cell wall. Given that animal cells lack a cell wall, it is easier for their cell membrane to be deformed and lead to a change in shape. This deformation is mainly promoted by crossed-linked actin filaments that form a more rigid structure capable of perturbing the cell membrane, causing the cell to locally change shape (Pruyne et al., 2004). An equivalent mechanism of cell shape change is not possible in yeast, because the actin cytoskeleton cannot exert sufficient force to deform the rigid cell wall of yeast cells. Instead, the turgor pressure is responsible for changes in cell shape (Harold, 2002). A turgor pressure exists in yeast cells because the concentration of solutes inside the cell is larger than that on the outside, resulting in the flow of water down its concentration.
gradient to the inside of the cell via channels in the plasma membrane, which increases cytoplasmic volume. The rigid cell wall keeps this water flow from continuing and eventually leading to a cell burst. The turgor pressure is then created, in which the plasma membrane presses against the cell wall.

An instance when yeast cells are required to change shape is when they form a bud during proliferation. The rigidity of the cell wall requires a modification of this structure before the cell can change shape. In this case, proteins responsible for weakening the cell wall, synthesizing new cell wall, and various other components necessary for bud emergence are packaged in vesicles and transported through actin cables to the site where the bud is going to form (Pruyne et al., 2004). That way, instead of providing a rigid structure to mechanically disturb the membrane as observed in animals, actin provides a path for delivery of exocytic vesicles containing cell wall modifying proteins from the Golgi to the incipient bud site. The turgor pressure continues to push the plasma membrane against the cell wall, but once the cell wall is weak enough at the incipient bud site, the pushing cannot be resisted, and a protrusion forms, which becomes the tip of the bud.

Before the bud can form, secretory vesicles, actin, and a type V myosin work together to delivery proteins necessary for budding to the future bud site. Novick et al. (1980) showed that when temperature sensitive mutants of genes required for the export of secretory enzymes from their site of synthesis to the cell surface were shifted to restrictive temperature, bud growth stopped. Moreover, Johnston et al. (1991) demonstrated that when temperature sensitive mutants of a specific gene encoding a type V myosin protein (Myo2) were shifted to restrictive temperature, that led to arrest of proliferation, with cells displaying a uniform unbudded morphology. Finally, Lillie and Brown (1994) showed that mutants with unpolarized actin failed
to localize Myo2 to the presumptive bud site. Together, these findings show that secretion and transportation of vesicles to the incipient bud site are necessary for bud emergence.

But how do actin cables target vesicle delivery to the incipient bud site? Here, proteins that nucleate actin called formins play an essential role. Yeast cells have two types of formin, Bni1 and Bnr1, and previous research has demonstrated that loss of both genes BNI1 and BNR1 is lethal for cells (Imamura et al., 1997). Bni1 polarizes several minutes before Bnr1, with recruitment of Bnr1 occurring only once a septin ring is well established, suggesting that Bnr1 polarization does not normally contribute to initial actin polarization (Chen et al., 2012). One of the downstream effectors of Cdc42, Gic2, seems to be involved in the regulation and localization of Bni1 to the incipient bud site. Research by Chen et al. (2012) suggests that Gic2 binds directly to Bni1 and brings the formin to the polarity patch, where active Cdc42 is concentrated. Further research also found that no bud is observed in yeast cells treated with Latrunculin-A, an F-actin inhibitor that prevents the formation of actin cables (Ayscough et al., 1997). These results indicate that yeast cells require formins and the actin cytoskeleton to survive and form a bud.

**Scaffold-mediated symmetry breaking polarization pathway**

In wild-type yeast cells Cdc42 polarization occurs through a well-defined axis determined by environmental cues or makers in the cells that accompany them since birth. However, polarization can also occur in the absence of such cues, with Cdc42 getting randomly activated at a region on the plasma membrane and other Cdc42 following it through a process called symmetry breaking. The primary pathway through which Cdc42 polarizes involves a positive feedback loop and is denominated scaffold-mediated symmetry breaking (Irazoqui et al., 2003). In order to achieve polarization to a single site through this pathway, Cdc42 interacts with
another set of proteins, one of which is indispensable for polarization: Bem1 (Chenevert et al., 1992).

Bem1 is a scaffold protein that binds to key polarity factors and is localized to the presumptive bud site as well as to the bud tip (Peterson et al., 1994). This protein brings together the GEF Cdc24 and Cdc42-GTP through direct binding, and also downstream effectors of Cdc42 called p21-activated kinases (PAKs), the main ones being Ste20 and Cla4. Effectors are cellular proteins that act downstream of a signaling pathway by binding active forms of GTPases, usually leading to a cellular response, such as wound healing in platelets. The complex PAK-Bem1-Cdc24 is recruited by the randomly activated patch of Cdc42, and then further activates neighboring Cdc42 proteins, producing a larger polarized patch (Kozubowski et al., 2008).

**Figure 2.** Schematic of stochastic polarization of Cdc42 through a positive feedback loop involving the GEF Cdc24, effectors Bem1, Ste20, Cla4, and others. The role of CDK activity in this pathway is still being investigated and was partially addressed in this research study. Adapted from (Moran et al., 2019).
Early studies reported the discovery of two other Cdc42 effectors, Gic1 and Gic2, homologous proteins that bind specifically to Cdc42-GTP through their conserved Cdc42- and Rac-interactive binding (CRIB) domain (Brown et al., 1997). They have no affinity to Cdc42-GDP and colocalize with Cdc42-GTP to the future bud site. Gic2 specifically may be involved in recruiting Bud6 (an actin- and formin-interacting protein) and Bni1 to active Cdc42 at bud emergence, emphasizing that Gic2 may play a role in the assembly of the actin cytoskeleton at the polarity site (Jaquenoud and Peter, 2000; Chen et al., 2012).

The positive feedback loop suggested above is thought to be the main mechanism responsible for the polarization of Cdc42 and effectors to a region of the plasma membrane prior to bud emergence. The timing of Cdc42 polarization is another factor that needs to be taken into consideration when discussing the polarization of this protein. The time at which proteins get polarized and interact with one another during the cell proliferation cycle is also crucial for the successful formation of a bud. The main protein responsible for regulating timing of the cell cycle is Cdc28. Cdc28 is also referred to as cyclin-dependent kinase (CDK), a protein kinase encoded by the CDC28 gene, whose first mutant allele was isolated in the early 1970s (Hartwell et al., 1970).

Cdc28 is dependent for its activity on the binding of positive regulatory proteins called cyclins. Nine cyclins that bind Cdc28 have been identified and different cyclin genes are transcribed during different times of the cell cycle, creating periodic patterns of accumulation (Malumbres, 2014). Cyclins (Clns) associate with and activate Cdc28 to regulate distinct processes (Howell and Lew, 2012). When bound to G1 cyclins, the complex formed between Cdc28 and G1 cyclins can be called G1 CDK. One way through which these complexes regulate progression through the cell cycle is by becoming active or inactive at specific checkpoints, in
which the progression of a cell to the next stage in the cycle can be halted until conditions are favorable. There are several checkpoints and the one involving G1 CDK activation is called Start. Start is the point at which cells commit to a round of budding rather than mating or switching to quiescence or meiosis. Start occurs around 15 to 20 minutes prior to bud emergence and it was suggested that the G1 CDK activity at Start might contribute to the polarization of Cdc42 (Lew and Reed, 1993), but the mechanism through which this occurs is not yet fully understood.

Gic1 and Gic2 (Gulli et al., 2000), Bem1 (Butty et al., 2002), Cdc24 (Gulli et al., 2000), and Cdc42 (Wedlich-Soldner et al., 2004) were all believed to require G1 CDK activity to be able to polarize. That is, they were not detectably polarized in cells depleted of G1 cyclins – which prevented the G1 CDK complex to form at Start – but became polarized following CDK activation. However, a recent study published by members of the Lew Lab showed that daughter cells can polarize Cdc42, Cdc24, Bem1 and Ste20 prior to Start, that is, prior to G1 CDK activity (Moran et al., 2019). Even though these proteins polarized before Start, the cells did not form a bud until after Start. Moreover, it was observed that mother cells were not able to polarize before Start, indicating that there is a difference between mother cells and daughter cells that allows pre-Start polarization to occur only in daughters.

A possible explanation for this difference between mothers and daughter cells stems from the fact that mothers spend less time between cytokinesis and Start compared to daughters, given that daughters need more time to grow to a certain size before proceeding through Start (Johnston et al., 1977; Di Talia et al., 2007). However, even in synchronized cell populations, mother cells proceed through Start earlier than daughter cells, with daughter cells still able to
polarize prior to Start (Moran et al., 2019). Thus, there is something related to the identity of mothers and daughter cells that leads to this difference in timing of Start.

Having this difference between mother and daughter cells in mind and results that showed some effector proteins polarizing before Start, Moran et al. (2019) further examined if other downstream effectors of Cdc42 such as Exo70, Cla4, and Bni1 were also unable to polarize prior to G1 CDK activity and found that these effectors were only able to polarize after Start. The purpose of this present research project was to continue the efforts of other members of the Lew Lab and examine the timing of polarization of two other effectors of Cdc42 relative to Start. Polarization of Gic1 and Gic2 was studied in order to further elucidate how the cell-cycle controls the components of the scaffold-mediated symmetry breaking polarization pathway.

The timing of G1 CDK activity was determined by monitoring the nuclear exit of an inhibitory protein called Whi5. Whi5 directly inhibits the transcription factor SBF, which then prevents G1-specific transcription of cyclins Cln1 and Cln2 (Costanzo et al., 2004; de Bruin et al., 2004). However, in the presence of Cln/CDK complexes, Whi5 gets phosphorylated, thereby dissociating from SBF and exiting the cell nucleus. Cln1 and Cln2 cyclins can then be transcribed and translated, and work to further phosphorylate Whi5, leading to a positive feedback loop. The timing of Start coincides to when 50% of the Whi5 probe has exited the nucleus of the cell (de Bruin et al., 2004; Costanzo et al., 2004). Time-lapse microscopy was used to compare when these effectors were polarized compared to the time of G1 CDK activity.
**Figure 3.** Phosphorylation by Cln/CDK causes Whi5 to dissociate from the transcription factor SBF, which then leads to the translation of Cln1 and Cln2 proteins. These two Clns then phosphorylate Whi5 through a positive feedback loop, promoting its export from the nucleus.

**Materials and Methods**

**Yeast strains**

Movies of the Gic1 probe were made with yeast strain DLY22856, of genotype GIC1-mNEON:LEU2/GIC1-mNEON:LEU2; WHI5-mCherry:kan^R/ WHI5-mCherry:kan^R. Gic1-mNeonGreen was generated by ligating a BglII-HindIII fragment containing a pRS305 backbone and the GIC1 gene (from pDLB4401 = pRS305-GIC1-mScarlett) with a BamHI-HindIII digested PCR fragment containing mNeonGreen (from pDLB4393 = pRS305-BEM1-mNeonGreen). The resulting plasmid was digested with SphI to target integration at GIC1. Whi5-mCherry was generated by creating a PCR fragment using primers that contained 50 bases upstream and downstream of the stop codon of the WHI5 gene and pFA sequence from the plasmid pFA6a-mCherry:kan (pDLB2774, from pBS34, Yeast Resource Center via Dr. K.Bloom) and
transforming that into wild-type yeast (DLY5732). The fragment integrated at the WHI5 locus after homologous recombination and transformants were then selected using selective media.

The first Gic2 strain imaged was DLY22747, of genotype GIC2-GFP:kanR /GIC2-GFP:kanR; WHI5-tdTomato:URA/WHI5-tdTomato:URA. Gic2-GFP was generated by creating a PCR fragment using primers that contained 50 bases upstream and downstream of the stop codon of the GIC2 gene and pFA sequence from the plasmid pFA6a-GFP(S65T)-KanMX6 (pDLB53) and transforming that into a rsr1 delete strain (DLY9139). This strain was sporulated and then crossed to DLY21123 to make GIC2-GFP:kanR /GIC2-GFP:kanR; WHI5-tdTomato:URA/WHI5-tdTomato:URA.

The second Gic2 strain imaged (DLY23272) was constructed from DLY22747, with an extra copy of GIC-GFP at the LEU2 locus. DLY23272, of genotype GIC2-GFP:kanR /GIC2-GFP:kanR; GIC2-GFP:LEU2/leu2; WHI5-tdTomato:URA/WHI5-tdTomato:URA. Gic2-GFP was generated by introducing an extra copy of GIC2-GFP was to DLY22747 by transformation with DLB4468 (pRS305-GIC2-GFP) at LEU2 using PpuMI restriction enzyme.

Table 1. Strain genotypes and corresponding DLY strain numbers

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<tr>
<th>DLY number</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>22856</td>
<td>GIC1-mNEON:LEU2/GIC1-mNEON:LEU2; WHI5-mCherry:kanR/ WHI5-mCherry:kanR</td>
</tr>
<tr>
<td>23272</td>
<td>GIC2-GFP:KAN/GIC2-GFP:KAN; GIC2-GFP:LEU/leu2; WHI5-tdTomato:URA3/WHI5-tdTomato:URA3</td>
</tr>
<tr>
<td>9139</td>
<td>rsr1::TRP1/wt</td>
</tr>
<tr>
<td>21123</td>
<td>Whi5-tdTomato:URA3</td>
</tr>
<tr>
<td>22747</td>
<td>GIC2-GFP:kanR /GIC2-GFP:kanR; WHI5-tdTomato:URA3/WHI5-tdTomato:URA</td>
</tr>
</tbody>
</table>

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Cell culture and cell synchronization with hydroxyurea

Yeast strains grew in yeast extract peptone dextrose (YEPD) plates at 30˚C and were then cultured at the same temperature until mid-log phase (around $10^7$ cell/mL) in complete synthetic media (CSM) with 2% dextrose before microscope images were taken. For experiments using the synchronization protocol, yeast cells were cultured in complete synthetic media (CSM) at 30˚C until mid-log phase (around $10^7$ cell/mL) and treated with 0.2 M hydroxyurea for 1 h, washed three times with fresh CSM media, and cultured for another 1 h in fresh CSM media to recover.

Microscopy and image analysis

Cells were mounted onto slabs with 1% agarose in CSM+dextrose media and cover slip edges were sealed with petroleum jelly. Movies were made using the Andor Revolution XD spinning-disk confocal microscope (Olympus) with Andor Ixon3 897 512 electron-multiplying charge-coupled device (EMCCD) camera using MetaMorph software (Universal Imaging). A 100×/1.4 UplanSApo oil-immersion objective was used. Images of cells with the Gic1-mNeonGreen probe were made using asynchronous cell populations grown at 30˚C, with 90 second intervals and 15 z-planes, at a spacing of 0.5 μm. A laser power of 10% was used for the 488-nm illumination and a laser power of 20% was used for the 561-nm illumination. Both channels were set to an EM gain of 200 Hz and an exposure time of 200 ms. Images of cells with the Gic2-GFP probe were made using a synchronous cell population grown at 30˚C and treated with hydroxyurea for 1h, followed by a 1h-release. The remaining settings were identical to those used in movies of the Gic1 probe.

Movies were first deconvolved using SVI Huygens deconvolution and montages of maximum projections were created using ImageJ (Fiji; National Institutes of Health). Movies were then analyzed using a customized graphical user interface (GUI; ROI_TOI_QUANT_V8)
in MATLAB as described by Lai et al. (2018). The GUI calculates the coefficient of variation (CV) of pixel intensity in a cell from a max projection of all z-stacks. The user can delineate an elliptical shape around the cell to determine the region of interest (ROI). The CV reflects the proportion of the total cellular probe that accumulates at the polarity site. When the CV value is large, it means that the probe of interest is concentrated at a specific location rather than diffused in the rest of the cytoplasm. Whi5 50% nuclear exit was determined by the coefficient of variation, while the polarization time of the polarity proteins was determined by visual examination of montages. The time of polarization was determined to be the first time point when the probe seemed to be accumulated at a region on the plasma membrane.

Whi5 50% nuclear exit was determined the following way: the highest CV measurement for Whi5 is normalized to 100%, which is when Whi5 is still in the cell nucleus. When this CV value drops down to 50% of the highest value measured during the time of interest, this time point is determined to be the point of Whi5 50% nuclear exit, which correlates with cell commitment to the cell cycle. The time of interest recorded for both channels (488-nm and 561-nm) included from 40 to 15 time points before Start to guarantee that the CV of Whi5 was at 100% normalized intensity so that 50% of the CV could be accurately determined. Time points after the formation of a bud were not included in the analysis and non-smoothed CV data was reported for Gic1 and Gic2 probes.

Statistical analysis

P-values were calculated via a two-tailed t test for the null hypothesis that mothers and daughters of the same strain exhibit the same mean timing of polarization relative to Start. The differences in time of polarization between mother cells and prestart daughter cells were large and
significant at P < 0.001. Small differences between mother cells and post-Start daughter cells were not significant.

Results

One of the first steps in investigating the ability of Cdc42 effectors to polarize independently of cell cycle regulation and of the activity of G1 CDK was to compare the timing of polarization of some of these effectors relative to Start. Members of the Lew Lab imaged cells containing fluorescently labeled probes of Bem1 and Whi5 and compared the timing of polarization of Bem1 relative to Start, which was determined to occur when 50% of the Whi5 probe exited the nucleus. The timing of polarization differed between mother and daughter cells, with Bem1 polarizing after Start in mother cells, but before Start in daughter cells (Fig. 4). This difference in timing of polarization of Bem1 relative to Start (G1 CDK activity) lead to the idea that other effectors of Cdc42 could have a similar behavior, that is, a polarization that is not regulated by the cell cycle. This study surveyed two other effectors of Cdc42 to assess their timing of polarization relative to Start: Gic1 and Gic2.

Figure 4. Normalized CV of Whi5 and Bem1 over time in daughter cells (left) and mother cells (right). The orange arrow indicates the time of polarization of Bem1, which corresponds to the frame in the montage with an orange asterisk. The blue arrow indicates the time of Start, when
50% of the Whi5 probe has exited the nucleus. Montage strips on top of graphs show individual
cells over the course of time, with the top strip showing the Whi5 probe and the bottom strip
showing the polarization of the Bem1 probe. Adapted from (Moran et al., 2019).

Individual cells containing fluorescently labeled Gic1 and Whi5 proteins were analyzed
in order to compare the timing of the Gic1 protein polarization and 50% Whi5 nuclear exit. The
same analysis was conducted with Gic2 as the probe. Gic1 movies were captured using a
population of unsynchronized diploid cells. Similar attempts to image an unsynchronized
population of diploid cells with the Gic2 probe were made but were unsuccessful because the
probe was not bright enough, preventing a precise determination of polarization. In order to try
to increase the brightness of the Gic2 probe, an extra copy of the gene tagged with a GFP was
added to the diploid strain, generating cells with three copies of the GIC2 gene with attached
GFP fluorophore. The new strain yielded a brighter probe but bleaching of the probe was
observed early in the movies. An 1 h hydroxyurea arrest treatment followed by a 1 h release were
performed before imaging this newly-made strain in order to try to reduce bleaching, resulting in
a successful movie of the Gic2 probe. Images were later analyzed using the methods described
by Lai et al. (2018). Each movie had four different stage positions, and each stage position an
average of 10 cells. Cells were subdivided into mother and daughter cells in each stage position
in order to compare the timing of polarization between these two groups. 50% Whi5 nuclear exit
was determined by tracking the coefficient of variation (CV) of pixel intensity of the Whi5 probe
and polarization of the probes (Gic1 and Gic2) was determined by visual examination of
montages.

Given that the timing of Start and the timing of polarization were determined for each
cell, the comparison between these timepoints allowed for the classification of the probe in either
the pre-Start or post-Start categories. The Gic1 probe did not polarize before Start in either mother or daughter cells. That is, the protein did not polarize until after 50% of the Whi5 exited the nucleus. The traces in Figure 5 show the CV of the Whi5 probe on the y-axis and the time in minutes on the x-axis. The time point zero marks Start, which is also shown by a vertical dotted line. Negative time points refer to moments before Start and positive time points refer to moments after Start. Gic1 seems to polarize post-Start in either mother or daughter cells (p > 0.5, indicating there is no significant difference between the timing of polarization relative to Start between mothers and daughters cells).

**Figure 5.** A) Normalized intensity of the CV of Whi5 over time in mother cells. The time point zero marks Start. B) Normalized intensity of the CV of Gic1 over time in mother cells. The time point zero marks Start. Gic1 in mother cells polarizes after Start, as observed by the increase in the coefficient of variation after time point zero in all the cells. C) Normalized intensity of the CV of Whi5 over time in daughter cells. The time point zero marks Start. D) Normalized
intensity of the CV of Gic1 over time in daughter cells. The time point zero marks Start. Gic1 in daughter cells polarizes after Start, as observed by the increase in the coefficient of variation after time point zero in all the cells. \( p > 0.5 \).

In order to confirm the results obtained using the quantification method described above, montages of single cells were created and the polarization of the Gic1 probe was observed by eye and by an individual trace determined by ROI TOI (Fig. 6). The timing of 50% Whi5 nuclear exit is indicated by an asterisk on one of the frames in the montage. The arrows indicate the time of polarization of Gic1 as observed by eye. The polarization of Gic1 compared to 50% Whi5 nuclear exit in the montages agrees with the CV results, again reinforcing the finding that Gic1 polarizes after Start in both mothers and daughter cells.
Figure 6. A) Montages of a single daughter cell. Top panel shows the Whi5 probe and bottom panel shows the Gic1 probe. There is an interval of 1:30 minutes between each frame. The asterisk indicates the time point where 50% of the Whi5 protein exits the nucleus (Start) and the arrow indicates the time of Gic1 polarization. The Gic1 probe polarized 5 time points (7:30 min) after Start. B) Normalized intensity of the CV of the Whi5 probe compared to the Gic1 probe. The vertical line indicates the time of Start. Post-Start polarization is observed in the single-cell level. The arrow indicates the time of Gic1 polarization as observed by eye.

For Gic2, however, some daughter cells seem to polarize prior to G1 CDK activity. Similar image collection and analysis methods were used to compare the timing of polarization of Gic2 and the time of Start. Initially, several trials in imaging the Gic2 probe with two copies of GFP (DLY22747) - one in each endogenous locus of a diploid - were made, but the probe was not bright enough, preventing image analysis and confident determination of polarization. In order to try to increase the intensity of the probe, several constructs were made, including Gic2-GFP probes, GIC2-mNeonGreen probes, and GIC2-sfGFP probes. In almost all of them, Gic2 failed to polarize, with either the probe being dispersed in the cytoplasm, not sufficiently bright, undergoing photobleaching in the beginning of the movie, or only detectably polarized in a couple of cells (Fig. 7). The only construct that showed the Gic2 polarizing with a brightness that was enough to determine when polarization occurred was the one made with an extra copy of the GIC2 gene attached to GFP, GIC2-GFP:KAN/GIC2-GFP:KAN; GIC2-GFP:LEU/leu2; WHI5-tdTomato:URA/WHI5-tdTomato:URA. Even in this last construct the fluorophore bleached at around 1 hour after the start of the movie. Because of this, only cells that polarized within 1 h from the beginning of the movie were scored.
Analysis of cells with the Gic2 probe showed that Gic2 polarized before Start in some daughter cells, while the remaining cells polarized at around Start, and the mothers polarized Gic2 after Start (Fig. 8) (p < 0.001, indicating that there is a significant difference between mothers and daughter cells regarding their time of polarization relative to Start). It also seems like there are two polarization events of Gic2, one before Start and one after Start. This is demonstrated by a decrease in the CV of Gic2 after its first polarization event pre-Start followed by an increase of the CV right after Start (Fig. 9). In order to confirm the results obtained above, montages of single cells were created and the polarization of the Gic2 probe was observed by eye and by an individual trace determined by ROI TOI. Some cells polarized Gic2 several time points before Start (Fig. 9), shortly before Start (Fig. 10), and at around Start (Fig. 11).

**Figure 7.** A) Max projection of GIC2-mNeonGreen cells showing that the probe was not be detectably polarized. B) Max projection of GIC2-GFP cells showing that the fluorescent probe was very faint and was only seen in a couple of cells.
Figure 8. A) Normalized intensity of the CV of Whi5 over time in mother cells. The time point zero marks Start. B) Normalized intensity of the CV of Gic2 over time in mother cells. The time point zero marks Start. Gic2 in mother cells polarizes after Start, as observed by the increase in the coefficient of variation after time point zero. C) Normalized intensity of the CV of Whi5 over time in daughter cells. The time point zero marks Start. D) Normalized intensity of the CV of Gic2 over time in daughter cells. The time point zero marks Start. Gic2 in daughter cells polarizes before Start in some cells and after Start in some cells, as observed by some cells showing an increase in the coefficient of variation before time point zero and some cells showing an increase in the coefficient of variation after time point zero. p < 0.01.
**Figure 9.** A) Montages of a single daughter cell. Top panel shows the Whi5 probe and bottom panel shows the Gic2 probe. There is an interval of 1:30 minutes between each frame. The asterisk indicates the time point where 50% of the Whi5 protein exits the nucleus (Start) and the arrow indicates the time of Gic2 polarization. The Gic2 probe polarized several time points before Start. B) Normalized intensity of the CV of the Whi5 probe compared to the Gic2 probe. The vertical line indicates the time of Start. Pre-Start polarization is observed in the single-cell level. The arrow indicates the time of Gic2 polarization as observed by eye.
**Figure 10.** A) Montages of a single daughter cell. Top panel shows the Whi5 probe and bottom panel shows the Gic2 probe. There is an interval of 1:30 minutes between each frame. The asterisk indicates the time point where 50% of the Whi5 protein exits the nucleus (Start) and the arrow indicates the time of Gic2 polarization. The Gic2 probe polarized one time point before Start. B) Normalized intensity of the CV of the Whi5 probe compared to the Gic2 probe. The vertical line indicates the time of Start. Pre-Start polarization is observed in the single-cell level. The arrow indicates the time of Gic2 polarization as observed by eye.
Figure 11. A) Montages of a single daughter cell. Top panel shows the Whi5 probe and bottom panel shows the Gic2 probe. There is an interval of 1:30 minutes between each frame. The asterisk indicates the time point where 50% of the Whi5 protein exits the nucleus (Start) and the arrow indicates the time of Gic2 polarization. The Gic2 probe polarized around the same time as Start. B) Normalized intensity of the CV of the Whi5 probe compared to the Gic2 probe. The vertical line indicates the time of Start. Post-Start/at-Start polarization is observed in the single-cell level. The arrow indicates the time of Gic2 polarization as observed by eye.
Discussion

Previous studies had shown that Cdc42 polarization in *Saccharomyces cerevisiae* was dependent on G1 CDK activity and thus that this main protein involved in cell polarization would only polarize after Start. Members of the Lew Lab have shown that this is not the case, and that Cdc42 polarization differs between mother and daughter cells, with mother cells polarizing post-Start, but daughter cells polarizing pre-Start (Moran et al., 2019). The symmetry-breaking polarization pathway involves various other proteins and effectors of Cdc42, and some of them were also tested in their ability to polarize before Start. The scaffold protein Bem1, the PAK Ste20, and the GEF Cdc24 were also able to polarize before Start. However, other effectors such as the PAK Cla4, the formin Bni1 and a subunit of the exocyst complex Exo70 were unable to polarize before Start in both mothers and daughter cells, suggesting that CDK activity might control the ability of these specific effectors to localize to sites with concentrated Cdc42-GTP.

This study investigated the ability of two other effectors of Cdc42, Gic1 and Gic2, to polarize prior to G1 CDK activity. Both the quantification of the coefficient of variation of the probes and visualization of the montages pointed to the same result regarding the polarization of Gic1, which was found to be post-Start in both mother and daughter cells, suggesting that the cell cycle (G1 CDK activity) could be controlling the polarization of this effector. On the other hand, the Gic1 paralog Gic2 was surprisingly able to polarize prior to Start in daughter cells, suggesting that this effector is not regulated by the G1 CDK. This is an interesting result, given that the only other effectors of Cdc42 that polarized before Start up until now were Ste20 and Bem1. Even the other similar PAK Cla4 seems to require G1 CDK activity in order to polarize. It is important to note that the results for Gic2 were obtained from a strain with 3 copies of the GIC2 gene, which could have disrupted the normal timing of polarization of the Gic2 protein. If
this is the case, the observed results can possibly differ from what would be observed in cells with a wild-type number of copies of this gene.

A major difference in the timing of polarization between Gic1 and Gic2 was observed in this study. In all mother cells, Gic1 polarized around the same time and in all daughter cells Gic1 also polarized at similar time points, with mother cells polarizing further from Start than daughter cells. After Gic1 polarization was first observed, the protein was still observed to be polarized for several time points past Start and bud emergence, when Gic1 tracks with the tip of the bud. On the other hand, Gic2 polarized at a variety of time points, including time points before and at Start. Moreover, the Gic2 protein disappears shortly after Start, which could be explained by findings presented by Jaquenoud et al. (1998). They showed that the interaction between Gic2 and active Cdc42 is necessary for the phosphorylation of Gic2, which eventually leads to ubiquitination and degradation of Gic2 by the proteasome. This could be the explanation for the short life of polarized Gic2, which gets degraded after its interaction with active Cdc42 at the polarity patch.

How does the CDK regulate the ability of Gic1 to polarize? One possibility for the function of G1 CDK in localizing effectors like Gic1, which only polarize post-Start, is their direct phosphorylation. It could also be that the G1 CDK complex phosphorylates other interacting proteins to enable binding of the effectors to Cdc42-GTP. The fact that Ste20 was able to polarize even in cdc28 temperature sensitive mutants indicates that the activity of the G1 CDK is in not necessary in the case of this effector. Following this observation, another hypothesis for the polarization of effector proteins can be presented, which consists in the idea that effectors might be able to bind to the active form of Cdc42 at any moment in time but need
additional regulation to be able to bind the plasma membrane. That way, the localization of these effectors could be regulated by protein-protein interactions and protein-membrane interactions.

Evidence for this hypothesis was presented by Takahashi and Pryciak (2007), who discovered a membrane-binding domain common in some of the Cdc42 effectors such as Ste20, Gic1, and Gic2. This domain is localized next to the CRIB domain in these proteins, through which the effectors interact with GTP-Cdc42. This region is named the basic-rich domain (BR) and interacts directly with membrane phospholipids. Mutations of basic or hydrophobic residues in this domain abolished the polarized localization of Ste20, indicating that not only the binding of this effector to Cdc42 is necessary for polarization, but also the binding to the membrane itself. Similar experiments were performed for Gic1 and Gic2, which surprisingly showed a difference in the function of the BR domain between these two proteins. Mutations in the BR domain of Gic1 did not disrupt the polarity localization of this protein and indicated that this region does not serve primarily to facilitate the polarization of Gic1. However, when the same mutations were applied to the BR domain of Gic2, the localization of this protein to presumptive bud sites was abolished, indicating that the function of this domain in localizing both Ste20 and Gic2 to the membrane and to Cdc42 in the polarity patch could be similar.

This study presented further evidence for the hypothesis of members of the Lew Lab (Moran et al., 2019) that G1 CDK regulates the polarization of most effectors, but not all of them. Future studies might want to design non-phosphorylatable mutants of Gic1 and Gic2 to elucidate if the phosphorylation by G1 CDK is necessary for the polarization of these effectors. Another possibility is to observe the polarization of these effectors under a temperature sensitive cdc28 mutation. In order to understand if the BR membrane-binding domain is necessary for other effectors to polarize, the same mutations described by Takahashi and Pryciak (2007) could
be applied to effectors such as Cla4 and Exo70. This study classified two effectors of Cdc42, Gic1 and Gic2, according to their ability to polarize prior to G1 CDK activity. Further classification of other effectors of Cdc42 regarding their time of polarization relative to Start and structural differences may suggest potential ways that G1 CDK regulates effector localization.
References


