A Role for Gic1 and GIC2 in Promoting Cdc42 Polarization

by

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Stefano DiTalia

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Cancer Biology in the Graduate School of Duke University

2018
ABSTRACT

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Abstract

The Rho GTPase Cdc42 is a master regulator of cell polarity that orchestrates reorganization of the cytoskeleton. During polarity establishment, active GTP-Cdc42 accumulates at a part of the cell cortex that becomes the front of the cell. Localized GTP-Cdc42 orients the cytoskeleton through a set of “effector” proteins that bind specifically to GTP-Cdc42 and not GDP-Cdc42. A family of Cdc42 effectors, called GICs in yeast and BORGs in mammals, have been implicated in regulation of both the actin cytoskeleton and the septin cytoskeleton. Yeast cells lacking both Gic1 and Gic2 are able to polarize and grow at low temperatures, but many mutant cells fail to polarize the cytoskeleton at high temperature. This led to the conclusion that GICs communicate between Cdc42 and different cytoskeletal elements.

To better characterize the role of GIC proteins in yeast, we utilized time-lapse fluorescent microscopy to examine morphogenetic events in living single cells. Surprisingly, we found that not only the cytoskeleton but also Cdc42 itself failed to polarize in many gic1 gic2 mutant cells at high temperature. This observation indicates that GICs may act upstream of polarization rather than downstream.

Polarization of Cdc42 is triggered by cell-cycle progression, and in particular by G1 Cyclin-dependent kinase (CDK) activity. Using a live-cell reporter for G1 CDK activation, we found that cells lacking GICs were not defective in CDK activation, but showed a specific defect in polarization downstream of the CDK. Previous work had implicated the scaffold protein Bem1 in a positive feedback loop important for
polarization. Cells lacking GICs failed to polarize Bem1 as well as Cdc42 at high temperature. Future work will be directed at understanding how GICs contribute to polarity establishment. Because many of the mechanisms and proteins involved in polarization are highly conserved, we anticipate our findings will help inform how this process regulated in higher eukaryotes.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenine diphosphate</td>
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<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
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<tr>
<td>Bem1</td>
<td>Bud EMergence 1</td>
</tr>
<tr>
<td>Bni1</td>
<td>Bud Neck Interactor 1</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42 and Rac Interactive Binding domain</td>
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<tr>
<td>GAPs</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GDP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>GEFs</td>
<td>guanine nucleotide exchange factors</td>
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<tr>
<td>Gic1</td>
<td>GTPase interacting component 1</td>
</tr>
<tr>
<td>Gic2</td>
<td>GTPase interacting component 2</td>
</tr>
<tr>
<td>Gics</td>
<td>GTPase interacting components</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>HbS</td>
<td>variant of the beta-globin gene that causes Sickle cell disease</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NPF</td>
<td>nucleation promoting factors</td>
</tr>
<tr>
<td>PAKs</td>
<td>p21-activated kinases</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>SNAREs</td>
<td>Soluble N-ethylmaleimide-sensitive fusion protein Attachment Protein Receptors</td>
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Acknowledgements

I would like to dedicate this work to the friends and family that have supported me throughout this process and to my advisor for his guidance.
1. Introduction

1.1 Cell shape is critical to cell function

Cells are able to assume distinct shapes depending on their surrounding environment. Changes in environmental conditions often trigger cells to alter their morphology in response. Shapes are adapted to allow cells to perform specialized tasks efficiently. Cells that perform similar functions often have similar shapes. Some examples that illustrate the strong association between cell shape and function can be seen in cells that travel through the blood to reach tissues to at distant sites, cells that change shape to control permeability and cells that swim.

Red blood cells are responsible for absorption and delivery of fresh oxygen throughout the body and the removal of carbon dioxide. In order to reach all parts of the body, red blood cells must routinely enter and exit narrow capillaries to travel to distant sites. This task is made easier by their small, flat, disc shape and elasticity. The center of the disc-shaped cells is slightly concave which increases the surface area and the amount of oxygen that can be transported. Additionally, in most species red blood cells lack organelles, allowing even more space for oxygen storage[1]. The importance of red blood cell shape in enabling its function is underscored by the defects observed in the autosomal recessive disease, sickle cell anemia. The disease pathology is due to a single point mutation that causes an amino acid change in intracellular hemoglobin[2]. In both normal cells and cells harboring the mutant hemoglobin allele, HbS, hemoglobin is uniformly distributed around the cell. However, during red blood cell deoxygenation
HbS forms polymers [3]. When polymers pierce the membrane it leads to transient calcium permeabilization and dehydration, which cause the flexible. HbS polymerization also causes cells to stretch and become rigid. The combination of membrane dehydration and elongation produce the characteristic “sickled” shape. This aberrant shape is not conducive for entering and exiting small blood vessels and often results in hallmarks of the disease such as vaso-occlusion in capillaries and arterioles [4]. Blockage of vessels in this way can cause excruciating pain in individuals with the disease [5]. Although the shape change is typically reversed when reoxygenization induces break down of polymers; repeated cycles of sickling and unsickling eventually cause the membrane to lose flexibility, locking cells in the sickled confirmation [5]. This pathology not only illustrates how shape can be favorable for carrying out specific cellular functions, but also how function can be impaired if shape becomes compromised.

Another example of the importance of cell shape can be seen in the function of plant guard cells. Guard cells are found in the epidermis of leaves. These cells promote plant growth and survival by sensing changes in environment and altering their volume in response[6]. Guard cells are arranged in pairs facing opposite directions. This arrangement leaves a minute pore in the center called the stoma. The stoma is the site of gas exchange with the atmosphere and water transpiration[6]. Microbes are also capable of entering the cell via the stoma[7]. Because this pore serves as a major gateway to the cell interior, it is important that it is able to open and close at the right time in order to
conserve resources and prevent infection. Guard cells are responsible for controlling stomatal aperture[8]. Guard cells sense internal and external factors such as light intensity, carbon dioxide, humidity, calcium, plant hormones and even microbe associated molecular patterns [6, 7, 9-11] [12, 13]. These stimuli trigger the vacuoles of guard cells to regulate osmotic uptake of water and protons producing rapid changes in vacuolar and cellular volume concomitant with changes turgor pressure. Increases in water uptake cause an increase in volume and opening of stomatal pores. Loss of water decreases vacuole volume and turgor pressure, making cells more flaccid enabling stomata closure. The volume of guard cells can change by more than 40% during stomatal movement from the time of opening to closure as a result of changes in vacuolar volume and morphology [14]. Tanaka et al. showed that decreases in vacuolar volume occur in accordance to changes in environmental conditions. They observed stomal closure in Arabadopsis exposed to darkness[15]. These changes in the structure and orientation of guard cells and stomata shape allow plants to respond to changes in their environment by altering their rate of water and gas uptake.

Spermatoza are part of the male reproduction machinery. Morphology plays an important role in their ability to function. Although there are slight variations in aspects of the morphology between species, such as relative length and head shape, number of mitochondria, the same basic elements make up sperm in most species [16]. Sperm consist of three main parts, a head that contains half of the genetic material that will be transferred to the zygote upon fusion with oocyte. The midpiece contains mitochondria
wrapped around axial filaments of flagella that provide energy for propulsive force [17-21]. Energy from this section is propagated along the flagella tail region to propel the cell through its environment. Structural integrity of these components is critical for enabling the sperm to traverse hostile microenvironments such as swimming through viscous uterine fluid, navigation of physical barriers and filtration through the cervix in order to reach the oocyte[22]. Studies of infertile men have shown a multitude of aberrant morphologies including having two tails and large heads. These changes in morphology make sperm less efficient at swimming and at fertilization. These morphological defects impair cell motility [23]. The female reproductive tract has multiple mechanisms for selecting sperm with particular morphological traits [24, 25].

Sperm that display abnormal morphologies often fail to reach the fertilization site [26, 27]. Studies by Pertrunkina showed that interactions with epithelial cells play a role in selecting for sperm with proper morphology. These cells preferentially bind to sperm with normal morphology. Binding to epithelial cells helps facilitate transport through binding to adjacent cells sperm can swim along epithelial surfaces. Biased binding provides an example of how the female reproductive system exerts selective pressure against mutant sperm [28]. Cell shape affects the efficiency of transport to and fusion with the oocyte and hence, chances of successful gamete formation.

The cell types described highlight specific examples of how cell shape governs function. Although drastically different in form, each of the described cell morphologies plays a critical role in enabling cells to undertake specific tasks.
In studies performed by Siefert and Fox, common shapes were mapped onto prokaryotic phylogenetic trees. They found that many trends in morphology have arisen and accumulated multiple times in separate lineages [29]. Notwithstanding the differences in the resulting morphologies, the process that produces them is also highly conserved.

1.2 Cell shape is largely determined by the cytoskeleton

The cytoskeleton provides a structural framework for the cell. Animal cells achieve diverse shapes by rearranging cytoskeletal elements. The cytoskeleton is composed of several types of filamentous polymers: microtubules, intermediate filaments, septins and actin microfilaments [30, 31]. The various polymers that make up the cytoskeleton are highly dynamic and constantly undergo cycles of assembly and disassembly with aid from a host of accessory proteins that regulate length, crosslinking and organization. The collective action of these proteins organizes polymeric filaments into higher order structures that enable changes in morphology. Collectively these distinctive structures establish internal organization, provide mechanical support and define shape[32].

Microtubules are filamentous polymers that are formed when heterodimeric globular subunits, alpha and beta tubulin, in a head to tail orientation. Monomers are added to the plus end of the growing filament so that elongation occurs in the direction of the cell periphery. Microtubules are the stiffest of the cytoskeletal polymers. When
assembled, microtubules are rigid structures that provide mechanical support and form to the cell.

Assembled microtubules also cycle between stages of assembly and disassembly, which allows them to alter their structure in response to stimuli or cellular cues. One example can be seen in organization of the filopodia on migratory cell fibroblasts[32]. Filopodia act as sensors that allow cells to detect changes in environmental conditions. In these fibroblasts, microtubules are targeted towards the filopodia. Studies with fibroblasts using pharmacological agents to artificially to induce filament growth and shrinkage in fibroblasts led to dramatic changes in morphology. Induction of filament shrinkage with nocodazole resulted in a reduction in filopodium merging[33]. Additional studies conducted using mutant forms of tubulin, rather than pharmacological inhibition, produced similar findings. Cell lines bearing mutant copies of tubulin displayed reduced microtuble dynamics and motility as assessed by wound healing assays[34]. Hence the organization of this structural component has great influence on cell morphology.

Pressure and tension can also influence cell shape. Cells are able to detect and respond to changes in substratum stiffness and external pressure. Intermediate filaments are nonpolar, highly flexible and harbor high tolerance to bending[35]. These properties make intermediate filaments well-suited for their role in regulating mechanical properties of the cell.
The third cytoskeletal elements, septins, are a family of heteromeric filament-forming GTPases that are rearranged into several higher order structures such as gauzes and rings. Septins function by directly binding to membranes and are required for cytokinesis[36-39]. Septins play multiple roles in ensuring proper cell shape and membrane adaptability.

In mammalian cells septins have been shown to cooperate with other cytoskeletal networks to create local alterations in cell shape. In this regard, septins have been shown to colocalize with and stabilize microtubules and mitotic spindles[40]. Disruption of septins causes morphological defects[41].

Septins also play a role in controlling cortical rigidity. Cell rigidity can affect whether cells are able to form stable contacts with neighbors or participate in activities that require more dynamic organizations such as phagocytosis. Septins have also been widely implicated as sensors of micron-scale membrane curvature. Upon detection, septins accumulate at curved sites proportionally to the degree of curvature[42]. These sites include but are not limited to cleavage furrows, neuron branches and phagocytotic cups. Septins are able to spatially organize signaling by recruiting and stabilizing proteins at these sites.

Actin filaments form mesh-like networks under the cell surface that are heavily implicated in providing structure and shape to the lipid bilayer. Actin filaments are less stiff than microtubules, but are still able to form stiff structures by being crosslinked and organized into higher order structures such as cables and branches.[32] Actin and its
associated motor protein, myosin causes constrictions in cells. Contractility drives processes such as membrane ingression for endocytosis and constriction of the actomyosin ring that cleaves a single cell into two [43]. Regardless of stimulus, reorganization of the cytoskeleton is a requirement for obtaining and changing cell shape.

Actin plays an especially important role in this regard. Actin is a major constituent of the cytoskeleton; it is required for shape determination, vesicle trafficking, adhesion, and generation of protrusive forces for locomotion.

Actin exists in monomeric subunits termed globular actin (g-actin). G-actin monomers combine in a head to tail fashion to form polymers. These polar polymers have a “pointed” end and a “barbed” end. Each end has different affinity for binding proteins. Adenine triphosphate (ATP)-bound g-actin is preferentially incorporated at barbed ends extending it into the polymeric form, filamentous actin (f-actin). Adenine diphosphate (ADP)-monomers and inorganic phosphate (P_i) are released from filament. Cycles of simultaneous growth and shrinkage produce treadmilling of actin monomers within a growing filament [44, 45]. G-actin can spontaneously self-assemble into dimers and trimers under physiologic conditions, but these intermediates are highly unstable and typically dissociate [46-48]. This would seem to make spontaneous self-assembly incredibly slow and not feasible for cells to rely on as a mechanism for reorganization in response to environmental cues.
Contrarily, Mallavearpo performed experiments that tracked the fiduciary marks on labeled actin in filament growth cones of neuroblastoma cells lines. The studies showed that actin structures are quite dynamic and undergo rapid assembly and disassembly. This assembly is responsible for the filopodia-like extensions and retractions of these cells. Additionally, these experiments showed that the dynamics of actin filaments are highly variable within and between filopodia[49].

Swift turnover permits cells to quickly choreograph the structural changes that underlie surface growth as needed. These findings were corroborated by results from studies done in other systems using varied techniques such using fluorescence recovery after photobleaching, fluorescence loss in photobleaching and pharmacological actin inhibitors. These results of these studies were consistent in showing rapid actin assembly and disassembly [50-54].

Bulk solution assays allowed for the discernment of some of the molecular details of actin assembly. However, it remained poorly understood how cells were able to overcome kinetic barriers to actin assembly. Much advancement in understanding was gleaned from the use of total internal reflection fluorescence microscopy (TIRF). Experiments involved reconstituting actin filament assembly in vitro tethered to microscope slides. Addition and removal of different actin regulatory components at the initiation of assembly and/or after assembly was underway allowed assessment of changes in dynamics. Together use of both of these technologies enabled researchers to define the necessary components for actin assembly and the roles of accessory proteins.
The process was broken down into four main steps: 1) Nucleus formation/ nucleation 2) elongation 3) capping and 4) disassembly. Each stage has a unique set of cofactor requirements. Nucleation is the initial step. The timing of nucleation can vary greatly. The rate at which nucleation and the subsequent steps occur is largely determined by the concentration of unassembled g-actin available for incorporation into dimers and trimers. The term critical concentration refers to the measure of the ability of g-actin in solution to initiate spontaneous polymerization [47]. Bulk assays showed slight increases in g-actin levels above the critical concentration lead to increased elongation rates. [54-57]. These in vitro studies helped to characterize relationship between free actin and filament growth rate and emphasize the importance of g-actin rich pools for fueling spontaneous assembly. Actin behavior in vivo mirrors this trend. Cells require large cytoplasmic pools of actin in order to sustain dynamic turnover. Cells typically contain very high actin concentrations that in some cases can greatly exceed the 1uM critical concentration [58, 59]. This discovery helped resolve the kinetic barrier conundrum. When the concentration of actin is high enough, nucleus formation and spontaneous assembly occur more quickly. As follows, active regulation of the internal ratios of g-actin to f-actin is critical for actin to grow and restrict growth at proper times.

One way cells achieve this level of regulation is through proteins that bind actin monomers and prevent their incorporation into filaments. This sequestration helps to create and maintain reservoirs of free g-actin in excess of critical concentration. Profilin performs this function by binding ADP-bound unpolymerized g-actin. Profilin remains
bound to the monomer, preventing subsequent monomer addition to nucleus [60]. In this way profilin prevents spontaneous nucleation through sequestration of g-actin in cytoplasmic pools until needed for assembly.

Once the kinetic barrier of nucleus formation is overcome, elongation ensues. Elongation is a much more energetically favorable reaction and occurs quickly. While profilin plays an inhibitory role in regulating actin nucleation, profilin performs a separate role that promotes filament elongation. Profilin-ATP-bound monomers are able to bind the barbed ends of filaments. Profilin has a lower affinity for monomers after incorporation into filaments. Profilin-ATP-monomers that bind barbed ends undergo a conformational change that causes profilin to relinquish the bound monomer [61]. As f-actin becomes longer, the concentration of g-actin goes down. In order to sustain actin growth dynamics, the cell must have ways to prevent depletion of the cytoplasmic actin pool.

The actions of profilin in promoting filament elongation are balanced by the activities of proteins that bind and inhibit assembly and/or lead to disassembly. The action of these proteins is also required for maintenance of the g-actin pool. This diverse set of proteins influences different aspects of actin polymerization through directly binding and performing roles such as nucleus stabilization, promoting elongation, bundling, crosslinking, capping, and even severing of actin structures. Their coordinated actions help cells spatially and temporally control actin polymerization and modulation of respective actin pools.
Regeneration of g-actin pools is achieved through the action of ADF/cofilin. The nucleotide bound state modulates affinity for this regulator[62]. There is a well-documented lag between the time ATP-bound subunits are incorporated at the barbed end and the timing ATP hydrolysis and P\(_i\) dissociation by the incorporated actin subunits[63]. This lag in hydrolysis and P\(_i\) dissociation results in enrichment of ATP-actin at barbed ends, long-lived intermediate species of ADP-P\(_i\) bound actin in the center and ADP actin towards the pointed filament ends. ADF/ cofilin preferentially binds to ADP-actin subunits so depolymerization primarily occurs at pointed ends [64]. ADF/cofilin enhances the rate of actin monomer dissociation at pointed ends by weakening interactions between ADP-bound subunits and induce severing within filaments [64, 65] Disassembly and severing of actin filaments enhances actin turnover rate and replenishes g-actin by releasing ADP monomers back into the cytoplasmic pool. This allows monomers to be recycled by associating with profilin again [64, 66]

In addition to factors that promote elongation and disassembly, the cell also relies on capping protein to stabilize assembled filaments. Filament capping limits the amount of free barbed ends available for elongation. Capping proteins are heterodimers that prevent assembly and disassembly by sterically inhibiting further binding of monomers or secondary regulatory proteins[67-70]. This stalls filament elongation and prevents depolymerization. This step is necessary to regulate the length of actin filaments.
Filament length is an important property because it affects the flexibility of a given filament. Certain functions of actin require rigid filaments that tend to be shorter in length, while others functions utilize longer, more flexible filaments. In Dictyostelium, overexpression of capping protein increased the rate of motility whereas decreasing expression of capping protein led to reduced motility[71]. These results were recapitulated in other systems such as fibroblasts[71].

The direction of subunit polymerization determines where polarized growth will occur. Rapid actin polymerization produces the directional forces to drive plasma membrane protrusions. Platelets undergo bursts of polymerization and corresponding shape change driven by rapid, localized actin polymerization by actin at barbed ends that become exposed after uncapping and/or severing[71].

The force generated by individual filaments is not sufficient to overcome the resistance of the plasma membrane for protrusion formation. The limitation of individual filaments is partially overcome by arranging filaments into higher order structures to produce larger forces. Actin networks are capable of generating forces that greatly exceed that of individual filaments. A specific type of nucleator organizes each type of actin structure.

Because of this increase in force generated, it was long assumed that polymerization alone led to protrusion of membranes. However, this mechanism of action left many open-ended questions. One of the most intuitive being, how force generated from polymerization is transduced into membrane propulsion? Another being
how polymerization can continue to occur once it reaches the cell barrier. Even if polymerization led to a slight push forward, it would fail to explain how additional subunits could be added to form a protrusion. A group of mathematicians and physicists sought to answer these and other lingering questions through a series of papers proposing thermal dynamic solutions. Simply stated, the model is grounded on the observation that polymerization occurs towards the cell membrane until there is no more space for additional monomers. The model proposes that the membrane is undergoing thermal dynamic fluctuations, which cause slight inward and outward movement. Each outward fluctuation produces a tiny gap in between the filament end and the membrane barrier. Additionally, this model presumes that actin monomers are tethered to rigid filament networks of preexisting filaments, but that the tips of these filaments are able to undergo thermal dynamic fluctuations that cause rapid bending motions. In instances where the gap created between the membrane and the barbed filament end is large enough, a monomer is added to the filament, sterically preventing the membrane from fully moving back but not from moving forward. Lastly, their model necessitated that filaments be short and stiff, as long filaments may become bent and fail to protrude. Completion of this model was a true tour de force for the scientific community. Not only did their model provide explanations for prior observations, but also it was also able to predict aspects of membrane and polymerization dynamics that were not proven experimentally until years later[72-74]. Studies corroborating their model showed that an actin nucleator, Arp2/3, was capable of generating short filaments
in an orientation consistent with the model.

Arp2/3 is part of a 7-subunit complex that leads to the formation of branched actin networks from actin filaments. Polymerization of these networks towards the cortex, rather than individual filaments, helps generate the force to drive large-scale conformational changes that push the membranes of cells that move forward. Individual Arp2/3 complexes bind the sides of preexisting “mother” filaments and initiate nucleation of daughter filaments at 70° from the mother[72, 75, 76]. Newly formed daughter filaments in turn act as a substrate for formation of more angled branch formation. Before Arp2/3 can engage in this activity it must first become activated via association with a member from a conserved class of Wiskott-Aldrich Syndrome Proteins (WASP)[77].

WASPs contain a Verprolin Central Acidic (VCA) domain that simultaneously binds to monomers and Arp2/3 leading to acceleration of nucleation of daughter filaments. The subunits of the Arp2/3 are separated by distance, preventing complex activation. The C and A domains bring Arp2/3 subunits together converting them to a closed confirmation that is primed for actin binding.

The VCA domain also plays an inhibitory role in actin assembly. VCA has high affinity for Arp2/3 but a lower affinity for Arp2/3 bound to branches. WASP must detach before initiation of daughter filament nucleation. In vitro studies reconstituting these reactions revealed VCA does not bind actin after branch formation has started. When mutations were introduced to alter the interaction between VCA and Arp2/3, the rate at
which VCA dissociated directly paralleled the changes in efficiency of branch formation. Thus, Arp2/3 binding VCA primes actin branching through delivery of monomers, activating Arp2/3 and releasing the brakes to initiate nucleation. Arp2/3 also dissociates from daughter filaments after a few seconds resulting in patches made up of short-branched networks. These dynamic structures combined with thermal dynamic fluctuations support the formation of membrane protrusions such as lamellipodia.

The formation of protrusive structures occurs when actin polymerization is concentrated at the leading edge of the cell, balanced by depolymerization at the rear[78]. As the protrusion extends, it forms new connections with the extracellular matrix that serve as anchorage points. Attachments are stabilized long enough for the cell to build up tension. Coordination of actin and myosin leads to cell contraction that allows the trailing edge to glide forward. This series of events is an iterative process that leads to translocation of the cell body via a crawling motion that propels single cells forward. Persistence refers a cell's ability to travel in one direction.

1.3 Walled organisms utilize different mechanisms to achieve shape

Unlike animal cells, plant cells and many fungi are surrounded by an exterior cell wall. The cell wall provides protection from sudden changes in osmolarity and other environmental stresses. Additionally, it provides support to oppose the high internal turgor pressure of the cell [79]. Much of the characteristic stiffness of the cell wall is due
to the high turgor pressure. Turgor pressure from inside the cell pushes outwards against the membrane, stretching the wall. This outward pushing force is counterbalanced by tension build up in the cell wall to resist turgor pressure. Whereas animal cells are able to induce deformation of the plasma membrane through force generated by the cytoskeleton, this force becomes negligible when opposed by the stiff barrier of a cell wall. Consequently, walled organisms must employ different mechanisms undergo morphogenesis.

Walled cells tend to grow either diffusely by extending radially over a wide area or by tip growth, in which growth is restricted to the apex[80]. Regardless of the growth mechanism, walled cells require a certain series of steps to occur to enable growth. Tension of the cell wall must be relieved to allow for expansion. This can be carried out by altering the compaction of proteins called expansins, changing cellular pH, severing of load-bearing structural components, local degradation by secreted enzymes and other methods[79, 81, 82]. Each of these methods serves the purpose of making the cell wall more malleable to deformation. Once the cell wall polymers are loosened, local delivery of cell wall matrix polysaccharides and glycoprotein synthesis enzymes are released and promote local degradation, biosynthesis and expansion by incorporation of newly delivered cell wall proteins at the plasma membrane.

Root hairs are tube shaped extensions that grow on the surface of trichoblast cells in the plant epidermis. Their structure enables them to increase the surface area of roots, increasing the amount of nutrient and water uptake. These cells grow by unidirectional
tip growth [83-85]. This is achieved by creating distinct zones that are permissive for specific directional growth but restrictive to lateral expansion. In root cells cellulose microfibrils bear brunt of the tensile stress. Microfibrils are synthesized by enzyme complexes in the plasma membrane where they become embedded in a network of polysaccharides[86, 87]. These polymers are closely aligned with each other and cross-linked by other matrix polymers[88, 89]. Cellulose deposition is highly ordered by the actions of cortical microtubules and the localization of cellulose synthase enzymes[90, 91]. Microtubules determine the location of cellulose microfibril deposition. Interactions between cortical microtubules and the cellulose microfibril synthesis machinery that restrict its movement also contribute to dictating the site of cellulose microfibril deposition [87]. Both of these polymers co-align parallel to each other and cross-linked to neighboring filaments. Localized loosening of the matrix polysaccharides that bind neighboring microfibrils results in biased apical growth perpendicular to cortical microtubules[81, 87]. Treatment of Arabidopsis root cells with agents that bind tubulin led to disruption of the transverse orientation of microtubules. Though not targeted by the reagent, cellulose microfibrils also lost their orientation and became randomly arranged. As a result, the region of elongation began to expand laterally. This experiment helped uncover the role of microtubules in guiding microfibril localization and in restricting the zones of cell expansion [86].

Actin is also heavily involved in driving tip growth. In these cells actin is organized into linear bundles of filaments that lay parallel to the root cell shank.
Secretion guides the place of deposition to the appropriate location. Vesicles containing cell wall assembly materials are trafficked through an actin-based mechanism called cytoplasmic streaming and deposited at the apex. Release of vesicular constituents deposits enzymes that engage in weakening the cell wall for insertion of newly assembled polymers. Secretory vesicles fuse with the membrane and fusion of vesicles with the membrane at the cell tip [92]. Continuous targeted vesicle delivery leads to formation of the characteristic tubular morphology. Actin is required for this polarized cell wall deposition and tip morphogenesis.

Filamentous fungi use the same steps to produce hyphae. Growth is restricted to the tip where new cell wall components and degradation machinery are continuously delivered by vesicles. Mutations in actin-associated proteins led to isometric growth [93]. Pharmacological disruption of actin in prevents tip delivery and leads to tip swelling of hyphae and an associated loss of tip growth [94-96]. In some fungi vesicle delivery is mediated by microtubules. In cells that rely on microtubule for vesicle delivery, disruption of microtubules leads to similar defects.

*Saccharomyces cerevisiae* / budding yeast are ellipsoidal/sphere shaped fungi that participate in tip growth at certain stages of their life cycle; during the formation of buds and mating projections. Under conditions of starvation yeast can also induce pseudohyphal growth. Single mutations in actin or its regulatory proteins (ACT, MYO2, TPM1, PFY1) were sufficient to disrupt the asymmetric organization of actin, impair vectorial secretion and result in uniform expansion [97-101].
These examples provide evidence that even though additional steps may be necessary to overcome the rigidity of the cell wall, cell shape is still heavily dictated by the cytoskeleton. In each case exocytosis is paramount to generation of polarized growth.

The exocytosis pathway is best understood in budding yeast. Schekman, and Novick pioneered much of the work of dissecting this process [102-104].

In yeast, proteins required for remodeling the extracellular matrix are sorted and packaged into secretory vesicles that are assembled in the golgi body. The vesicles undergo processing by addition of polypeptide and lipid tags that designate their sorting and delivery to distinct target membranes. In order for targeted vesicle delivery to yield apical growth vesicles must be delivered, tethered and fuse with the target membrane to release their contents. Successful completion of each of these steps is critical for polarized growth and bud emergence.

The orientation of the actin cytoskeleton is the major determinant of where vesicle delivery and growth will occur. This point has been illustrated through studies in which yeast were treated with the microtubule inhibiting agent nocodazole. Treated cells were still able to engage in targeted vesicle delivery and produce buds [105]. Whereas this same experiment repeated using an actin inhibiting agent, latrunculin, caused loss of polarized secretion and budding [106]. These results were recapitulated in genetic studies in strains with various mutant alleles of the microtubule subunit tubulin. Mutants containing no microtubules were still able to transport secretory vessels, form
buds and secrete enzymes [98]. Contrarily, a conditional, temperature sensitive mutant of the actin stabilizing protein, tropomyosin, experienced reversible loss of actin and vesicle delivery when shifted between the permissive and restrictive growth temperatures [107]. Collectively these results support the assertion that actin is essential for polarized growth in yeast, while the role of microtubules is dispensable.

Vesicles are transported along actin cable structures assembled by the nucleator Bud Neck Interactor 1 (Bni1). Bni1 is a member of the formin family and produces actin short, parallel filaments that are bundled into cables. Cables anchored at the cortex extend towards the presumptive bud site [108, 109]. Actin cables serve as the tracks for targeted vesicle secretion from the golgi body, to the growth site, and to the tips of new buds [110-112]. Vesicles are docked at the polarity site through the action of the Rab GTPase protein Sec4. Sec4 resides on secretory vesicles where it promotes physical association between vesicles and the type V myosin motor protein Myo2, that fuels vesicle delivery [113-116]. This was shown in studies in which mutations introduced into the tail region of Myo2 were produces cells that were able to polarize, but failed to transport vesicles [113]. The ability of Myo2 to transport vesicles depends on the presence of intact actin cables [107, 113, 117-119]. The loss of actin cables observed in temperature sensitive Tropomyosin mutants also caused delocalization of Sec4 and Myo2[120].

The exocyst is an effector of Sec4 that contributes to post-golgi vesicle delivery. The exocyst complex is made up of 8 subunits and involved in exocytosis. The exocyst is also on the surface of vesicles[121]. During exocytosis it transported along vesicles
within the cell from the emerging bud, to large buds, to cytokinetic rings. Local activation of exocyst function leads to vesicle docking with the plasma membrane via polarized actin cables. The exocyst concentrates at the fusion site and tethers vesicles to specific lipids in the plasma membrane[122, 123].

Soluble N-ethylmaleimide-sensitive fusion protein Attachment Protein Receptors (SNAREs) mediate fusion of vesicles with the plasma membrane at the growth site [124, 125]. SNARE complexes assemble and bring membranes intended to fuse in close proximity to each other. Vesicular-SNARES are paired with their cognate Target membrane-SNARE by the exocyst [126]. Because of the vast variety of proteins in the SNARE family, and their concentration on distinct vesicles, it has been postulated that SNARES impart specificity between vesicle and their target membrane. However, studies have shown promiscuity amongst cognate SNARES[126].

When vesicles fuse they discharge their components into the space in between the cell wall and the plasma membrane, the periplasm, in order for them to carry out their functions. Glucanases degrade the cell wall locally, allowing for incorporation of new plasma membrane proteins.

Mutation of different nodes in the yeast exocytosis pathway has helped determine the sequence and roles of many of the involved proteins. Exocyst mutants have defects in fusion. Vesicles are delivered, but because they cannot fuse they accumulate at the growth site. It was also shown that the SNARE complex fails to assemble in these mutants. This finding placed exocyst activity upstream in the pathway
from SNAREs and prior to vesicle fusion[127]. Sec4 mutants have widespread secretion defects. They accumulate vesicles, plasma membrane components and have decreased growth rates[128]. These mutants display blocks in delivery at multiple stages suggesting they act more upstream in post-golgi vesicle delivery. Loss of Bni1 does not produce a dramatic phenotype because much of its role is compensated by the redundant formin isoform; Bnr1. Bnr1 performs a similar role of nucleating actin cables at the bud neck [129]. However, simultaneous deletion of both formins is lethal, indicating that assembly of actin cables is an essential process[130]. The phenotypes of these mutants reinforce the necessity of actin in ensuring secretion to target sites and polarized growth through exocytosis.

Formins are responsible for generating cables and focusing regions of growth. In order to perform this role, formins must translocate from the cytoplasm to the plasma membrane. A family of highly conserved proteins called Rho GTPases binds, and recruits actin nucleators, including the formin Bni1, to the polarity site[131-133]. The site of Rho GTPase localization and accumulation establishes the site on the surface where growth will occur. In this way Rho GTPases are able to direct the location of polarized growth by directing the orientation of the cytoskeleton. Cdc42 is the Rho GTPase that conducts these rearrangements and determines polarity in yeast; hence it is often referred to as the “master regulator of polarity”[134-136].

Cdc42 is capable of polarizing in the absence of Bni1 polarization but the converse does not occur, indicating that Cdc42 indeed is an upstream regulator of
Bni1[137, 138]. Cdc42 polarization is required for the polarization of Bni1. The C188 allele of Cdc42 disrupts the membrane anchorage motif. This sole disruption is sufficient to prevent Cdc42 polarization; and instead lead to diffuse localization throughout the cytoplasm. Consequently these cells fail to localize formins and actin cables[139]. Additionally, temperature sensitive mutant alleles of Cdc42 that are unable to polarize also fail to localize formins and hence all downstream components of the secretory pathway[105]. The septin cytoskeleton also becomes delocalized in these mutants[140].

The act of choosing a site is called polarity establishment. During polarity establishment, cells designate a front and a back and asymmetrically distribute components along this single axis, creating distinct domains within the cell.

### 1.4 Rho GTPases determine the orientation of the cytoskeleton

The proteins and pathways that regulate the process of polarity establishment are highly conserved across species from plants to humans. Regardless of species, polarization is governed by the same general hierarchal scheme.

Cdc42 is part of a family of highly conserved proteins that regulate a suite of diverse biological processes within the cell such as cell proliferation, differentiation, shape, adhesion, migration and division[141]. In humans, polarization allows for the development of cardiac, neuronal and other body systems. Cdc42 was discovered during a genetic screen. Its dramatic phenotype at restrictive temperatures helped pinpoint it as an integral component of the cell polarization machinery. Essentially all
aspects of polarized growth are lost when Cdc42 function is compromised. Cells arrest as large, unbudded cells that fail to polarize or bud [141-143].

Much of what we know about human Rho GTPase function was determined by studies using yeast as a model, including the discovery of Cdc42[141]. Cdc42 sequence and function are highly conserved amongst species. The human and yeast genes are 80% identical and functionally interchangeable [144].

Cdc42 is conformationally regulated through cofactors that render it active or inactive. When Cdc42 is bound to guanine triphosphate (GTP) it is active, while binding to guanine triphosphate (GDP) leads to inactivation. Active Cdc42 bind proteins called GTPase activating proteins (GAPs) [145]. Binding accelerates the slow intrinsic GTPase rate of Cdc42 thus prompting conversion to the inactive state by hydrolysis of GTP into GDP. In contrast, association with the guanine nucleotide exchange factor (GEF), which binds the inactive, GDP-bound form, facilitates the exchange of GDP for GTP [146, 147].

In its active state Cdc42 binds and recruits multiple effector proteins that initiate distinct signaling cascades that facilitate reorganization of the cytoskeleton and morphogenesis[105, 148, 149].

The timing of Cdc42 activation and localization to specific sites are regulated in relation to key morphogenic life cycle events by the cell cycle[150].
1.5 The cell cycle

The Cell cycle is a series of events in the cell that result in the division of one cell into two. Proper ordering and localization of cellular events is essential for production of progeny with identical genetic material and organelles.

 Certain changes in cell morphology are correlated with cell cycle progression. Coordination of this sort is necessary for cells to maintain shape and size[151]. Support for this is seen in experiments performed by Hartwell using various methods to arrest cells at different stages of the cell cycle showed. Cells arrested at the same stage of the cell cycle displayed uniform morphology[152]. The scope of this study focused on the events that take place in G1 as this is the stage at which Rho GTPases polarize and targeted secretion occurs. These are formative events in shaping subsequent cell behavior and fate.

The cell cycle consists of 4 main stages Gap1 (G1), Synthesis (S), Gap2 (G2) and Mitosis (M). The cell cycle is followed by a period of division called cytokinesis. Completion of cytokinesis splits one cell into a genetically identical mother and daughter. Cells assume unique morphologies as they traverse the cell cycle. In early Gap1 (G1) cells are small, round and unbudded with Cdc42 located throughout the cytoplasm and on membranes[150]. The cell cycle is initiated by passage of a checkpoint referred to as “start” that commits the cell passage through the cell cycle. Start regulates pre-budsite assembly. Activation of proteins called Cyclin-dependent kinases (Cdks) is thought to trigger passage through start and promote bud emergence. Support for this
claim comes from experiments in which premature activation of Cdk induced premature bud emergence [153-155]. Similarly, temperature sensitive alleles of Cdk failed to undergo bud emergence at restrictive temperatures, instead cells arrested cell cycle progression before start[156]. Cdks are serine/threonine kinases that function by forming heterodimeric complexes with other protein subunit called cyclins. Cyclins are proteins that undergo rounds of synthesis and degradation throughout the cell cycle. When expressed, cyclins bind and activate Cdk.

The timing and order of Cdk induced events is events are well characterized. Cdk activation occurs 15-20 minutes prior to bud emergence [101, 157]. Unpolarized cells start to polarize Cdc42 ~ 11 minutes prior to bud emergence[67]. The GEF for Cdc42 also localizes at the bud site during this time to activate Cdc42[158]. Cdc42 activation is quickly followed by initiation of actin-mediated secretion ~1 minute later [67, 101, 159-161]. In addition to Cdk activation, cyclins also direct Cdk activity to specific targets involved in cell cycle progression. Phosphorylation by Cdks can lead to inhibition or activation of substrates. Phosphorylation of Cdc42 regulators indirectly enables an additional way to temporally fine-tune Cdc42 activity. Cdc42 has 4 GAPs, 2 of which have been shown to undergo inhibitory phosphorylation by Cdk in a cell-cycle dependent manner[143, 158, 162-165]. Concomitantly, GTP-Cdc42 levels peak in late G1 [166]. The timing of Cdk-induced inhibition of GAPs ensures that Cdc42 becomes activated at the right time to promote polarization and bud growth. Single deletion of a GAP is well tolerated suggesting they share at least partial overlapping roles[167].
However, deleting multiple GAPs causes more pronounced defects in morphology, elongated buds due to hyperactivation of Cdc42. Terminating the GTPase activity of Cdc42 also causes defects in morphology. Further support of a role for Cdk in regulating GAPs, overexpression of wild type GAPs still under Cdk control did not cause dramatic effects, in comparison to non-phosphorylatable alleles which were highly toxic. This suggests a requirement for temporal control of GAP expression by Cdk[162].

Cdks can associate with various types of cyclins. Different cyclins affect different stages of the cell cycle. For example, G1 cyclins (Clns) 1-3 are associated with promoting events in G1 while Class B cyclins (Clbs) 1-6, (also periodically expressed) are responsible for regulation of events in S and G2. In this way Cdk-regulator binding serves as the timer that sets the pace for subsequent cell cycle events and orchestrates transitions between cell cycle phases. The transition from S to G2 marks the transition of the cell from apical to isotropic growth. Cdc42 remains polarized until G2 [168]. Deleting all 3 G1 cyclins in a single strain showed evidence for the requirement of cyclins for this type of regulation. This strain contained a single cyclin under the expression of an inducible promoter. When the promoter was off, the strain lacked expression of all 3 G1 cyclins. The phenotype of this strain mimicked the temperature sensitive Cdk phenotype at restrictive temperature. Polarization was inhibited and cells arrested in G1. Reintroduction of the single cyclin under the inducible promoter was sufficient to restore polarization[158, 169, 170].
In the Synthesis phase DNA is replicated in preparation for cell division. In Gap 2 (G2) cells continue to grow. These phases are referred to as interphase; they serve to ensure the cell meets certain requirements before entering mitosis. In mitosis, duplicated DNA is separated and partitioned between mother and daughter cell. Cells undergoing mitosis are visually distinct from those in interphase. In cytokinesis septins drive cleavage, which physically separates the mother and daughter into two distinct cells[171].

Although Rho GTPases such as Cdc42 become stably concentrated at one site, the individual molecules are not stationary. On the contrary, studies using fluorescence recovery after photobleaching revealed that exchange of Cdc42 between the plasma membrane and cytoplasm is highly dynamic, occurring at $T_{1/2} = 4-5s$ [170, 172]. Yet, Cdc42 still remains clustered despite molecules continually diffusing outward from the patch. Logically, the question arises “how do cells maintain a cluster at one site”? Proper localization is vital for the function of Cdc42 and proteins involved in polarity establishment. Experiments that artificially targeted Cdc42 regulators uniformly on the plasma membrane, or sequestered them in the cytoplasm were not able to induce polarization [173].

1.5.1 Bud site selection

The bud site in yeast is not random. Positioning is determined by the placement of inherited “bud scars” or cytokinesis scars from the previous cell division and the localization of integral membrane proteins called landmarks[174]. Landmarks are
prelocalized at or near sites where budding may ultimately occur[175, 176]. Diploid cells bud in a bipolar fashion, at the opposite poles, while haploids bud axially by forming new buds at a site adjacent to the previous bud [177]. Bud site selection is largely determined by the GTPase RaS Related 1 (Rsr1). Rsr1’s main role, when active, is to recruit the Cdc42 GEF[178]. Inactive Rsr1 is distributed all over the membrane, but its GEF Bud5, colocalizes with landmark proteins[175, 176]. Thus landmark proteins are able to dictate the presumptive bud site by defining the available sites for Rsr1 activation [179].

**1.5.2 Positive regulation of Cdc42**

The GEF Cdc24 is responsible for localized activation of Cdc42. Cdc24 uses a highly conserved catalytic “Dbl” domain to facilitate the exchange of GDP for GTP. Cdc42 also contain a Plekstrin homology domain. Temperature sensitive mutants of Cdc24 display a similar phenotype to that of temperature sensitive Cdc42 mutants. Cells display uniform expansion and arrest as large round, unbudded cells at the restrictive temperature[105, 141, 149]. This phenotype was the basis for discovery of several of the essential polarity proteins in yeast. As previously stated, in its active state, Cdc42 recruits and activates target proteins that control actin polarity[110, 131]. Activation is an essential component of Cdc42’s ability to function. Polarization of Cdc42 requires that a significantly higher concentration of Cdc42 be present at one site on the cortex[110, 130, 150, 161]. A mechanism utilizing regulator-effector interplay exists for rapidly amplifying active Cdc42 signal to lead to polarization. This mechanism involves
initiation of a positive feedback loop that allows for propagation and reinforcement of the active Cdc42 signal[180]. Membrane localized spatial cues are inherited by cells. These cues, called landmarks, determine where the subsequent bud will form.

Landmarks have intracellular domains that recruit and bind the GEF for Rsr1. Rsr1 localizes and becomes active at this site, followed by recruitment of Cdc24 and GDP-Cdc42. Rsr1-mediated colocalization of Cdc24 and its substrate GDP-Cdc42 allow for local activation. The pathway promotes asymmetric accumulation of GTP-Cdc42 at discrete sites on the plasma membrane[175, 176]. Studies using cytoskeletal-depolarizing agents revealed that this recruitment occurs independently of actin and microtubules[181]

Deletion of Rsr1 effectively eliminates this pathway[182]. Surprisingly, \textit{rsr1}Δ cells are still able to polarize Cdc42 by an alternate pathway called symmetry breaking. A theoretical concept proposed by Alan Turig, is often applied to the yeast system to conceptualize how polarization can arise from a homogenous population of freely diffusing molecules. In its application, the theory posits that molecules of Cdc42 may still become activated by chance encounters with GEF due to Brownian motion. In the absence of Rsr1 these collisions will no longer be restricted to landmarks, but will instead be able to occur anywhere on the cortex. As a result, \textit{rsr1}Δ cells form buds in random locations. Symmetry breaking relies on the scaffold function of Bem1 to support amplification of fluctuations of active Cdc42. As in wild type cells, Bem1 forms cytoplasmic complexes with the Cdc42 GEF and effector[183, 184]. Stochastically
activated Cdc42 recruits cytoplasmic complexes with GEF and effector to site. Recruited complexes position the GEF in close enough proximity to activate neighboring inactive molecules of Cdc42[184]. Newly activated Cdc42 molecules then go on to recruit more Bem1 complexes initiating a positive feedback loop[183]. Because each complex is capable of activating multiple molecules of Cdc42, the concentration of locally active Cdc42 increases quickly (Figure 1.1).

*bem1Δ* cells are highly compromised in their ability to polarize Cdc42, but they are viable[170]. *rsr1Δ bem1Δ* cells however, are not viable due to inability to polarize Cdc42[183]. This genetic interaction reveals that the Rrs1 and Bem1 pathways have a shared role in polarization of Cdc42 via localizing the GEF, and that cells must have at least one functional pathway. Kozubowski et al. performed experiments artificially fusing the GEF to the effector and showed that this was sufficient to restore Cdc42 polarization in *rsr1Δ bem1Δ* cells[184]. These experiments also reveal that the sole role of Bem1 in polarity establishment is to link the Cdc42 GEF and effector at the polarity site.
Figure 1.1: Positive feedback mechanism in yeast
A) Gdi1 recycling of Cdc42 provides Bem1 complex linking GEF and PAK B) PAK associates with GTP-Cdc42 and brings the GEF proximal to neighboring GTP-Cdc42 C) Newly activated GTP-42 is now able to recruit more GEF-containing Bem1 complexes. Over time this cycle enriches the concentration of GTP-42 at the membrane

1.5.3 Negative feedback

Just as it is important for Cdc42 activation to be regulated, it is equally important to regulate when the signal should be attenuated. It may seem counter intuitive, but negative regulation of Rho GTPases by GAPs and negative feedback enhances the robustness of polarization to fluctuations in protein concentration[164]. The negative feedback loop was discovered when oscillations of Bem1, Cdc24 and Cdc42 polarity...

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clusters were observed during filming of live cells[185]. Positive feedback alone leads to clustering but not dispersal of polarity components. Treatment with an agent that blocks actin polymerization did not reduce oscillations[164]. This result ruled out the possibility of oscillations being due to dilution of the polarity site by deposition of vesicle contents[164]. A series of models based on this result led to the finding that incorporation of a negative feedback was sufficient to reproduce the observed oscillations[164]. Later studies helped unveil the mechanism. Negative feedback is initiated shortly after polarity establishment is underway. The Cdc42 effector kinase Cla4 is recruited to the polarity site to promote actin polarization. Cla4 has also been shown to exert action on the Cdc24 GEF. Cla4 phosphorylates the GEF rendering it inactive. Cdc42 activation is curtailed as more molecules of Cdc42 undergo inhibitory phosphorylation[185]. The non-phosphorylatable mutant allele of Cdc24 is hyperactive in vivo and leads to excess accumulation of Cdc42[185].

These experimental results highlight the importance of negative regulation of Rho GTPase in addition to positive, but also the importance of being able to cycle between the active and inactive state.

1.5.4 Rho GTPase recycling

Although not as essential as the role of GAPs or negative feedback, a third pathway exists to aid in enrichment of active Cdc42 at the cortex. This pathway is dependent on the action of Rho GTPase dissociation inhibitor Rdi1[186, 187]. Cdc42 undergoes posttranslational prenylation at its carboxyl terminus. This modification
promotes the association of Cdc42 with membrane surfaces[144, 188]. Rdi1 preferentially binds to GDP-bound Cdc42 and masks the prenylation site that confers association with the plasma membrane[189]. Once membrane association is disrupted Rdi1 is able to extract inactive Cdc42 from the membrane by binding and chaperoning it into the cytoplasm[190]. Cdc42 bound to Rdi1 is more mobile than GTP-bound Cdc42 at the membrane. GTP-Cdc42 activation of neighboring molecules of GDP-Cdc42 at the surface can quickly lead to depletion of the local concentration of surrounding GDP-Cdc42. In this instance there would be no more substrate available for conversion into GTP-Cdc42, thus stalling local activation. The differential mobility imparted by Rdi1 binding allows for the local pool of GDP-Cdc42 near the polarity patch to be replenished. This mechanism provides the fuel for further local activation. In this way Rdi1 contributes to increasing the local concentration of Cdc42. rdi1Δ cells are viable, but polarization dynamics are dramatically slower. Cdc42 exchanges at $T_{1/2} = 15-20s$ [172]. This indicates that yeast have alternative mechanisms of recycling Cdc42 independently of Rdi1. Deletion of rdi1 doesn’t cause a severe phenotype but overexpression can cause lethality[186].

### 1.6 Effectors

The copious mechanisms that have evolved to spatially and temporally regulate the activity of Cdc42 allude to the importance of Cdc42 as a master regulator. Although Cdc42 is fairly upstream in the polarization cascade, it is able to induce far-reaching changes in downstream cell functions and behavior. Cdc42 controls actin organization,
septin assembly, polarized surface growth, bud emergence, shmoo formation, intracellular trafficking and many other processes[180]. This is possible due to engagement with numerous effectors (Figure 1.2). Interactions with different effectors have different biological outputs. The term “effector” refers to proteins that preferentially bind GTP-Cdc42. For most effectors, this occurs via a highly conserved 16 amino acid sequence called the Cdc42 and Rac Interactive Binding (CRIB) domain[191]. When bound to GTP-42, effectors are able to exert their function in divergent pathways. In this way Cdc42 is able to link upstream cues to elicit various cellular responses. Numerous studies have isolated partial function alleles of Cdc42 that harbor impairments in individual pathways while others remain intact. For example, the Cdc42-6 allele is able to polarize actin cables, but has defects in exocytosis[121]. These mutants accumulate vesicles at the polarization site that fail to fuse. This phenotype implicates Cdc42 in regulation of exocytosis in a role that is independent of its role in actin polarization. Similarly, Gladfelter et al. isolated several cdc42 mutants that had normal actin polarization but failed to assemble septins[192]. These and other partial function alleles helped illuminate different pathways under regulation by Cdc42 and the effectors involved.
1.6.1 PAKs

One set of effectors is a family of P21-activated serine threonine kinases called PAKs. In yeast there are 3, Ste20, Cla4 and Skm1. Each PAK contains a CRIB domain that binds GTP-Cdc42. PAK localization changes throughout the cell cycle in accordance with sites of growth, from the cortex to the incipient bud site and to bud or shmoo tips. Deletion of the CRIB domain disrupts association with Cdc42 and mutants fail to localize. Interaction with Cdc42 also relieves auto-inhibition by leading to conformational change that unveils the kinase domain.

Ste20 plays distinct roles in the yeast mating and budding pathways. Haploid yeast reproduce by mating with a strain of the opposite mating type. Individual haploid cells are able to locate potential mates through secretion and sensing of pheromone gradients. Pheromones engage surface receptors on cells of the opposite mating type. Surface receptor activation leads to cell cycle arrest in G1, activation of Mitogen
Activated Kinase (MAPK) signaling and polarized growth towards the potential mating partner in the form of a shmoo[139, 193]. Ste20 is an intermediate in the MAPK signaling cascade. \textit{Ste20Δ} cells are sterile and fail to arrest or produce shmoos in the presence of mating pheromone due to disruption of MAPK signaling. Thus Ste20 is a critical mediator in the yeast-mating pathway.

The role of Ste20 in the budding pathway is less understood. Ste20 has been implicated in bud site selection and regulation of the actin cytoskeleton. In regard to actin organization, Ste20 phosphorylates the type I myosin motor protein, Myo3. Myo3 is involved in regulation of endocytosis. Replacement of endogenous Myo3 with an allele containing a mutation at the phosphorylation site is not capable of rescuing Myo3 null cells. This indicates that phosphorylation by Ste20 is required for this function[194, 195].

Yeast form buds in predictable patterns based on their ploidy. Yang et al partially positioned by the cytoskeleton. Each cytoskeletal structure led to the organization of a particular bud site selection pattern. Septins were implicated in determining axial patterning while actin dictated that of bipolar diploids. Further, perturbation of either cytoskeleton caused cells to adopt the opposite bud site selection pattern. Because of these observations, defects in budding pattern can be used as an indicator of potential protein involvement in regulation of a specific cytoskeletal pathway[196]. Along this vein, it was observed that homozygous \textit{ste20Δ} cells bud in a
unipolar rather than a bipolar pattern. This lends further support for Ste20 involvement in regulation of actin.

With the exception of the Pleckstrin Homology domain, Ste20 and Cla4 share many of the same motifs. The Pleckstrin Homology domain binds multiple phosphoinositides and is believed to play a role in mediating association with the plasma membrane[197]. Simultaneous deletion of Cla4 and Ste20 is lethal, which indicates a shared essential function[198].

Unlike Ste20, Cla2 is heavily involved in regulation of septins. Cla4 directly phosphorylates the Cdc3 and Cdc10 septin subunits. In the absence of Cla4, septins are aberrantly localized at bug tips instead of the mother-daughter bud neck[198]. Cla4 is also involved in initiating negative feedback by phosphorylating Cdc24 (discussed in section 1.5.3). Null haploids have abnormal morphology, elongated buds and defects in cytokinesis. Overexpression of Cla4 leads to hyperphosphorylation of Cdc24[158].

Skm1 is a paralog of Cla4 that originated from whole genome duplication. Deletion of Skm1 does not yield an obvious phenotype. Unlike dual deletions of Ste20 and Cla4, combining deletion of Skm1 with that of another PAK is not lethal. This suggests that despite their similar homology, Skm1 performs a function that does not fully overlap with that of Ste20 and Cla4[198].

**1.6.2 Formins**

Formins are a family of actin nucleators that produce actin cables. These multidomain effectors of Cdc42 lack a CRIB domain. Instead, formins associate with
GTP-Cdc42 through an amino terminal GTPase binding domain. Formins also contain two conserved Formin homology domains, FH1 and 2, at their carboxyl terminus[199, 200]. The FH2 domain is a hoop–shaped dimer that assumes a closed confirmation until encounters with actin monomers. The hoop structure encircles the barbed end of the filament, blocking the action of capping proteins[201]. The dimers function by making transient contacts as they process along a growing filament[202]. Mutations that prevent dimerization have been shown to compromise the efficiency of formin-mediated elongation in multiple systems[203]. In contrast to the Arp2/3 complex, formins remain associated with growing actin filaments as they elongate.

FH1 is a largely unstructured domain, which contains multiple adjacent polyproline motifs that bind profilin[43, 130, 133]. The abundance and positions of these motifs allow for capture and local concentration of diffusing profilin-bound actin monomers. The flexible nature of FH1 facilitates changes in its orientation that permit delivery of “captured” profilin-bound actin monomers to the catalytic FH2 domain. This cooperative action between FH1 and FH2 promotes accelerated elongation of filaments[201]. Filaments nucleated by formins are bundled into cables and crosslinked through the concerted action of the NPFs Actin binding protein 140, Fimbrin and Tropomyosin 1 and 2[108, 201].

Yeast have two functionally redundant formins, Bni1 and Bnr1. Bni1 and Bnr1 are structurally similar but function at different locations within the cell. Bni1 nucleates cables at the budsite whereas Bnr1 nucleates actin cables at the mother-bud neck. Each
formin associates with different Rho GTPases and has a unique set of regulatory proteins. Bni1 is regulated primarily by Cdc42, Rho1, Rho3 and Rho4 whereas Bnr1 binds Rho4[130, 131, 204]. Single deletions of either formin have minor defects but are viable[204, 205]. Loss of both formins is lethal.

1.6.3 Bem1

Bud Emergence Protein1 (Bem1) is a scaffold responsible for localization of and stabilization of components necessary for activation of Cdc42. Bem1 is another effector that does not contain a CRIB domain. Bem1 has multiple protein-protein interaction motifs that link polarity establishment proteins. The second SH3 domain associates with PAKs as well as another set of effectors called BOIs. Cdc42 binds the adjacent CI domain and Cdc24 binds to the carboxyl terminus PB1 domain[184]. Bem1 dynamically relocates throughout the cell cycle starting from diffuse throughout the cytoplasm in early G1, polarized to the growth site in late G1, bud tips and later to the mother-bed neck during M. Proper localization of Bem1 is essential for promoting local activation of Cdc42. Multiple studies that force mislocalization of Bem1 were shown to block polarization (described in section 1.5.2). Binding to Cdc24 is required for apical growth but not for localization of Bem1. Bem1 mutants defective in binding to Cdc24 localize to the same sites as wild type cells[206]. However, Cdc24 binding to Bem1 is required for Cdc24 to initiate negative feedback. Cdc24 binding is also required for linking Bem1 to the downstream pheromone receptor machinery during mating. Bem1Δ cells display severe
polarization defects, grow slowly at room temperature and are less efficient at polarizing Cdc42[207]. This is hypothesized to be due to failure to maintain polarization rather than defects in polarity establishment.

### 1.6.4 Exocyst

The exocyst is a complex that consists of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 and is required for exocytosis[208]. The complex localization is highly dynamic. The exocyst localizes at the prebud site, bud tips and disperses during isotropic growth[123]. Components of the exocyst complex associate with post-golgi vesicles and tether them to the membrane. Docking and fusion with the membrane allows for the delivery of cell wall remodeling components to be delivered to growth sites. Particular subunits within the complex, Exo70 and Sec3 are effectors of Cdc42 and Rho3[209]. Cdc42 binding leads to activation of the exocyst and helps to localize them. Out of the complex, these subunits are targeted to the bud tip even in the absence of actin[112, 208].

### 1.6.5 Boi 1 and 2

Boi1 and 2 (Bois) are effectors with redundant function for polarization and cytokinesis. Bois lack a CRIB domain, but bind Cdc42 via its PH domain. Bois also bind Bem1 and Cdc24[151]. Double deletion causes defects in polarization and inhibits exocytosis of a specific vesicle type. Several lines of evidence indicate a role of Bois in regulating exocytosis[210, 211]. Bois bind multiple exocyst components and the SNARE component Sec1 in vitro and in vivo. boi1Δ boi2Δ phenocopy other exocytosis mutants in
that they accumulate a specific vesicle type. Lastly, gain of function mutations in certain exocyst or SNARE components sufficed to restore exocytosis in boi1Δ boi2Δ.[212]

More recently, Bois have been shown to perform a second function that may, or may not be linked to its role in promoting polarized growth. In this role, Bois are assumed to be inhibitors of bud abscission during late mitosis. In yeast, the “Nocut” checkpoint allows cells to halt bud formation in the event of improper chromatin segregation. Although this aspect of Boi function is carried out by a different binding domain than required for their function in bud growth, exocytosis required to target Bois to the neck therefore there may be links between these two functions[212].

1.6.6 Gic1/Gic2

A family of conserved, homologous proteins called GTPase Interactive Component 1 and 2 (GICs) in yeast (Binder of Rho GTPase in humans) are effectors that remain poorly understood. GICs contain a consensus CRIB domain that binds GTP-Cdc42. A single deletion of either GIC is well tolerated, but simultaneous deletion produces a dramatic temperature-sensitive phenotype in haploid cells. At low temperatures, double deletion mutants appear as wild type however; elevating temperature causes vast defects in polarization of the actin cytoskeletons[213-215]. This phenotype is consistent with that of other, more characterized, polarization mutants and led to the postulation that GICs regulate the actin. The generally accepted model of actin cable assembly posits that Cdc42 recruits Bni1 to the polarization site through direct binding of Bni1’s GTPase binding domain. A caveat to this model was introduced by
Gao et al studies that showed solely expressing the catalytic carboxyl terminal of formins, lacking the Cdc42 binding region, was sufficient to lead to asymmetric actin cable organization[216]. This finding suggests that another entity may be responsible for localizing Bni1. Yeast two hybrid assays revealed Gic2 is capable of binding to Bni1 and one of its NPFs, Bud6[169]. These results support the hypothesis that GICs play a role in actin regulation. This phenotype coupled with the ability to bind preferentially bind to GTP-Cdc42 makes GICs ideal candidates for functioning in this capacity.

Similarly, data from electron microscopy studies uncovered direct interactions between GICs and septins. In order for septins to properly assemble into higher order structures each of the 4 septin units must be in tact and recruited to the cortex. Sadian et al. showed that GIC1 is able to bind the Cdc10 subunit of septins directly[217]. Removal of gic1Δ gic2Δ led to failure of septins to polarize or organize into rings.

Collectively, these findings implicate GICs in regulation of both cytoskeletons. As GICs are only essential at high temperatures, a compensatory pathway likely exits that is able to perform the role of GICs at low temperatures in gic1Δ gic2Δ cells. Msb3 and 4 are homologous proteins that have been proposed to function in parallel with GIC1s. GICs and Msb3/4 share interesting opposing phenotypes. As previously stated, GICs associate with GTP-Cdc42. Biochemical assays revealed that Msb3/4 bind preferentially to the inactive GDP-Cdc42. MSb3/4 belong to the TBC/PTM/GYP family of Rab GTPases Msb3/s serves as a Gap for the Rab GTPase Sec4 in vivo and in vitro and is required for efficient exocytosis[190]. Deletion of Msb2/3 is synthetic lethal with deletion of Gic1/2
which suggests a genetic interaction. The amino terminus of Msb3/4 term binds to another Bni1 binding protein, Spa2, directly. Double deletions grow slower than single deletes. Similarly to Gic1/2, both proteins localize to sites of polarized growth. In small buds Msb3/4 localizes to bud tips while in large buds they spread across the cortex.[218]

Although strong evidence for direct binding of Msb3/4 to Bni1 is still lacking, several studies have shown physical interactions between Msb3/4 and other Bni1 regulators Bud6 and Pea2 by yeast-two hybrid and pulled down in complexes[190].

While these findings are also interest, exploration of the potential involvement of MSB3/3 of their role as a compensatory pathway is beyond the scope of this work.

The study will examine the upstream function of GICs in promoting Cdc42 polarization and polarity establishment.
2. A Role for Gic1 and Gic2 in Promoting Cdc42 Polarization

2.1 Introduction

Regulation of cell shape is central to cell proliferation as well as many aspects of cell function. Cell shape is in large part governed by the cytoskeleton, which itself is regulated by multiple signaling pathways. Among the most prominent and ubiquitous cytoskeleton-regulating pathways are those mediated by evolutionarily conserved small GTPases of the Rho family, including Rho, Rac, and Cdc42 [219]. These GTPases are thought to act as molecular switches, toggling between an inactive GDP-bound state and an active GTP-bound state. Intrinsic rates of activation (GDP/GTP exchange) and inactivation (GTP hydrolysis) are slow, and can be greatly enhanced by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively [220]. Rho-family GTPases are prenylated and reside primarily on the cytoplasmic leaflet of cellular membranes, although they can be extracted to the cytoplasm by guanine nucleotide dissociation inhibitors (GDIs) [221, 222]. Signaling pathways controlling cell shape often act by regulating and localizing the activities of GEFs and GAPs, leading to specific spatiotemporal patterns of GTPase activity.

Information encoded by the abundance and spatial pattern of GTPase activity is decoded by a set of GTPase-specific “effectors”, which are proteins that bind to the active but not the inactive form of the GTPase. Most known effectors are cytoplasmic proteins whose activity and localization within the cell can change as a result of GTPase
binding. Effector localization and activity can also be regulated by other signals (e.g. phosphoinositides), allowing for complex combinatorial control of the cytoskeleton. Among the most intensively studied effectors are the p21-activated kinases (PAKs) [223], the WASP and WAVE regulators of branched actin nucleation by Arp2/3 complexes [224], and the formins that nucleate and accelerate polymerization of unbranched actin filaments [225]. In aggregate, GTPase signaling via effectors is responsible for sculpting the cytoskeleton.

One major role for Cdc42 and Rac concerns the establishment of cell polarity [226]. Studies of polarity establishment in the model yeast Saccharomyces cerevisiae led to the identification of both positive feedback and negative feedback loops built into the polarity circuit [164, 227]. In the positive feedback loop, effector PAKs are recruited to bind GTP-Cdc42, and they bind a scaffold protein called Bem1, which in turn binds to Cdc24, the yeast GEF for Cdc42 [184]. These interactions mean that wherever there is a slight local accumulation of GTP-Cdc42, recruitment of PAK-Bem1-Cdc24 will lead to enhanced GEF activity, leading to further local Cdc42 activation in a positive feedback loop [228]. Once GTP-Cdc42, PAKs, and Cdc24 co-accumulate to high levels due to positive feedback, the active PAKs promote multi-site phosphorylation of Cdc24 [146, 229, 230]. This phosphorylation reduces GEF activity [185], possibly by more than one mechanism [231], yielding a negative feedback loop. Thus, in addition to signaling to the cytoskeleton downstream of the GTPase, some effectors can also act as feedback transducers to regulate the local activation of the GTPase itself.
Analysis of several Cdc42 and Rac effectors, including the PAKs, led to the identification of a conserved Cdc42/Rac interactive binding (CRIB) motif that recognizes GTP-Cdc42 and GTP-Rac [232]. Bioinformatic searches for other CRIB-containing proteins identified the GTPase interacting components (GICs), Gic1 and Gic2, in *S. cerevisiae* [213, 214]. GICs are small proteins that encode membrane-binding amphipathic helices [233] and a short conserved GIC motif of unknown function [169] in addition to the CRIB domain. The mammalian binder of Rho GTPase (BORG) proteins have a similar organization and may constitute homologs of the GICs [234]. In yeast cells, GICs are concentrated at polarity sites marked by active Cdc42 [213, 214]. Deletion of either GIci or GIc2 does not produce a dramatic phenotype, but cells lacking both GICs are large and misshapen, and (in haploids) fail to proliferate at high temperature (37°C)[213, 214].

Subsequent work implicated GICs in regulating both the actin and septin cytoskeletons. In yeast cells, filamentous actin is present in actin cables (linear filament bundles oriented towards the polarity site that enable type V myosin-mediated cargo delivery to the bud) and in cortical actin patches (branched actin structures that promote invagination of the plasma membrane at sites of endocytosis)[235, 236]. In polarized cells, actin patches accumulate near the polarity site and cables are oriented towards that site. However, in *gic1Δ gic2Δ* haploids at 37°C, most cells display randomly distributed actin patches, and fail to form a bud [214]. Moreover, Gic2 interacts with and helps to
localize the formin Bni1 to the polarity site, providing a potential mechanism for actin regulation [237].

Septins are conserved filament-forming proteins that assemble into a ring surrounding the polarity site following polarity establishment in yeast [238, 239]. However, in gic1Δ gic2Δ haploids at 37°C, most cells fail to recruit septins to the polarity site [215]. GICs were shown to bind septins and affect interactions between septin polymers in vitro, providing a potential mechanism for septin regulation [215, 217].

In addition to the studies implicating GICs as mediators of Cdc42-induced actin and septin rearrangements, a genetic interaction was identified between GICs and the Ras-family GTPase Rsr1 [240]. Rsr1 mediates communication between various transmembrane “landmark” proteins, which mark preferred sites for subsequent polarization, and the Cdc42-based polarity establishment pathway [180]. Unlike gic1Δ gic2Δ or rsr1Δ mutants, which are viable at 24°C, gic1Δ gic2Δ rsr1Δ triple mutants were lethal. This suggested that GICs might act upstream of Cdc42, in parallel with Rsr1, as well as downstream of Cdc42.

Here, we have investigated the gic1Δ gic2Δ phenotype in greater detail, using live-cell imaging of cells bearing probes for polarity regulators Cdc42 and Bem1. We found that at 37°C, a majority of gic1Δ gic2Δ cells failed to polarize at all. This finding provides an alternative interpretation for previous findings in which the mutants failed to polarize actin or septins: these defects could be secondary effects stemming from a more fundamental lack of Cdc42 polarization. A subset of the gic1Δ gic2Δ did polarize
Cdc42 and Bem1 at 37°C, and those cells did not display any obvious difficulty in forming a bud, suggesting that downstream cytoskeletal defects (if present) were quite mild. We conclude that, as suggested by Kawasaki et al. [240], a major role of the GICs is to promote Cdc42 polarization.

2.2 Materials and methods

2.2.1 Yeast strains and growth conditions

The yeast strains used in this study are in the YEF473 strain background (his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52)[241] and are listed in Table 2.1. Standard yeast molecular and genetic manipulations were used to construct strains, with additional precautions due to the high propensity of strains lacking GICs to become genetically suppressed. GIC1 and GIC2 deletions were generated by the one-step PCR-based method [242] with pRS304 as template for gic1::TRP1 and pRS403 as template for gic2::HIS3.

Table 2.1: Yeast strains used in this study

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Deletions were introduced into diploid strains, and diploids containing at least one wild-type GIC gene were used as strain construction intermediates to avoid selection for suppressors. In cases where strain construction involved a haploid gic1::TRP1 gic2::HI3 intermediate, we introduced a URA3-marked 2 \(\mu\)m plasmid (pDLB2693) carrying wild-type GIC2 into the parent diploid strain and maintained the plasmid in the derived haploid so as to avoid selecting for suppressors. Loss of the plasmid was induced when needed by growth on plates containing 5-fluoro-orotic acid (5-FOA)[243].
Gene tagging at endogenous loci was previously described for Whi5-GFP [244], Whi5-tdTomato [245], Bem1-tdTomato [164] and Bem2-12myc [246]. The GFP-linker-Cdc42 probe was expressed in addition to endogenous untagged CDC42 (as the GFP-tagged version is not fully functional on its own) as described [185].

The Cdc24-3HA allele was generated by the PCR-based gene modification method [247]. Briefly, primers with 50 bp of CDC24 C-terminus and 3'UTR homology were used to amplify the pFA6 3HA kanMX cassette. The PCR product was purified and transformed to tag CDC24 via standard transformation methods. Proper integration was confirmed by PCR and sequencing.

The Bem3-12myc, Rga1-12myc, and Rga2-12myc constructs were made by cloning PCR products encoding the C-termini of the proteins into a pRS306-based integrating plasmid (pSWE1-myc) containing 12 myc tags and the SWE1 terminator [248]. Digestion at a site within the gene was used to target integration of the plasmid at the endogenous loci, and proper integration was confirmed by PCR checks.

Cells were grown on rich YEPD media (1% yeast extract, 2% peptone, 2% dextrose) or complete synthetic medium (CSM; MP Biomedicals) with 2% dextrose at 24°C as described below.

2.2.2 Isolation and analysis of suppressor mutants

The MATa gic1Δ gic2Δ strain DLY20961 was streaked for single colonies on YEPD plates at 24°C. 10 colonies were picked using sterile toothpicks, and the cells were resuspended in 1 mL of sterile distilled water and counted. 1 million cells from each
colony were spread onto individual YEPD plates. Each plate was incubated at 37°C for 3
days, after which plates displayed growth of numerous microcolonies and a few large
colonies. A large colony was picked from each of the 10 plates, and mated to non-
suppressed MATα gic1Δ gic2Δ strain DLY21941. Mating was conducted by mixing cells
on a YEPD plate, with a large excess of the MATα strain, so that most MATα cells would
mate. Cells from the mating mix were spread on YEPD plates containing 2 µM α-factor
to arrest unmated MATα cells, and colonies were tested to determine whether they
could sporulate (indicating successful diploid formation) when transferred to 2%
Potassium Acetate plates and allowed to grow for 5-7 days.

Asci from sporulating diploids were digested by treatment with lyticase for 5
min. Tetrads were diluted in sterile distilled water, spread on YEPD plates, and
dissected with a micromanipulator. Tetrads were incubated at 24°C or 37°C as
indicated for 3 days. Images of plates were taken on day 3. Spore colonies were replica
plated to relevant selective media plates to test for auxotrophic markers. Suppressor
strains were then crossed to each other and tetrads were analyzed using a similar
procedure.

2.2.2.1 Cell synchronization

For imaging experiments, the cells were first synchronized by G1 arrest/release.
MATα cells were grown overnight at 24°C in CSM+D, adjusted to 1.5x10⁷ cells/mL, and
treated with 2 µM α-factor (Genesee Scientific) at 24°C for 3 h. G1-arrested cells were
released from arrest by washing two times with fresh medium, and placed on microscope slabs at 37°C for imaging.

**2.2.2.2 Microscopy and image analysis**

Cells were mounted on a 250 μL slab solidified with 2% agarose on a microscope slide. After putting a cover slip on top, the edges were sealed with petroleum jelly to prevent evaporation. Image acquisition was done using an Andor XD Revolution spinning-disk confocal microscope (Olympus) with a Yokogawa CSU-X1 5000 r.p.m. disk unit, and a 100x/1.4 UPlanSApo oil-immersion objective controlled by MetaMorph software (Universal Imaging). The microscope is enclosed in a temperature-controlled chamber that was set to 37°C 1 h prior to imaging. Fluorophores were excited with 488 nm and 561 nm diode lasers. Images (stacks of 17 z planes spaced 0.5 μm apart) were collected at 1 min intervals using a iXon3 897 EM-CCD camera with 1.2x auxiliary magnification (Andor Technology). Laser power was set to 10% maximum output to reduce phototoxicity. Exposure time was 200 ms for each image. An EM-Gain setting of 200 was used for the EM-CCD camera.

Collected images were deconvolved using Hyugens Essential software (Scientific Volume Imaging). Images were then processed using ImageJ (National Institutes of Health). Z-stacks were collapsed into maximum projection images. Polarization was scored by eye as the first detection of a cluster of the polarity probe (GFP-Cdc42 or Bem1-tdTomato). Whi5 nuclear export was scored using a custom MATLAB graphical user interface (GUI; NucTrackV3.3) as described [249]. This tool allows for designation
and tracking of a region of interest at specific times of interest during the course of the time-lapse. For our purposes, regions of interest were individual cells. The coefficient of variation of Whi5 signal intensity between pixels in each cell was measured and used to determine the time point at which 50% of Whi5 exited the nucleus. Calculated values are normalized to peak intensity for each track. This tool is available upon request from Dennis Tsygankov (ude.hcetag.emb@voknagysT.sined).

2.2.2.3 Immunoblotting

Cells were grown overnight in YEPD at 24°C, and where indicated shifted to 37°C for 6 h prior to harvesting. Cell pellets (about 10⁷ cells) were resuspended in 225 μL cold Pronase buffer (25 mM Tris-HCl, pH 7.5, 1.4 M Sorbitol, 20 mM NaN₃, 2 mM MgCl₂) and 48 μL of 100% TCA. Pellet-buffer mixture was stored frozen at -80°C. Once thawed on ice, cells were lysed by vortexing with 280 μL of sterile acid-washed glass beads at 4°C for 10 min. Beads were washed twice with 5% TCA. Lysate was collected and precipitated proteins were pelleted by centrifugation at maximum speed in an Eppendorf centrifuge for 10 min at 4°C. Pellets were solubilized in Thorner buffer (40 mM Tris-HCl, pH 6.8, 8 M Urea, 5% SDS, 143 mM β-mercaptoethanol, 0.1 mM EDTA, 0.4 mg/ml Bromophenol Blue). 2 M Tris base was used to adjust the pH to 8. Samples were heated at 42°C for 3 min prior to loading on a 10% Acrylamide/Bis gel and run for 1 h at 40 mA. Following transfer, membranes were probed with anti-cMyc, or anti-HA (12CA5) (Roche) monoclonal antibodies and anti-Cdc11 polyclonal antibodies (Santa Cruz Biotechnologies) used at 1:1000 and 1:2000 dilution respectively. Secondary
antibodies IRDye800-conjugated anti-mouse IgG (Rockland Immunochemicals) and Alexafluor680-conjugated goat anti-rabbit IgG (Invitrogen) were used at 1:10,000 dilution. After washing, Western blots were visualized using the ODYSSEY imaging system (Li-COR Biosciences).

Western blot quantification was done using ImageJ to measure band intensity in individual color channels. Mutant and wild-type bands were always compared from the same blot using lanes with comparable Cdc11 loading controls. After dividing by the loading controls, bands were normalized to the wild-type signal.

2.3 Results

Polarity establishment in yeast is regulated by the cell cycle. In particular, activation of G1 cyclin-dependent kinase (CDK) complexes at a commitment point called start in G1 promotes Cdc42 polarization [229]. G1 CDK activation at start occurs through a transcriptional positive feedback loop in which rising CDK activity promotes the inactivation and nuclear export of the repressor Whi5, allowing more transcription of G1 cyclins [250-252]. Commitment to enter the cell cycle (i.e. start) occurs when 50% of nuclear Whi5 has been exported, at which point the positive feedback loop becomes self-sustaining [244]. We used Whi5-GFP or Whi5-tdTomato as a probe for start, and Bem1-tdTomato [164] or GFP-Cdc42 [185] as a probe for polarization. Haploid cells were grown at 24°C and arrested in G1 by treatment with mating pheromone. Arrested cells were released to proceed into the cell cycle by washing out the pheromone, and placed
on microscope slabs at 37°C. Live cell imaging by confocal fluorescence microscopy was then employed to monitor probe localization.

In wild-type cells under these conditions, Whi5 nuclear export was closely followed by Bem1 (Figure 2.1A, video 1) or Cdc42 (Figure 2.1B, video 2) polarization. However, in \textit{gic1Δ gic2Δ} cells there was a heterogeneous phenotype: a majority of cells failed to polarize either Bem1 (Figure 2.1A, video 1) or Cdc42 (Figure 2.1B, video 2) after Whi5 nuclear exit. A substantial minority of cells did polarize the probes, although polarity establishment occurred somewhat later than in wild-type cells. Quantification revealed that 30%-40% of \textit{gic1Δ gic2Δ} cells were able to form buds, but compared to wild-type cells the start-to-budding interval was longer (Figure 2.2A). Similarly, 30%-40% of \textit{gic1Δ gic2Δ} cells were able to polarize Bem1 or Cdc42, but with a longer and more variable interval between start and polarization (Figure 2.2B). For the subset of \textit{gic1Δ gic2Δ} cells that did polarize, the interval between polarization and budding was similar to that in wild-type cells (Figure 2.2C). Thus, the major defect exhibited by \textit{gic1Δ gic2Δ} cells at 37°C was an inability to establish polarity.
Figure 2.1: Delayed or blocked polarity establishment in gic1Δ gic2Δ mutants at 37°C.

(A) Schematic depicting Whi5 and polarity protein distributions as cells proceed through the cell cycle. In early G1 phase (pre-start), Whi5 is concentrated in the nucleus (green) and polarity factors are dispersed. As CDK activation occurs, Whi5 is exported from the nucleus (when 50% of Whi5 has been exported the cells commit to enter the cell cycle at “start”). CDK activation triggers localization of polarity factors to a cortical site (red: polarization) from which the bud later emerges (bud).

(B) Inverted maximum projection montages of selected timepoints for representative cells from movies of wild-type (WT: DLY19654) or mutant (gic1Δ gic2Δ: DLY20961) cells progressing through the cell cycle at 37°C. The cells express Whi5-GFP (top row) and Bem1-tdTomato (bottom row) probes. Cells were synchronized in G1 by pheromone arrest-release, and time relative to start is indicated. Scale bar, 5 µm.

(C) Display as for (B) but with strains expressing Whi5-tdTomato (top row) and GFP-Cdc42 (bottom row). Wild type: DLY21726. gic1Δ gic2Δ: DLY21728. For both sets of strains, wild-type cells polarized shortly after start, whereas gic1Δ gic2Δ cells either failed to polarize (cell 1) or polarized after a delay (cell 2).
Figure 2.2: Quantification of polarity establishment in gic1Δ gic2Δ mutants at 37°C.
Time intervals between start and bud emergence (A), between start and polarization (B),
and between polarization and bud emergence (C) were scored from the time-lapse
movies described in Figure 2.1. Top: schematics as in Figure 2.1A, indicating the interval
scored (red box). Bottom: graphs showing the cumulative % of cells (y axis) that
completed the interval by the indicated time (x axis). The number of cells scored for each
plot is indicated (n). (A-C) plot data for strains expressing Bem1-tdTomato (as in Figure
2.1B), while (B) additionally plots data for strains expressing GFP-Cdc42 (as in Figure
2.1C). For (C), we only scored the subset of gic1Δ gic2Δ cells that polarized (hence the
lower n).

Polarity establishment requires activation of Cdc42, which is promoted by the
GEF Cdc24 and antagonized by the GAPs Bem2, Bem3, Rga1, and Rga2 [180]. Thus, one
possible basis for the defect in polarity establishment in gic1Δ gic2Δ cells is that they
have insufficient GEF or excess GAP activity. As an initial attempt to test that hypothesis, we compared the abundance of these regulators in wild-type and \( \text{gic1}\Delta \text{gic2}\Delta \) cells. We noted no significant differences, either when the cells were grown at 24°C (Figure 2.3A) or 37°C (Figure 2.3B). Many of the regulators undergo phosphorylation, which is thought to regulate their activity [146, 162, 163, 185, 229, 230]. Although we detected altered-mobility species in many of the blots, we did not find any systematic difference between wild-type and \( \text{gic1}\Delta \text{gic2}\Delta \) cells.
Figure 2.3: Abundance of Cdc42-directed GEF and GAPs in gic1Δ gic2Δ mutants.

(A) Anti-HA Western blot to compare the abundance of Cdc24-3HA expressed at the endogenous locus in wild-type (DLY15429) and gic1Δ gic2Δ (DLY21815) strains (left). Anti-myc Western blots to compare abundance of Bem2-12myc (wild-type, DLY8228; gic1Δ gic2Δ, DLY22229), Bem3-12myc (wild-type, DLY11483; gic1Δ gic2Δ, DLY22232), Rga1-12myc (wild-type, DLY21093; gic1Δ gic2Δ, DLY22235), and Rga2-12myc (wild-type, DLY11847; gic1Δ gic2Δ, DLY22232) expressed at the endogenous loci. Loading control is a blot of Cdc11 (a septin) in the same lysates. Cells were grown to mid-log phase and lysates were prepared as described in Methods. Quantification of each blot (fluorescence intensity of secondary antibody for each regulator normalized to its corresponding loading control) is shown in the bar graph below each blot. When independent Western blots were performed, the number of blots is indicated and the bar graphs show mean and standard error of the mean. (B) Western blots were repeated using lysates from cells that were shifted to 37°C for 6 h prior to lysate preparation.
We next attempted a genetic approach to test whether mutations in regulators might enhance or suppress the phenotype of \( \text{gic1}^\Delta \text{gic2}^\Delta \) cells. Although \( \text{gic1}^\Delta \text{gic2}^\Delta \) mutants are lethal at 37°C following tetrad dissection (Figure 2.4A), this approach was thwarted by a high frequency of spontaneous suppression of the lethality. We speculated that because a subpopulation of mutant cells was able to bud (Figure 2.1 and 2.2), strong selection pressure could be applied to the expanding population, yielding a high spontaneous suppression frequency. Such suppression might occur at many loci or just a few, and we reasoned that in the latter case, identification of the basis for spontaneous suppression might be informative with regard to the specific molecular defect that prevents polarization of a majority of \( \text{gic1}^\Delta \text{gic2}^\Delta \) cells.
Figure 2.4: gic1Δ gic2Δ mutants spontaneously acquire a Mendelian suppressor mutation.

(A) gic1Δ gic2Δ mutants are inviable at 37°C. A diploid strain with the indicated genotype (DLY21711) was sporulated and tetrads (four spores in a vertical column) were dissected onto plates that were incubated at the indicated temperature. Tetrads contain two GIC1 gic2Δ spores and two gic1Δ gic2Δ spores. At 24°C all four spores were viable and gave rise to colonies, but at 37°C two spores from each tetrad died. Replica plating confirmed that the dead spores were the gic1Δ gic2Δ cells. (B) Isolation and genetic characterization of spontaneous gic1Δ gic2Δ suppressors. Cells of a gic1Δ gic2Δ strain (DLY20961) were streaked for single colonies. One million cells from each colony were plated on rich media and incubated at 37°C for 3 days. Although most cells died, several heterogeneously sized colonies were able to grow (example plate, bottom left), and one large colony from each independent plate was picked for further analysis. Suppressed cells were mated to a non-suppressed gic1Δ gic2Δ strain of opposite mating type (DLY21941), and the resulting diploids were sporulated and dissected as in (A). Tetrads showed 2:2 viability (middle panels) at 37°C indicating segregation of the suppressor as a single Mendelian locus. Independent suppressed strains (from different initial colonies) were then mated to each other and the resulting diploids were sporulated and dissected as in (A). All tetrads showed 4:0 viability at 37°C (right panels) indicating that the suppressors all map to the same locus. Sequencing confirmed that suppressed strains retained the gic1Δ and gic2Δ mutations.
We picked 10 independent unsuppressed haploid \textit{gic1}Δ\textit{gic2}Δ colonies growing at 24°C, and spread a million cells of each colony on a rich media plate that was incubated at 37°C. Multiple colonies arose spontaneously on each plate, ranging from large to tiny in size (Figure 2.4B). We picked a large colony from each plate, and mated them to an unsuppressed \textit{gic1}Δ\textit{gic2}Δ of the opposite mating type. Upon sporulation of the resulting diploids, viability at 37°C segregated 2:2 in tetrads in 9 cases, showing that suppression was due to a single Mendelian locus in these independently derived strains (Figure 2.4B and Table 2.2).

<table>
<thead>
<tr>
<th>Suppressor</th>
<th>% of tetrads segregating 2:2 for viability at 37°C</th>
<th>Number of tetrads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>92</td>
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<td>4</td>
<td>78</td>
<td>18</td>
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<td>5</td>
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<td>8</td>
<td>89</td>
<td>19</td>
</tr>
<tr>
<td>9</td>
<td>95</td>
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To assess whether the independent suppressors occurred at the same or different loci, we performed pairwise crosses between \textit{gic1}Δ\textit{gic2}Δ mutants carrying the different suppressors. In all cases, diploids generated by crossing one suppressed strain to
another showed 4:0 segregation for viability at 37°C (Figure 2.4B). This indicates that all suppressors are tightly linked, and likely to be in the same locus.

To characterize the suppressed phenotype, we performed live cell imaging of suppressed strains carrying Whi5-GFP and Bem1- tdTomato. We found that suppressed strains were similar to wild-type in terms of the efficiency and timing of polarization relative to start (Figure 2.5). Thus, suppression is highly effective in restoring the ability to polarize.
Figure 2.5: Suppressed *gic*Δ *gic*Δ mutants polarize like wild-type cells.

(A) Inverted maximum projection montages of selected timepoints for representative cells from movies of wild-type (WT: DLY19654) or suppressed *gic*Δ *gic*Δ (DLY22968) cells progressing through the cell cycle at 37°C. The cells express Whi5-GFP (top row) and Bem1-tdTomato (bottom row) probes. Cells were synchronized in G1 by pheromone arrest-release, and time relative to start is indicated. Scale bar, 5 µm. (B) Time intervals between start and polarization were scored from the time-lapse movies above as in Figure 2.2B. The number of cells scored for each plot is indicated (n). Data for unsuppressed *gic*Δ *gic*Δ cells is reproduced from Figure 2.2 to allow direct comparison to suppressed strain.

2.4 Discussion

Previous studies identified roles for Gic1 and Gic2 in regulating actin or septin organization downstream of Cdc42. As GICs are effectors that bind specifically to GTP-Cdc42, it was natural to expect roles of GICs acting downstream of Cdc42. However, the
discovery of synthetic lethality between rsr1Δ and gic1Δ gic2Δ mutants [240] indicated that GICs might also act upstream of Cdc42. Our major finding is that GICs are required for efficient and timely polarization of Cdc42 at 37°C, strongly supporting the conclusion that GICs act upstream of Cdc42.

There are several potential explanations for these findings. First, GICs might play dual roles, acting both upstream of Cdc42 and downstream of Cdc42 in separate pathways. A preliminary examination of the levels of known Cdc42 regulators did not reveal any differences between wild-type and gic1Δ gic2Δ mutant cells, but it remains possible that GICs affect the activity rather than the abundance of these regulators.

Second, GICs may simply act as downstream effectors of Cdc42, mediating cytoskeletal reorganization. Because Cdc42 is known to polarize even in the absence of F-actin [183, 253] or polymerized septins [254], this alone would not necessarily yield the observed defects in Cdc42 polarization. However, it could be that the particular cytoskeletal misregulation that occurs in gic1Δ gic2Δ mutants triggers a stress response that blocks effective Cdc42 polarization. Although stress pathways can act to block polarization [255, 256], we believe this scenario is unlikely.

Third, and perhaps most likely, GICs could operate as part of a positive feedback loop in which GTP-Cdc42 acts to promote further local accumulation of GTP-Cdc42. This would explain why cells lacking GICs have difficulties in polarizing Cdc42, and there is precedent for such feedback in the role of PAKs and Bem1 [184, 227]. However, the mechanism by which GICs might exert such feedback remains mysterious, and given
that cells already have one positive feedback pathway it is not immediately obvious why
they would require another.

Cells growing at 24°C do not require GICs for successful proliferation, indicating
that there are parallel pathways that can operate in the absence of GICs. Moreover, the
growth defect of haploid cells lacking GICs can be suppressed by overexpression of
Cdc42 [214], and diploid cells lacking GICs are able to proliferate successfully even at
37°C [257]. Other mutants (e.g. lacking the formin Bni1) display more severe phenotypes
in diploids than in haploids [257]. The basis for these differences is unclear. We found
that in our strain background, gic1Δ gic2Δ mutants frequently acquired spontaneous
suppressors, and a genetic analysis indicated that several independently isolated
suppressors all mapped to the same locus. Identification of the suppressor gene may
provide insight into the role of GICs in promoting Cdc42 polarization.
3. Conclusions

3.1 Upstream functions

3.1.1 Cdc42 activity

A major hypothesis arising from the results of this study is that GICs are involved in regulating Cdc42 activity. While the current data suggest this to be feasible, this question was not directly tested by any of the experiments performed. Future work on the function of GICs in promoting Cdc42 activity should include assays to assess the degree of Cdc42 activation in wild type and gic null strains.

3.1.2 Cdc42 regulator activity

3.1.3 Cdc24 subcellular localization

Cdc24 is known to be regulated at the level of nuclear-cytoplasmic shuttling (nuclear sequestration by Far1 reduces cytoplasmic GEF available for polarization [203] and inhibitory phosphorylation by Cla4[185, 203]. Future work will test whether GICs reduce nuclear sequestration of Cdc24 by examining Cdc24 localization in mutant cells. Future experiments may also test whether mutations that impair nuclear sequestration (e.g. cdc24-m1 or far1-H7 [258-260]) restore polarization in gic mutants.

3.1.4 Cdc24m1

3.1.5 Cdc24-38A

We will also assess whether a version of Cdc24 resistant to phosphorylation (Cdc24^{38A} [185]) restores polarization in gic mutants.
3.1.6 Non-phosphorylatable GAPs

This study showed GAP abundance to be unaltered in gic mutants. However, it could still be that GAP activity is increased, blocking polarization. The GAPs each have a C-terminal GAP domain and long N-terminal regulatory domains whose roles are not well understood. To assay GAP activity, we will use a recently validated [261] pull-down assay based on the idea that active (but not inactive) GAPs would bind tightly to the GTP-locked GST- Cdc42Q61L. Lysates from cell-cycle-synchronized populations of wild-type or gic mutant cells carrying epitope-tagged versions of each endogenous GAP will be incubated with beads carrying recombinant GST- Cdc42Q61L. The amount of GAP that remains on the beads after washing should reflect the level of active GAP in the cells. If the activity of one or more GAPs is increased in gic mutants compared to wild-types, that would suggest that GICs regulate GAP activity. We would proceed by asking whether GICs bind the relevant GAP (GICs are known to bind Rga1 but it is not clear whether they bind other GAPs), and if so whether that binding is important. This will involve making GIC mutants defective in GAP interaction but intact for binding Cdc42 (see above) and testing whether such GICs are capable of promoting polarization. We will also test whether deletion of the relevant GAP restores polarization in gic mutants, and if so we will pursue biochemical experiments directed at understanding how GICs regulate GAP activity.
3.1.7 Gic binding partners – do gics promote positive feedback?

As effectors of Cdc42, most previous studies have assumed that GICs act downstream of Cdc42 polarization [169, 213, 214]. However, my findings indicate that they can act upstream of Bem1 polarization. The simplest way to reconcile these views is that GICs participate in a positive feedback loop to help polarize Cdc42. Positive feedback is thought to be mediated by the ability of GTP-Cdc42 to recruit Bem1/Cdc24 complexes that then promote GTP-loading of neighboring Cdc42 [173, 184, 262]. The most obvious way that GICs could participate in positive feedback is by binding either Bem1 or Cdc24, so that a GIC binds simultaneously to both GTP-Cdc42 and Bem1 or Cdc24. Previous screens did not uncover physical interactions between GICs and Bem1 or Cdc24, but it remains possible that such interactions would only occur when GICs are bound to GTP-Cdc42, in which case they would have been missed by those studies. Thus, future work in this regard will be to test the ability of GICs to bind Cdc24 and/or Bem1 in the presence on GTP-locked Cdc42. If GICs do not bind Bem1 or Cdc24, they may still promote positive feedback. The best-characterized feedback loop involves interaction of GTP-Cdc42 with the PAK effector kinases Cla4 and Ste20, which in turn interact with Bem1. GICs can interact with Cla4 in the yeast two-hybrid assay [263], so it could be that GIC-Cla4 interaction increases recruitment of Bem1 and/or Cdc24 to the complex.

If GICs do play roles in positive feedback (whatever the mechanism), one question is why the already-characterized pathway is insufficient. We imagine that it
could be because the PAK-Bem1-GEF interactions are not strong enough on their own, so that one or more interaction needs to be assisted by GICs.

3.1.8 MSb3/4 relationship/ compensatory path

3.2 Downstream functions

3.2.1 Role of Gics in actin

GICs are required to polarize actin at high temperatures, but it remains unclear how. It is known GICs are able to bind Bni1; a formin that nucleates actin cables. However, gic-null mutants are unable to polarize Bni1 at high temperatures. To this end, it is worth exploring the filament dynamics in gic1- null cells in vitro. Biochemical to reconstituting filament dynamics in vitro, and genetic approaches to dissect how GICs contribute to Bni1 localization and activity.

3.2.2 Role of Gics in septin

GICs are required to polarize septins at high temperatures. Activated Cdc42 recruits GICs to the polarity site. GICs participate in multiple binding interactions in addition to binding Cdc42; GICs dimerize and bind septins. Presently the functional relevance of these interactions remains unknown. By determining which of these interaction(s) is required for recruiting septins to the polarity site, we can begin to distinguish competing models of how this process may occur. This question can be easily tested using the yeast two-hybrid system to isolate GIC mutants that are specifically deficient in interacting with septins, or in oligomerizing with other GICs.
Isolated mutants can then be used to assess whether these interactions mediate septin ring assembly \textit{in vivo}

\subsection*{3.3 Suppressor screen}

One of the outcomes of this study was the discovery of the high susceptibility of \textit{gic null} strains to spontaneous suppressor mutations. We successfully isolated 9 mutants that acquired spontaneous suppressors in the same gene. The high selection for this gene may indicate a potential protein that acts in parallel with GICs thus; future work will involve determining the identity of the gene involved in \textit{gic null} suppression.

\subsubsection*{3.3.1 Illumina sequencing}

Illumina sequencing is a highly robust platform that could be used to identify the differences in genomic loci between unsuppressed and suppressed strains. Sequencing results can then be validated through PCR and linkage analysis assays.

Once a gene has been identified, experiments can be performed to determine how the protein(s) encoded by the suppressed gene are able to circumvent the requirement for Gic1 and 2 at high temperatures. Sole identification of the gene involved would be valuable in generating new hypotheses regarding how GICs function in polarity establishment. Further, elucidation of the mechanism of suppression may provide insight to GIC function.
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**Biography**

Christine attended Emory University for her undergraduate studies in Anthropology with a concentration in human biology and a minor in global health. After graduating from Emory she was accepted into the Post Baccalaureate Research Education Program (PREP) at the University of Michigan. After completing the program she was accepted into the Cell and Molecular Biology Ph.D. umbrella program at Duke. After her first year she affiliated with the Pharmacology and Molecular Cancer Biology Department.