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OPTOGENETIC INVESTIGATION REVEALS ROBUST SYMMETRY BREAKING MECHANISMS IN *SACCHAROMYCES CEREVISIAE*

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Abstract

Cell polarization underlies many cellular and organismal functions. The GTPase Cdc42 orchestrates polarization in many contexts. In budding yeast, polarization is associated with a focus of Cdc42•GTP which is thought to self sustain by recruiting a complex containing Cla4, a Cdc42-binding effector, Bem1, a scaffold and Cdc24, a Cdc42 GEF. Using optogenetics, we probe yeast polarization and find that local recruitment of Cdc24 or Bem1 is sufficient to induce polarization by triggering self-sustaining Cdc42 activity. However, the response to these perturbations depends on the recruited molecule, the cell cycle stage, and existing polarization sites. Before cell cycle entry, recruitment of Cdc24, but not Bem1, induces a metastable pool of Cdc42 that is sustained by positive feedback. Upon Cdk1 activation, recruitment of either Cdc24 or Bem1 creates a stable site of polarization that induces budding and inhibits formation of competing sites. Local perturbations have therefore revealed unexpected features of polarity establishment.

Chapter 1: Introduction and overview of polarity establishment

Section1: Introduction to polarization

Cell polarity occurs in all kingdoms of life, from organisms as diverse as the prokaryotes *Escherichia coli* and *Caulobacter crescentus* to the eukaryotes *Saccharomyces cerevisiae*, *Caenorhabditis elegans, Drosophila melanogaster*, and vertebrate species (Dworkin 2009; Thompson 2012; Nance and Zallen 2011; Etienne-Manneville 2008). It is through polarization that cellular systems can execute such distinct events as asymmetric cell division, faithful segregation of cell fate determinants, and collective cell migration. Disparate species spatially organize specific cellular components to direct a myriad of cellular behaviors and morphogenetic events (Thompson 2012). For instance, epithelial cells polarize along their vertical axis to ensure tissue integrity and proper secretion (St Johnston and Ahringer 2010). Migrating cells polarize “front”-to-“back” to promote migration events necessary in wound closure and embryogenesis (Ridley et al. 2003). Further, single cells can spatially organize to undergo asymmetric cell division, often a critical early step in the development of a complex organism (Thompson 2012; Etienne-Manneville 2004). These processes are each coordinated by a common, evolutionarily conserved regulator, a molecular switch called Cdc42. Despite this conserved regulator, the deployment of both polarity determinants and the mechanisms that regulate them are diverse, yet converge to a single unifying idea: the formation of a stable asymmetry sufficient to orchestrate distinct cellular processes. How varying polarity schemes govern polarity determinants has been investigated in a suite of distantly-related organisms including *Caenorhabditis elegans, Drosophila,* and

Schizosaccharomyces pombe (Nance and Zallen 2011; Tepass 2012; F. Chang and Martin 2009).

Perhaps, one of the most well-studied polarization systems is bud site selection in *Sacchromyces cerevisiae* (Johnson 1999). Beginning as a symmetric cell early in the cell cycle, budding yeast develop a cortical patch of asymmetry in response to intrinsic signals. This small patch matures into a nascent bud, defining the axis of polarity and orienting growth to form a new daughter cell (F. Chang and Peter 2003; Howell and Lew 2012; Bi and Park 2012). As cellular polarization requires robust integration of dynamic signaling mechanisms, budding yeast represent a genetically tractable system in which more complex behaviors observed in higher organisms are present in a more simplified form. Therefore, investigations into the mechanisms that govern polarization in this single-celled organism can deliver insight into processes evolutionarily diverse organisms.

Section 2: Model Systems

The process of polarization is inherently complex as it requires the rapid formation of a stable asymmetry in response to signaling cues. To accomplish such rapid transitions, diffusive molecules must be employed (Thompson 2012). As such, active mechanisms must be in place to initiate - and subsequently maintain - asymmetries, despite the constant challenge of diffusion within a common a cytoplasm. Moreover, the axis of polarity is not necessarily permanent, as is the case in chemotaxing cells (Van Haastert and Devreotes 2004). In this instance, polarity mechanisms must withstand counteracting diffusive forces as well as retain plasticity. A prerequisite to understanding

the nature of such complex cellular programs, one must first identify the molecular players is requisite. Genetic analyses to identify these players have revealed a series of underlying principles in such wide-ranging cellular events as the asymmetric distribution of cell fate determinants, tissue integrity, and oriented growth. Here, we discuss several model systems that have been integral in the discovery and understanding of polarity schemes.

Section 2.1: Polarization in *C. elegans* embryos

Many important discoveries related to cell polarity emerged from genetic analysis of the one cell *C. elegans* embryo. In these large cells, intrinsic cues initiate polarization to establish two asymmetric domains, the interface of which delineates the site of cell division (Figure 1.1A). Cell fate determinants are asymmetrically distributed to each daughter cell, with the anterior daughter cell defining the lineage of multiple somatic tissues, while the posterior daughter cell is the ancestor to the germ line and other tissues (Guo and Kemphues 1996; Schubert et al. 2000). Early work employing genetic screens for maternal effect genes uncovered a group of proteins required for the establishment of these domains (Kemphues et al. 1988). Referred to as PARs (*par*titioning-defective), a defined subset concentrates anteriorly (anterior PARs) while a distinct group cortically accumulates in the posterior of the *C. elegans* embryo (posterior PARs). Surprisingly, this suite of diverse proteins initially had no obvious links to cytoskeletal machinery or other cellular mechanisms known to drive asymmetries (Goldstein and Macara 2007). Yet, the asymmetric distribution of these components is responsible for orchestrating the signaling events necessary to dictate the site of cell

division and ensure the localization of cell fate determinants, highlighting the critical role of PAR-mediated polarization in *C. elegans* embryonic development (Lyczak, Gomes, and Bowerman 2002; Hoege and Hyman 2013).

Prior to fertilization, the oocyte is symmetric with the anterior PAR module - consisting of two PDZ-containing proteins, PAR-3 and PAR-6, and a kinase, the atypical Protein Kinase C (aPKC) - extending across the entire cell cortex (Nance and Zallen 2011; Motegi and Seydoux 2013). Entry of the sperm pronucleus into the symmetric oocyte determines the posterior; this is the intrinsic symmetry breaking cue (Goldstein and Hird 1996). Polarization of the zygote ensues as the sperm aster orchestrates two critical events: 1) initiation of anterior-directed cortical flows, and 2) construction of radial microtubules that facilitate the deposition of the posterior PAR, PAR-2, a RING-finger domain-containing protein (Levitan et al. 1994; Motegi et al. 2011). Prior to symmetry breaking, the entire cortex is contractile. Upon microtubule contacts with the posterior end of the zygote, contractility is inhibited in the posterior, thereby driving the contractile network and the PAR-3-PAR-6-aPKC module toward the anterior (Munro, Nance, and Priess 2004; Motegi and Sugimoto 2006; Schonegg 2006). Simultaneously, PAR-2 recruits the posterior PAR, a kinase called PAR-1, whose primary function appears to phosphorylate and inhibit the accumulation of the anterior PARs on the cortex (Motegi et al. 2011; Hoege and Hyman 2013). Combining these efforts, the anterior PARs are locally excluded from the posterior end, thereby establishing anterior-posterior polarity.

Surprisingly, PAR-2 is not required for asymmetric concentration of anterior PARs, suggesting that cortical flows are sufficient to establish asymmetry (Cuenca 2003). However, asymmetric distribution of the anterior PARs is rapidly lost upon cessation of

cortical contractility (Nance and Zallen 2011; Hoege and Hyman 2013), indicating that establishment and maintenance of anterior-posterior polarity are distinct. Indeed, PAR-2 is required to maintain the asymmetric cortical domains. Moreover, both PAR-3 and PAR-6 are required for asymmetric distribution of PAR-2 (Boyd et al. 1996; Watts et al. 1996; Cuenca 2003). These data suggest that polarization in the one-cell *C. elegans* embryo can be temporally divided into two distinct phases: establishment and maintenance. Furthermore, maintenance of the asymmetric domains appears to engage a mutual exclusion mechanism. Specifically, the PAR proteins self-sort to promote the inclusion of domain-specific proteins while concomitantly excluding opposing proteins, thereby maintaining two stable domains within a freely diffusive cytoplasm (Goehring et al. 2011; Hoege and Hyman 2013). Indeed, recent work has uncovered that a dynamic cross-inhibitory pathway exists to maintain the polarized asymmetry (Sailer et al. 2015). Collectively, these data support a bimodal model of polarization in which cortical contractility and mutual exclusion cooperate to establish and maintain anterior-posterior asymmetry in the developing *C. elegans* embryo.

Section 2.2: Polarization in *Drosophila* epithelia

Despite the diverse make-up of PAR proteins, they are not unique to *C. elegans.* The PARs are evolutionarily conserved in *Drosophila* and vertebrate species (Suzuki 2006; McCaffrey and Macara 2009). While many cells and tissues polarize in these organisms, one of the better studied examples is the formation of apical-basal polarity in epithelial cells. Epithelia exist as an interconnected tissue, linked by specialized cell-cell junctions that both inhibit free diffusion of small molecules and ions through the tissue

as well as propagate morphogenetic, functional, and polarity information between cells (Baum and Georgiou 2011). During the formation of epithelial tissue, intracellular apical and basolateral domains are generated with cellular junctions at the interface of these two domains (Figure 1.1B) (Knust and Bossinger 2002; Thompson 2012).

Figure 1.1

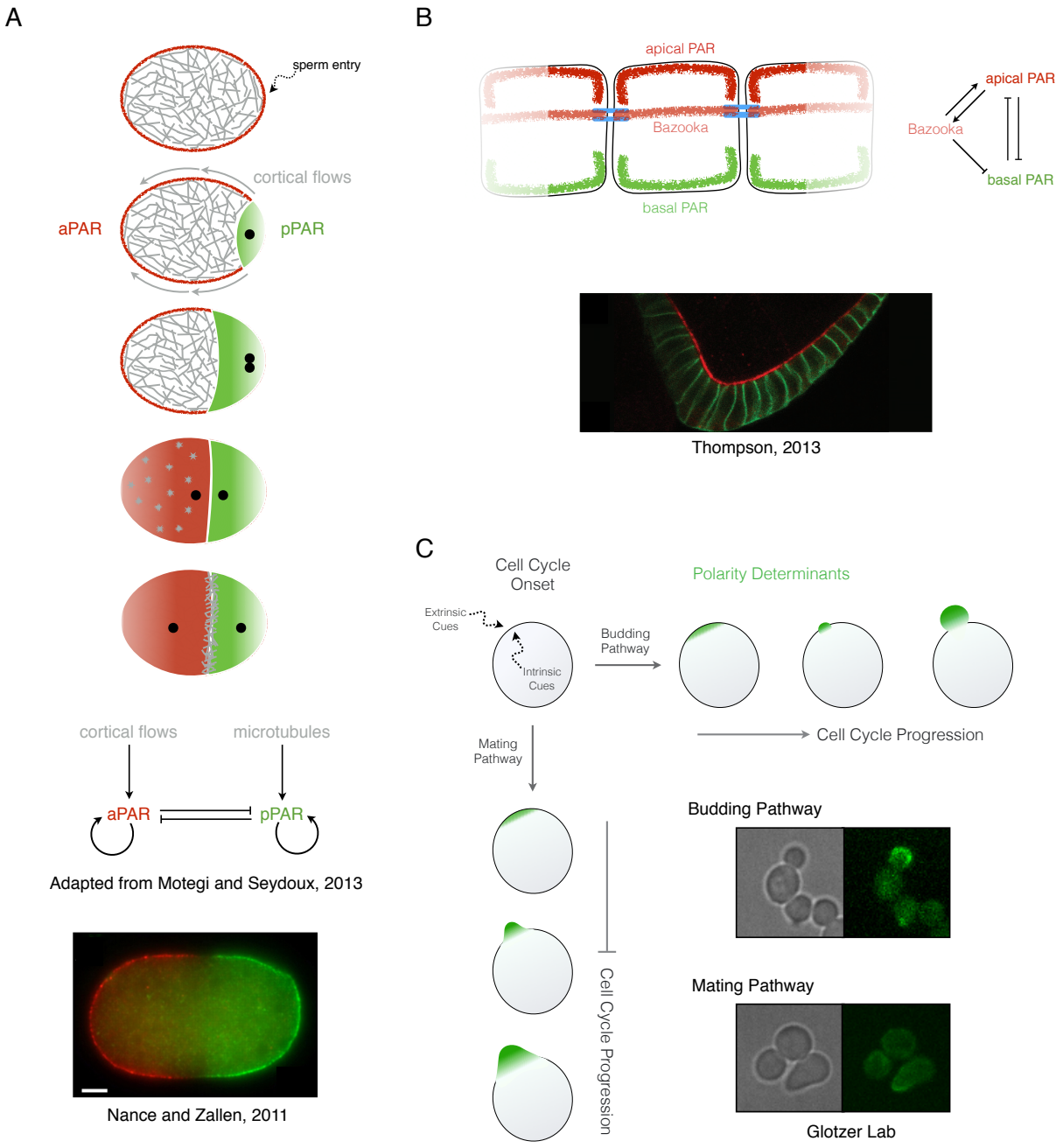


Figure 1.1 (continued): The process of polarization in *Caenorhabditis elegans* embryos, *Drosophila* epithelia, and *Saccharomyces cerevisiae.*
A) The one-cell *C. elegans* embryo polarizes along its anterior-posterior axis. Cortical flows and loading of a posterior PAR, PAR-2, drive the initially symmetric anterior PARs out of the posterior. Utilizing a mutual exclusion mechanisms, each PAR group reinforces its own accumulation while concomitantly opposing cortical association of opposing PARs, thereby establishing a stable asymmetry. At the interface of the polarized domains, the contractile ring assembles to induce an asymmetric cell division and promote the downstream events necessary for *C. elegans* embryonic development.
B) *Drosophila* epithelia employ the PAR module to establish apical-basal polarity. Bazooka, the PAR-3 homolog, concentrates at adherens junctions (blue), where it promotes concentration of apical PARs and inhibits the wandering of basal PARs into the apical domain.
C) Budding yeast undergo two alternative forms of polarization: bud site selection and mating projection formation. At the onset of the cell cycle, a symmetric cell receives either extrinsic or intrinsic cues to undergo mating or budding, respectively. In response to these cues, polarity factors concentrate to promote polarized growth. Entrance into the mating pathway halts cell cycle entry, whereas budding is intricately linked to cell cycle progression.

In *Drosophila*, the epithelial ectoderm is responsible for secreting components required for cuticle formation (Wieschaus, Nüsslein-Volhard, and Jürgens 1984), therefore it is critical that the epithelium polarize in a manner that facilitates secretion. Using a genetic screen for mutants defective in cuticle formation, a critical polarity determinant referred to as Bazooka was discovered (Wieschaus, Nüsslein-Volhard, and Jürgens 1984). Sequence homology and protein domain prediction later revealed Bazooka as the PAR-3 homolog (Kuchinke, Grawe, and Knust 1998). Similar methods were used to identify the *Drosophila* homologs of PAR-6, PAR-1, and aPKC (Petronczki and Knoblich 2001; Shulman, Benton, and St Johnston 2000; Wodarz et al. 2000). In accordance with a conserved role in mediating polarity, mutations in both PAR-6 and aPKC phenocopy that of Bazooka (Petronczki and Knoblich 2001; Shulman, Benton, and St Johnston 2000; Wodarz et al. 2000). Further work in mammalian epithelial cells revealed the existence of PAR homologs (Joberty et al. 2000; Qiu, Abo, and Steven Martin 2000; Johansson, Driessens, and Aspenström 2000), indicating that the PAR module is evolutionarily conserved across disparate species. How might PARs mediate

polarization in *Drosophila* and vertebrate epithelia? While the initial cue that directs polarization in epithelia is not well understood (St Johnston and Ahringer 2010), the dynamic interactions that establish and maintain apical-basolateral polarity in *Drosophila* epithelia have been well-defined (Müller and Wieschaus 1996; Plant et al. 2003; Yamanaka et al. 2003; Assémat et al. 2008).

Similar to *C. elegans* embryos, Bazooka (PAR-3) interacts directly with PAR-6 and aPKC to polarize their distributions toward the apical domain (Petronczki and Knoblich 2001; Wodarz et al. 2000; St Johnston and Ahringer 2010). However, unlike *C. elegans* embryos, components of the apical PAR complex does not constitutively colocalize (Hutterer et al. 2004; T. J. C. Harris and Peifer 2007). Prior to epithelia formation, *Drosophila* embryos begin as an unpolarized syncytium and establish polarity upon cellularization. Strikingly, Bazooka polarization is already apparent at the onset of cellularization, though the instructing cue is unknown (Mavrakis, Rikhy, and Lippincott-Schwartz 2009). Apically-enriched Bazooka coordinates the polarized accumulation of PAR-6 and aPKC. Subsequently Bazooka migrates basally, ultimately residing at the cell-cell junctions. Despite the lack of colocalization, the polarized distributions of PAR-6 and Bazooka are mutually-dependent, indicating a dynamic reinforcement amongst an unassembled apical PAR complex (Figure 1.1B) (Hutterer et al. 2004; K. P. Harris and Tepass 2008; Thompson 2012). Intriguingly, colocalization amongst the PARs is not observed in mammalian epithelia either (St Johnston and Ahringer 2010), suggesting that plasticity in the PAR module may have evolved.

Section 2.3: Polarization in budding yeast

Budding yeast exhibit asymmetric growth in two alternative cellular programs: 1) the construction of a mating projection or 2) the development of a new daughter cell. At the onset of the cell cycle, yeast cells are symmetric. Prior to cell cycle entry, extrinsic cues can halt cell cycle progression and direct polarized growth of a mating projection. Conversely, in the absence of extrinsic cues, cell cycle progression proceeds and intrinsic cues initiate bud formation at a unique cortical position (Figure 1.1C) (Etienne-Manneville 2004; F. Chang and Peter 2003); thus, asymmetric growth appears intricately linked to the cell cycle, suggesting that these two processes may be interdependent (Howell and Lew 2012). A seminal genetic analysis has been instrumental in elucidating these interdependent mechanisms (Hartwell, Culotti, and Reid 1970; Hartwell et al. 1973). The Hartwell screen was designed to identify regulators of the cell division cycle (*cdc*). By definition, all *cdc* mutants arrest at a particular stage of the cell cycle. An interesting class of these mutants failed to form nascent buds. Rather, these mutants arrested as large, unbudded cells that continued to grow in mass and volume until late in the cell cycle while retaining the ability to undergo DNA synthesis (Hartwell, Culotti, and Reid 1970; Hartwell et al. 1973; Adams et al. 1990). These observations indicate a specific defect at an early step in the budding process.

Two critical mutants emerged from this subclass: *cdc24* and *cdc42*. Phenotypic and molecular characterization of each revealed a necessary role in the formation of both mating projections and nascent buds, suggesting that Cdc24 and Cdc42 are essential for polarity establishment (Sloat, Adams, and Pringle 1981; Adams et al. 1990).

Molecular cloning revealed a high degree of similarity between Cdc42 and the Ras superfamily of GTPases (Johnson and Pringle 1990; Adams et al. 1990). Ultimately, identification of a mammalian Cdc42 homolog implicated in binding and hydrolyzing GTP validated Cdc42 as a Ras superfamily GTPase (Shinjo et al. 1990; Munemitsu et al. 1990).

Subsequently, Cdc42 homologs were identified in both *C. elegans* (W. Chen, Lim, and Lim 1993; Run et al. 1996) and *Drosophila* (Luo et al. 1994) and implicated in cell polarization, suggesting an evolutionarily conserved role throughout eukaryotes (Johnson 1999). Genetic perturbations of Cdc42 in *C. elegans* embryos induces symmetric cell division and uniform distribution of both anterior and posterior PARs, indicating that Cdc42 is required for proper polarization in *C. elegans* embryos. Indeed, Cdc42 binding to PAR-6 is necessary for maintaining anterior-posterior polarity (Aceto, Beers, and Kemphues 2006). Similarly, active Cdc42 and PAR-6 interact and polarize to the apical membrane of *Drosophila* epithelia. Furthermore, apical localization of PAR-6 and Cdc42 is required for proper polarization of both apical and basolateral polarity regulators (Hutterer et al. 2004; K. P. Harris and Tepass 2008; Thompson 2012). Thus, Cdc42 is a critical player in establishing and maintaining polarized domains in a wide variety of organisms and cell types (Johnson 1999; Etienne-Manneville 2004).

Section 3: Rho-family GTPases promote complex signaling events

Cdc42 is the master regulator of cell polarity throughout eukaryotic cells. It is part of the Ras superfamily of GTPases, proteins that regulate a host of diverse cellular programs. Specifically, Ras superfamily of GTPases function as molecular switches to execute

signal transduction pathways (Rojas et al. 2012). A subset of the Ras superfamily is the Rho-type GTPases (Hodge and Ridley 2016). These switches often govern cellular programs that involve the actomyosin cytoskeleton, such as cytokinesis (Kishi et al. 1993; Larochelle, Vithalani, and De Lozanne 1996; Drechsel et al. 1997), cell crawling (Nobes and Hall 1999; Ridley 2001), intracellular trafficking (Symons and Rusk 2003), and actomyosin-dependent shape changes (Etienne-Manneville and Hall 2002).

Rho GTPases primarily function at the the plasma membrane, their attachment to which is mediated by a C-terminal CAAX-box that can be post-translationally modified with a lipid anchor (Klooster and Hordijk 2007). They exist in either an “off” state, GDP-bound, or an “on” state, GTP-bound, with the “on” state sufficient to interact with downstream effectors that modulate signaling cascades; however, the intrinsic GTP hydrolysis and release of GDP is slow (Vetter and Wittinghofer 2001). Therefore, auxiliary proteins such as guanidine-exchange factors (GEFs) and GTPase activating proteins (GAPs) enable Rho GTPases to act as switches by accelerating the transition of the nucleotide state (Vetter and Wittinghofer 2001). Both GEFs and GAPs can localize to the plasma membrane in a regulated manner, thereby balancing the activation state of Rho GTPases. Once inactivated by GAPs, Rho GTPases are extracted from the plasma membrane by guanidine dissociation inhibitors to limit excessive GTPase activation (Dovas and Couchman 2005). The interplay of Rho GTPases and their respective auxiliary proteins suggests that GTPase activity can be elegantly fine-tuned by robust spatiotemporal dynamics (Hodge and Ridley 2016).

The most well characterized Rho GTPases are RhoA, Rac1, and Cdc42. While all predominantly function in cytoskeletal rearrangements, subdivisions amongst the

proteins are as follows: Rho is the central regulator of contractile actomyosin, Rac primarily regulates cell motility, and Cdc42 functions in cell polarization and filopodia formation (Ridley 2001). All are conserved throughout eukaryotic cells; however, Cdc42 retains close to 90% sequence conservation from yeast to humans whereas Rac1 and RhoA are not quite as well conserved, suggesting a role for Cdc42 that is particularly constrained (Johnson 1999; Park and Bi 2007).

Coordination amongst these GTPases can elicit a single biological response. For example, in fibroblasts undergoing wound healing, RhoA was responsible for the formation of contractile actomyosin structures and focal adhesions, Rac1 promoted lamellopodia extension, and Cdc42 facilitated filopoida dynamics (Nobes and Hall 1999). Additionally, Cdc42 has been shown to vectorially direct the activity of Rac1 in crawling cells (Allen, 1998), and an antagonistic relationship has been observed between Rac1 and RhoA under some conditions (Rottner, Hall, and Small 1999; Nimnual, Taylor, and Bar-Sagi 2003; Arthur and Burridge 2001; Ohta, Hartwig, and Stossel 2006). These studies highlight the complex relationships amongst Rho GTPases that promote a variety of complex biological processes. Frequently, Cdc42 is responsible for instructing the directionality of RhoA and Rac1, such as during cell migration (Hall 1998; Weiner et al. 2002; Kawasaki et al. 1999; Bi and Park 2012; O'Neill, Kalyanaraman, and Gautam 2016). Indeed, Cdc42 is required for directionality in migration, as cells without a functional Cdc42 are unable to migrate despite construction of the cytoskeletal machinery necessary for movement (Srinivasan et al. 2003; Allen et al. 1998; Etienne-Manneville 2008).

Section 4: Cdc42 in Polarization

The requisite nature of Cdc42 in polarity systems is conserved from yeast to vertebrate organisms, yet is critical to understand how Cdc42 locally directs the dynamic processes that establish polarity. Here, we investigate the mechanisms by which a localized patch of Cdc42 harnesses a suite of signaling cascades to establish an axis of polarity in *Saccharomyces cerevisiae*. Additionally, we discuss mechanisms that reinforce Cdc42 activation so as to maintain its asymmetric distribution.

Section 4.1: Polarity establishment in *Saccharomyces cerevisiae*

Cdc42 is required for polarity establishment in *S. cerevisiae* (Hartwell, Culotti, and Reid 1970). Specifically, it must localize to a discrete patch on the cell cortex where it then engages downstream pathways involved in exocytosis, actin assembly, and vesicle delivery (Johnson 1999). Budding yeast undergo two alternative forms of polarization each of which utilize similar machinery: mating projection formation and bud site selection.

Section 4.1.1: Cdc42 in the mating pathway

Budding yeast can proliferate as either haploid or diploid cells. Diploid cells are the fusion product of two haploid cells of opposite mating type, *MAT***a** or *MAT*α. Each mating type releases a pheromone that stimulates the opposite mating type to prepare for mating by undergoing numerous physiological alterations (Bardwell 2004). Yeast cells are so attuned to the pheromone, that concentrations in the low nanomolar range are sufficient to induce the mating program (Moore et al. 2008). One of the primary

morphogenetic changes that occurs is the construction of the mating projection ("shmoo") (Figure 1.1C). As yeast cells are non-motile, they translate the pheromone gradient into polarized growth toward their mating partner in a Cdc42-dependent manner (Reid and Hartwell 1977; Zhao et al. 1995; Simon et al. 1995). Strikingly, polarized cells will abandon shmoos in favor of a new, more concentrated pheromone signal, underscoring the plasticity of this polarization scheme (Dyer et al. 2013). When presented with a uniform gradient, cells will break symmetry by randomly orienting their axis of polarity (Madden and Snyder 1992; Dyer et al. 2013). What is the mechanism of polarity establishment in the budding yeast mating pathway?

Prior to cell cycle entry, cells can initiate the mating program in response to a pheromone gradient (Figure 1.2A) (Hartwell et al. 1974). Pheromones of the opposite mating type bind cell surface receptors known as heterotrimeric G protein receptors. Binding of the pheromone causes the Gα subunit to exchange GDP for GTP and release from the G$\beta\boldsymbol{\gamma}$ heterodimer (Dohlman 2002). The primary signaling cascades proceed through G$\beta\boldsymbol{\gamma}$, Ste4 and Ste18, respectively, with their output locally concentrated to promote shmoo growth. Specifically, Ste4 mediates two interdependent signaling pathways: 1) a mitogen-activated-protein kinase (MAPK) pathway, and 2) the Cdc42 polarization module (Bardwell 2004). The MAPK signaling cascade and the Cdc42 polarization module crosstalk through a variety of effector proteins. The Ste4 signaling pathway interacts directly with an adaptor protein called Far1, which binds to the Cdc42 GEF, Cdc24. The Far1-Cdc24 complex is sequestered into the nucleus of haploid cells early in the cell cycle (Butty et al. 1998; Nern and Arkowitz 2000; Shimada, Gulli, and Peter 2000). When the heterotrimeric G protein cascade activates in

response to a pheromone gradient, Ste4 recruits the Far1-Cdc24 complex to a discrete cotical patch, thereby localizing Cdc42 activation (Valtz, Peter, and Herskowitz 1995; Butty et al. 1998; Shimada, Gulli, and Peter 2000). Morevoer, activated Ste4 locally biases components of the the MAPK pathway by directly recruiting the scaffolding

Figure 1.2

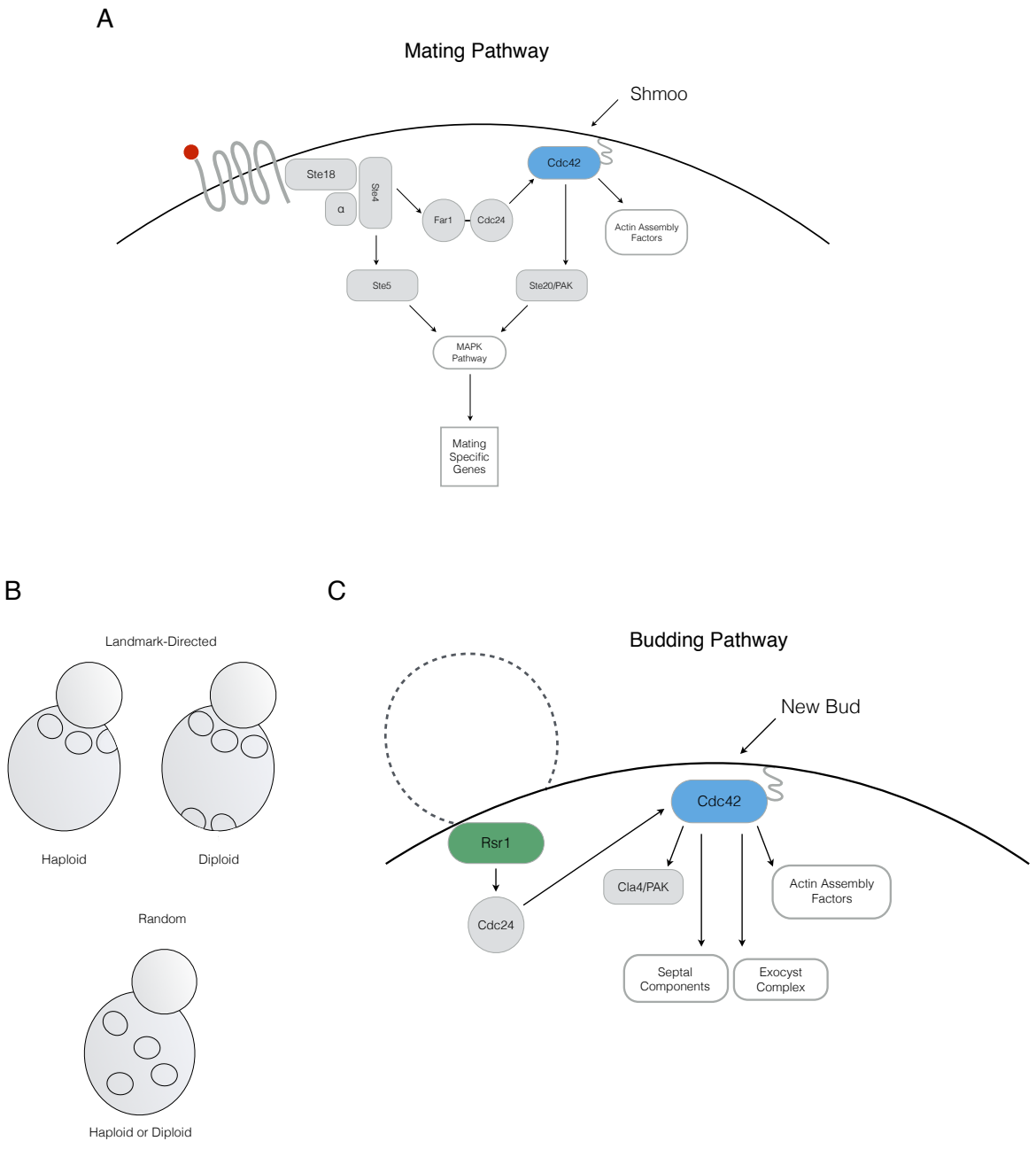


Figure 1.2 (continued): Polarized growth in budding yeast occurs in both mating and budding.
A) The mating pathway in budding yeast is activated prior to cell cycle entry in response to a pheromone gradient (red circle). Pheromone activates the heterotrimeric G protein receptor, causing dissociation of the Gα subunit from Gβγ, Ste4 and Ste18, respectively. Ste4 recruits the Far1-Cdc24 complex to locally activate Cdc42, which interacts with the PAK, Ste20. Ste20 and the scaffold protein Ste5 cooperate to induce MAPK signaling and expression of mating specific genes. Local activation of Cdc42 also recruits downstream effectors, such as cytoskeletal machinery, to construct the shmoo and promote mating.
B) Landmark-instructed budding leads to invariant budding patterns. Haploid cells bud axially, while diploid cells bud in a bipolar fashion. In the absence of landmarks or the signals that propagate those cues, both haploid and diploid cells bud from random cortical positions.
C) Rsr1 specifies the axis of polarity by instructing local accumulation of Cdc42-GTP through interactions with the GEF Cdc24. The local focus of Cdc42 interacts with downstream signaling components, including Cla4, cytoskeletal machinery, and the exocyst complex to promote bud growth.

protein Ste5. A downstream effector of Cdc42, the p21-activated kinase (PAK) - Ste20 in this case - activates members of the MAPK pathway that are assembled on Ste5, serving as yet another link between these two signaling cascades. Thus, Cdc42 activation directly promotes the mating program.

Temperature sensitive Cdc42 (*cdc42-ts)* mutants derived from the Hartwell screen (Hartwell et al. 1973), are not only unable to bud, but are also defective in mating. Early work showed that *cdc42-ts* cells do not construct a mating projection in response to pheromone treatment (Reid and Hartwell 1977). Later work confirmed that localized accumulation of both the GEF, Cdc24, and active Cdc42 were required for polarization of the actin cytoskeleton, polarized vesicle delivery, and subsequent polarized growth of the shmoo (Evangelista et al. 1997; Shimada, Gulli, and Peter 2000; Bardwell 2004). However, Cdc42 does not solely function in polarized growth. Through direct interactions with the Ste20-Ste5 module, Cdc42 regulates the MAPK signaling cascade which functions to induce expression of gene systems necessary for mating (Simon et al. 1995; Lamson, Winters, and Pryciak 2002; Bardwell 2004). Collectively, these results indicate that Cdc42-GTP is a critical orchestrator of the mating pathway. Cdc42 is

required for both polarity establishment in mating projection formation and for expression of mating-specific genes.

Section 4.1.2: Cdc42 in bud site selection

Saccharomyces cerevisiae propagate by budding, a process that begins at the start of the cell cycle. In wildtype yeast the axis of polarity is predetermined by the deposition of proteins at specific cellular domains. These proteins act as landmark-directed cues to yield an invariant budding pattern. Haploid cells bud in an axial fashion, where *de novo* bud sites cluster around the previous bud site (Figure 1.2B). Conversely, wildtype diploid cells bud in a bipolar pattern, alternating poles with each successive cell division (FREIFELDER 1960; Hicks, Strathern, and Herskowitz 1977; Sloat, Adams, and Pringle 1981). While there are several landmark proteins, the Ras-related GTPase, Rsr1, is essential for transducing the landmark signal to the polarity module (Bender and Pringle 1989). In its GTP-bound form, Rsr1 binds directly to the Cdc42 GEF, Cdc24, thereby locally concentrating Cdc42 activation toward a provided cue (Park et al. 1997). Recent work has shown that local concentration of Cdc42 is necessary for polarity establishment (Woods et al. 2015). Strikingly, cells lacking spatial cues efficiently establish polarity in a process known as symmetry breaking.

Disruption of landmark-directed cues randomizes the budding pattern (Figure 1.2B) (Bender and Pringle 1989). It is thought that Cdc42-GTP and Cdc24 locally concentrate to a random cortical domain, thereby establishing the axis of polarity (Irazoqui, Gladfelter, and Lew 2003; Woods et al. 2015). These data suggest that landmark-independent mechanisms exist to concentrate active Cdc42 at one and only one site. This is in contrast to the polarization scheme in the mating pathway which has the

capacity to reorient its growth after axis formation. How yeast form one and only one axis remains a critical question in the field.

Once stably associated to a discrete domain, active Cdc42 interacts with downstream effectors that are required for proper growth of the bud (Bi and Park 2012). These include, but are not limited to, the PAK Cla4, which is a functional and structural homolog to Ste20 (Cvrcková et al. 1995; Bagrodia and Cerione 1999), actin assembly factors such as the formins Bni1 and Bnr1 (Evangelista et al. 1997; Pruyne et al. 2004) the exocyst complex (X. Zhang et al. 2001; Casamayor and Snyder 2002), and scaffold proteins that promote septin ring assembly (G. C. Chen, Kim, and Chan 1997; Iwase et al. 2006; Bi and Park 2012). Thus, Cdc42-GTP not only establishes the axis of polarity, it integrates a multitude of biological processes necessary for bud formation and growth (Figure 1.2C).

Activated Cdc42 is necessarily required for successful polarization and maintenance of a single axis of polarity, a phenomenon known as singularity; thus, one must consider the mechanisms that locally concentrate Cdc42. Both membranes and cytoplasm are quite diffusive and studies have shown that polarized Cdc42-GTP rapidly exchanges between the plasma membrane and the cytoplasm (Wedlich-Soldner 2004; Slaughter et al. 2009). Therefore, successful polarization must engage active measures that can overcome diffusion.

Section 4.2: Initial cues, bias, and amplification: how cells establish and maintain polarity

How do cells concentrate factors within a symmetric cytoplasm, and subsequently retain those factors in a stable asymmetry? Ample evidence from diverse species suggest that this is achieved by a feedback system that amplifies the initial signals that inform the axis of polarity (Etienne-Manneville 2004; Thompson 2012). The initial signals can be provided extrinsically, intrinsically, or perhaps by stochasticities at the plasma membrane. In response to the initial cue, a bias of activated Cdc42 appears at that position. Active Cdc42 concentrates by interacting with downstream effectors, some of which likely encourage additional accumulation Cdc42. This process is referred to as positive feedback (Jilkine and Edelstein-Keshet 2011).

In his seminal work, Alan Turing showed mathematically that simple diffusion was sufficient to transform an equally mixed system of two interacting species into a stable asymmetric system (Turing 1952). Curious as to how patterns arise in biological systems, he set out to interrogate whether freely-diffusing morphogens in a zygote are sufficient to define the organismal body plan. He established the conceptual framework such that one species was a slow-diffusing activator while the other was a fast-diffusing inhibitor (Figure 1.3A). Under this scheme, activated molecules are slow to diffuse away from the activation site, thereby generating a focus of activated species. Activated molecules that exit the focus are rapidly inactivated by the inhibitor. Critically, this system is sufficient to amplify small perturbations within the symmetrical cytoplasm, thereby establishing and maintaining an asymmetric domain (Turing 1952).

Later theoretical work expanded upon Turing's findings - who faced an untimely demise in 1954 - as his linear solutions were unstable and unlikely to withstand the complexity of biological phenomena (Gierer and Meinhardt 1972). Turing's simple reaction-diffusion

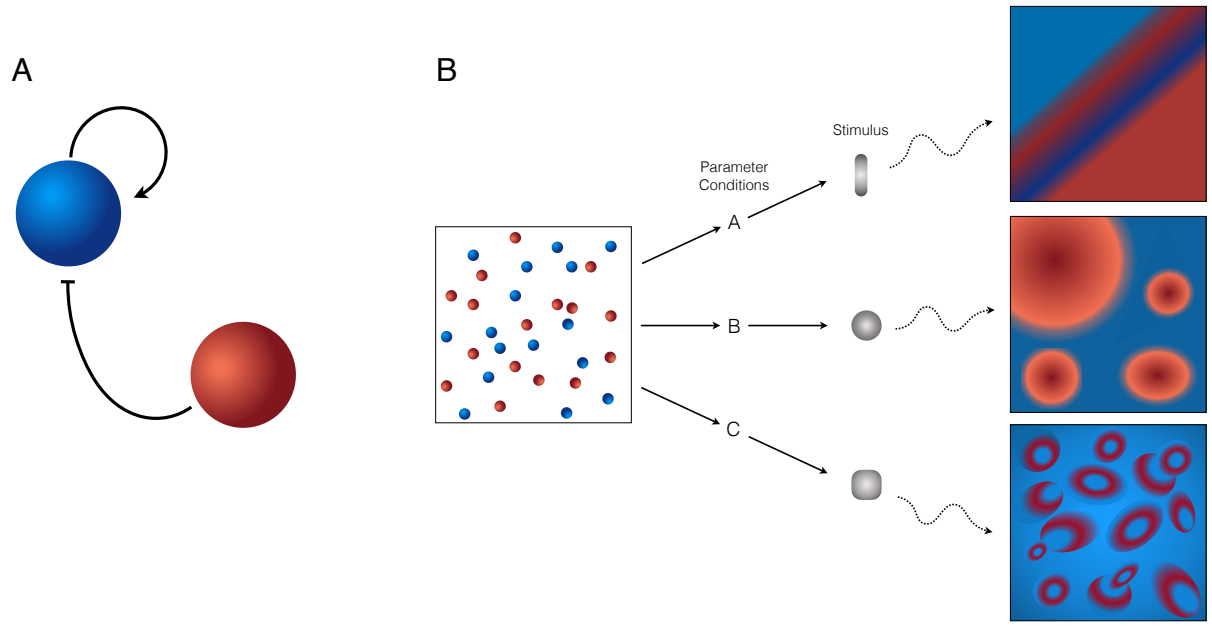


Figure 1.3: Turing-type reaction-diffusion mechanisms are sufficient to form complex patterns.
A) Schematic of two diffusing molecules, participating in Turing reaction-diffusion system. The blue molecule undergoes positive feedback while the red molecule inhibits the blue molecule
B) Diagram of Turing patterns that can emerge from an equally mixed system of molecules that react with each other upon a stimulus. Complex patterns including stripes, ovals, and unique shapes can emerge depending on parameter conditions.

mechanism were built upon by endowing the activator with local autocatalytic behavior, by giving the inhibitor long-range activity, and by accounting for the local density of both activator and inhibitor (Figure 1.3A) (Gierer and Meinhardt 1972). Autocatalysis can be achieved by a local increase in either the density or the activity of the activator, and it need not be solely dependent on the activator itself. Scaffolding or auxiliary components can provide the requisite domains necessary for autocatalysis. However, if left unfettered, the activator will ultimately spread throughout the cortex rather than remain discretely concentrated. Thus, inclusion of a long-range inhibitor - which in some cases may be a direct product of the activator - is necessary for a stable asymmetry (Gierer and Meinhardt 1972). Utilizing this simple scheme, exquisite patterns can emerge from the tuning of parameters and stimuli (Figure 1.3B). Indeed, almost all polarized cells

coordinate some type of positive feedback coupled to a slow-acting inhibitory response to establish and/or maintain their axis of polarity.

Section 4.3: The role of positive feedback in polarity establishment

The mechanics of positive feedback are well characterized in a variety of polarized biological processes and polarized cell types. This section will focus on the mechanisms by which two closely related yeasts, fission yeast and budding yeast, employ positive feedback in polarization.

Section 4.3.1: Positive feedback in fission yeast polarity

The mechanism of positive feedback in fission yeast contains the hallmarks of a traditional Turing-type reaction-diffusion system (Das and Verde 2013). Following cell division, the landmark protein Ras1 accumulates at the previous site of cell division (Hughes, Fukui, and Yamamoto 1990). It interacts directly with the primary Cdc42 GEF, Scd1, leading to local activation of Cdc42 (E. C. Chang et al. 1994). Cdc42-GTP recruits downstream effectors necessary for growth and elongation of the rod-shaped cell in a monopolar fashion (F. Chang and Martin 2009; Das and Verde 2013). Once the cell reaches a threshold length late in the cell cycle, a process termed New End Take Off (NETO) initiates bipolar growth until it achieves the a minimal length necessary for cytokinesis (F. Chang and Peter 2003). It is unclear how Cdc42-GTP begins to accumulate at the new end, though there is evidence that phosphorylation cascades mediated by Cdc42-GTP trigger NETO. For example, a downstream effector of Cdc42-GTP is the fission yeast homolog of PAK, Shk1. A non-lethal hypomorphic allele of Shk1

restricts growth to a single pole (Verde, Wiley, and Nurse 1998; Sawin, Hajibagheri, and Nurse 1999), suggesting a critical role in establishment of bipolar growth. Intriguingly, Shk1 interacts with a scaffold protein Scd2 that also directly interacts with the Cdc42 GEF, Scd1. Furthermore, accumulation of Shk1 to cell tips is dependent upon localization of Scd1 and Scd2 (Kelly and Nurse 2011). Thus, the Shk1-Scd2-Scd1 complex forms the requisite domain structure to mediate positive feedback (Figure 1.4A) (E. C. Chang et al. 1994; Wheatley and Rittinger 2005; F. Chang and Martin 2009).

Recent work that aimed to interrogate the events necessary for NETO, uncovered a striking phenomenon (Das et al. 2012). At the onset of Cdc42-GTP accumulation to the new end, activated Cdc42 displayed anti-correlative oscillations between the two poles (Figure 1.4A). Oscillations are necessarily derived from negative feedback (Houssine Snoussi 2011; Alon 2006), suggesting an active mechanism to minimize accumulation of Cdc42-GTP. To understand the oscillatory accumulations of activated Cdc42, the authors developed a mathematical model that invoked an initial fast-acting positive feedback loop and a delayed negative feedback loop. The results of the simulation captured the biological phenomenon as well as recapitulated the expected responses given biological perturbations (e.g. changes in the abundance or activity of Cdc42). Therefore, invoking positive and negative feedback in the model was sufficient to mimic the endogenous behavior. Furthermore, the authors found that both Scd1 and Scd2 undergo similar oscillations, indicating that their behaviors are linked to Cdc42-GTP. Finally, the authors interrogated the negative feedback arm of the model.

The PAK homolog in budding yeast, Cla4, has been suggested to inhibit the GEF activity of Cdc24 (Gulli et al. 2000; Kuo et al. 2014; Rapali et al. 2017); thus, a similar process may occur in fission yeast. The authors utilized the aforementioned hypomorphic allele of Shk1, which contains a point mutation that decreases its kinase activity (Verde, Wiley, and Nurse 1998; Sawin, Hajibagheri, and Nurse 1999; Das et al. 2012), and observed the localization of Cdc42-GTP, Scd1, and Scd2. In that background, all three components are retained in a monopolar orientation suggesting that kinase activity is required for bipolar, and thus oscillatory, behavior (Das et al. 2012). As such, these results are consistent with active Cdc42 mediating its own negative regulation through Shk1 kinase activity, thereby embodying a Turing-type reaction-diffusion mechanism (Figure 1.4A).

Section 4.3.2: The model of symmetry breaking in budding yeast

As polarization in *Saccharomyces cerevisiae* is one of the best-studied polarity examples, the molecular players involved in establishment and maintenance are well-defined. Primarily, research has focused in landmark-deficient backgrounds, as cells can potently break symmetry in the absence of these spatial cues. The current model of symmetry breaking suggests that the initial cue is provided by a stochastic cortical accumulation of Cdc42-GTP. The activated GTPase appears to induce a positive feedback loop, mediated by a tripartite complex consisting of the Cdc42 GEF, Cdc24, the scaffold protein, Bem1, and Cla4, which binds directly to activated Cdc42 (Figure 1.4B, C) (Gulli et al. 2000; Bose et al. 2001; Butty et al. 1998). These binding interactions are reminiscent of those observed in fission yeast and, as such, also provide the requisite domains to facilitate positive feedback. Pointed interrogation into

Figure 1.4

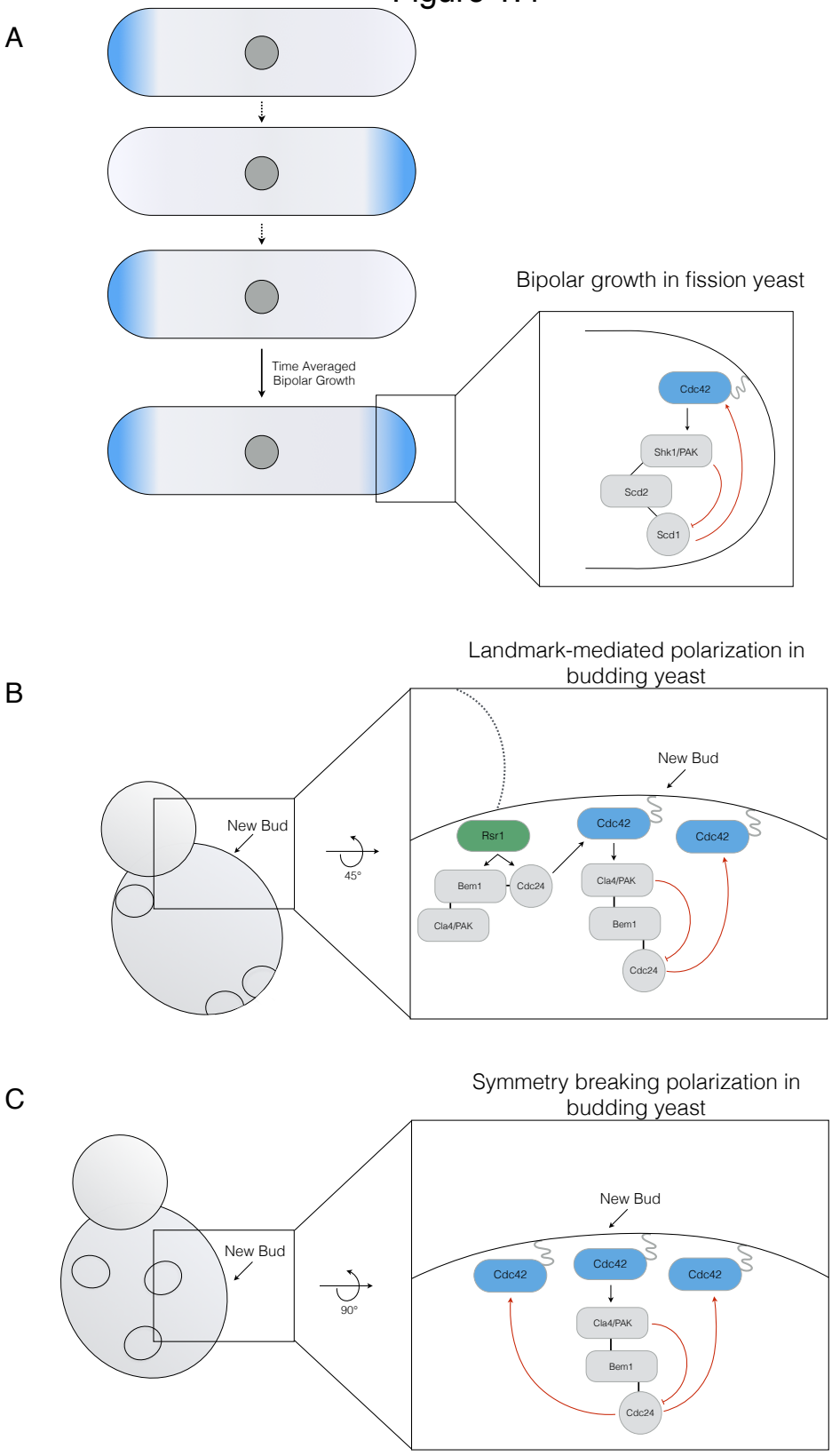

A
Time Averaged
Bipolar Growth
Bipolar growth in fission yeast
Cdc42
Shk1/PAK
Scd2
Scd1
B
Landmark-mediated polarization in
budding yeast
New Bud
45°
Rsr1
Cdc42
Cdc42
Bem1
Cdc24
Cla4/PAK
Cla4/PAK
Bem1
Cdc24
C
Symmetry breaking polarization in
budding yeast
New Bud
90°
Cdc42
Cdc42
Cdc42
Cla4/PAK
Bem1
Cdc24

Figure 1.4 (continued): Positive feedback in fission and budding yeasts.
A) Bipolar growth in fission yeast oscillates from pole to pole, with time-averaging leading to equal growth from each end. Cdc42 is locally concentrated at the poles via a positive feedback loop that engages the tripartite polarity complex Shk1-Scd2-Scd1. Scd1 activates additional molecules of Cdc42 where as Shk1 appears to participate in a phosphorylation-dependent negative feedback loop.
B) In landmark-mediated polarization in budding yeast, Rsr1 interacts directly with Bem1 and Cdc24 to locally activate Cdc42 proximal to the previous bud site. Cdc42-GTP interacts with downstream effectors, such as Cla4. The Cla4-Bem1-Cdc24 complex mediates positive feedback to establish the incipient bud site. A negative feedback loop appears to proceed through Cla4-dependent phosphorylation of Cdc24, thereby limiting growth of the nascent site.
C) The model for symmetry breaking polarization suggests that a stochastic accumulation of Cdc42 appears on the cortex where it engages the Cla4-Bem1-Cdc24 polarity complex in a potent positive feedback loop. Cla4-dependent phosphorylation appears to down-regulate Cdc24 GEF to hinder expansion of the Cdc42 focus.

this tripartite complex has revealed that the scaffold protein Bem1 is critical for bud emergence.

Early work showed that *bem1Δ* cells display severe defects in polarized growth and often become swollen, multinucleated cells (Bender and Pringle 1991; Chenevert, Valtz, and Herskowitz 1994), suggesting a Bem1-dependent defect in bud emergence. Furthermore, *bem1Δ/rsr1Δ* is synthetically lethal (Irazoqui, Gladfelter, and Lew 2003), and Bem1 has been shown to bind directly to Rsr1 (Park et al. 1997). Combined, these results indicate that Bem1 plays a critical role in bud emergence, potentially by modulating cortical association of the polarity complex. Indeed, Bem1 localizes to incipient sites of polarized growth, is required for stable accumulation of the GEF Cdc24, and must bind Cla4 for cells to undergo symmetry breaking (Gulli et al. 2000; Kozubowski et al. 2008; Howell and Lew 2012). Additionally, other work has suggested that Bem1 binding to Cdc24 may relieve the GEF of autoinhibition (Butty, 2001) (Shimada, 2004), suggesting that Bem1 engages in a multi-faceted approach to positively regulate bud emergence.

To determine whether a crucial function of Bem1 is to serve as a scaffold protein that links the Cdc24 GEF to an active Cdc42 binding protein, a fusion protein consisting of Cla4 and a variant of Cdc24 that lacks its Bem1-binding domain (Cdc24ΔPB1) was expressed in *bem1Δ/rsr1Δ* cells. The Cdc24ΔPB1-Cla4 fusion recovered viability, polarized growth, and cells proliferated with similar kinetics as *rsr1Δ* cells, thereby directly showing that an essential function of Bem1 is to link Cla4 and Cdc24 (Kozubowski et al. 2008). Collectively, these results support a model in which symmetry breaking proceeds by a positive feedback loop (Figure 1.4B, C). Complementary to this study, a computational model was developed that effectively recapitulated symmetry breaking in budding yeast by using positive feedback (Goryachev and Pokhilko 2008). By invoking a minimal set of molecular components, including Cdc42, Cdc24, Bem1, a GAP, and the Cdc42 GDI (Rdi1 in budding yeast), a Turing-type reaction diffusion model was sufficient to generate a stochastic accumulation of Cdc42-GTP that engaged the Bem1-Cdc24 complex to mature into a focus of Cdc42-GTP. Positive feedback necessitates that the local inhomogeneity will expand at the expense of other stochastic cortical associations of Cdc42-GTP. Therefore, if unharnessed, Cdc42-GTP will ultimately expand across the entire cortex (Jilkine and Edelstein-Keshet 2011). To combat the inherit drive for expansion, a GAP-dependent mechanism was included that would rapidly convert Cdc42-GTP that diffused out of the focus into Cdc42-GDP. Precocious activation of Cdc42-GDP outside the focus was inhibited by GDI-dependent (Rdi1 in budding yeast) extraction and recycling to the cytoplasm (Goryachev and Pokhilko 2008). Strikingly, this minimal system was sufficient to polarize a simulated cell

under a variety of parameter conditions, providing additional evidence to the positive feedback model.

Biological behaviors of the polarity complex and activated Cdc42 are also consistent with the positive feedback model. For instance, FRAP analysis has showed that activated Cdc42, Bem1, and Cdc24 exchange rapidly between the nascent bud and the cytoplasm (Wedlich-Soldner 2004). Additionally, traveling waves of polarity components have been observed in unpolarized cells prior to symmetry breaking (Ozbudak, Becskei, and van Oudenaarden 2005). Furthermore, cells are robust to multi-budding, as overexpression of the scaffold Bem1 does not drastically increase the observation of multi-budded cells (Howell et al. 2009). These data suggest that stringent inhibitory mechanism(s) may exist beyond Cdc42 recycling. Indeed, oscillatory accumulations of activated Cdc42 and Bem1 have been observed (Howell et al. 2012), which necessarily invoke negative feedback (Houssine Snoussi 2011; Alon 2006).

In the Goryachev model mentioned above, inhibition was administered through active cycling of Cdc42 and the Bem1-Cdc24 complex into the cytosol. Despite retaining the ability to undergo bud emergence, *rdi1Δ* cells display a broad expansion of activated Cdc42 (Slaughter et al. 2009; Freisinger et al. 1AD)(this work), suggesting that this mechanism contributes to focusing of the nascent bud. Indeed, the rate of Cdc42 exchange with the cytosol drastically reduces in *rdi1Δ* cells (Slaughter et al. 2009; Klünder et al. 2013); though it is unlikely Rdi1-mediated recycling is the sole inhibitory mechanism. Several recent studies have implicated Cla4-dependent phosphorylation of Cdc24 - which significantly reduces GEF activity - as a mode of negative feedback (Gulli et al. 2000; Kuo et al. 2014; Rapali et al. 2017). Finally, actin filaments have been

suggested to participate in negative feedback by diluting the focus through vesicle delivery (Layton et al. 2011; Savage, Layton, and Lew 2012). Intriguingly, some research has suggested that actin filaments participate in a positive feedback loop via vesicular delivery of Cdc42 to the nascent bud site; though it likely facilitates maintenance of the polarized bud rather than establishment (Wedlich-Soldner 2003; Slaughter et al. 2009; Freisinger et al. 2013; Jose et al. 2013; Woods et al. 2016). Likely, these mechanisms, and potentially more, cooperate to produce a potent inhibitory response. Thus, polarization in budding yeast appears to invoke a Turing-type reaction-diffusion system that employs fast-acting positive feedback and delayed inhibition to specify the axis of polarity once within the cell cycle. How does the cell cycle exert control over this diffusion-based system to ensure that only a single bud emerges within a cell cycle?

Section 5: Influence of the cell cycle on polarity

Section 5.1: Cdk1 - the conserved regulator of cell cycle progression

The cell cycle is divided into 4 distinct phases: growth phase 1 (G1), DNA synthesis (S), growth phase 2 (G2), and mitosis (M). Cells repeatedly cycle through these stages, with the end product generating a new daughter cell. The conserved regulator of cell cycle progression, cyclin-dependent kinase 1 (Cdk1), is indispensable for the switch-like transitions that drive entry into each stage. The primary function of Cdk1 is to activate a suite of cellular programs necessary for each switch-like transition (Hochegger, Takeda, and Hunt 2008). Critical to activation by Cdk1, are the Cdk1 adaptor proteins known as cyclins. Each transition is modulated by an alternate set of cyclins that are sufficient to

define Cdk1 substrate specificity (Loog and Morgan 2005). As polarity establishment in budding yeast occurs early in the cell cycle, the focus will be on the G1 events that promote entry into the cell cycle.

Toward the end of G1, entry and irreversible commitment to the cell cycle is coordinated by the transition known as Start (Lew and Reed 1993). Prior to Start, Cdk1 is minimally active via its interaction with Cyclin3 (Cln3) (Cross and Blake 1993). As cells continue to grow, the transcriptional inhibitor Whi5 is phosphorylated by Cdk1-Cln3 and ejected from the nucleus, allowing transcription of two additional cyclins, Cln1 and Cln2 (Costanzo et al. 2004). Cln1 and Cln2 amplify Cdk1 activation and in doing so, hyperactivate their own transcription in a Cdk1-dependent manner. Thus, a transcriptional positive feedback loop propels cells through Start (Skotheim et al. 2008). There is considerable genetic redundancy amongst the cyclins. While a *cln1Δ/cln2Δ/cln3Δ* triple mutant is lethal, both c*ln3Δ* and *cln1Δ/cln2Δ* strains are viable (Richardson et al. 1989), suggesting robust mechanisms to ensure Cdk1 activation. One such mechanism involves the process by which Whi5 is inactivated. Intriguingly, recent work has attributed Whi5 inactivation by Cdk1 to cell size increase. Specifically, the translation of Whi5 ceases in G1, resulting in its dilution as cells grow. Conversely, the translation rate of Cln3 mirrors the rate of cell growth such that its effective concentration is static. Therefore, cell growth increases the ratio of Cdk1-Cln3 activity to Whi5 concentration to efficiently drive entry into Start (Schmoller et al. 2015).

Section 5.2: Cell cycle regulation of polarity establishment in *Saccharomyces cerevisiae*

Bud emergence requires Cdk1 activation via interactions with Cln1, Cln2, and Cln3 (Richardson et al. 1989). Furthermore, Cdk1 activity is required to concentrate activated Cdc42, Bem1, Cdc24, and downstream Cdc42-GTP effectors at the presumptive bud site (Gulli et al. 2000; Jaquenoud and Peter 2000; McCusker et al. 2007). How Cdk1 regulates the accumulation of these factors and the events necessary for bud emergence is unclear; yet, a variety research points to an indirect role in licensing Cdc42 accumulation to a distinct cortical domain (Howell and Lew 2012; Woods and Lew 2017).

Under the landmark-directed regime, activated Cdk1 may facilitate either GTP loading of Rsr1 and/or promote Cdc24 binding to Rsr1-GTP (Howell and Lew 2012). Indeed, both Cdc24 and the Rsr1 GAP, Bud2, are putative targets of Cdk1 (Gulli et al. 2000; McCusker et al. 2007; Holt et al. 2009). Moreover, Cdc24 is sequestered into the nucleus prior to Start in haploid cells; therefore, Cdk1-dependent phosphorylation of Cdc24 may facilitate nuclear exit. However, Cdc24 does not concentrate in the nucleus in diploid cells, suggesting that Cdk1 may participate in alternative mechanisms to promote Cdc42 activation. Accordingly, Cdk1 has been implicated in down-regulating Cdc42 GAPs to promote polarity establishment (Howell and Lew 2012). The current model of symmetry breaking suggests that the tripartite polarity complex is constitutively complexed (Bose et al. 2001). As such, axis specification by Cdc42-mediated positive feedback is limited by the activation rate of Cdc42 (Howell and Lew 2012). Thus, down-regulation of global GAP activity would facilitate cortical stochasticities of Cdc42-GTP and induction of positive feedback (See Section 4.3.2).

There are four identified Cdc42 GAPs in budding yeast: Rga1, Rga2, Bem2, and Bem3 (Stevenson et al. 1995; Johnson 1999; Marquitz et al. 2002; Zheng, Cerione, and Bender 1994). Intriguingly, all four are putative substrates of Cdk1 (Ubersax et al. 2003; Holt et al. 2009). Overexpression of either Bem3 or Rga2 with mutationally-ablated Cdk1 consensus sites induces cytoxcity. Specifically, these experiments generated an abundance of enlarged, depolarized cells, which could be recovered by mutationally inactivating the GAP activity (Knaus et al. 2007; Sopko et al. 2007). These results support a model in which GAP down-regulation by Cdk1 is required for proper Cdc42 polarization. While these data suggest a mechanism by which Cdk1 can regulate polarity establishment, it need not be the only mode of Cdk1-dependent promotion of polarization.

Intriguingly, budding yeast cells can polarize in the absence of Cdk1 activation. Prior to Start, haploid cells are challenged with a decision to either engage the mating program or to commit to cell cycle entry (Hartwell et al. 1974). In the presence of a pheromone (See Chapter 4, Section 4.1.1), cells will initiate the mating pathway and orient polarized growth toward the pheromone gradient (Bardwell 2004). Intriguingly, growth of the mating projection requires Cdc24 and Bem1 (Adams and Pringle 1984; Chenevert et al. 1992; Chenevert, Valtz, and Herskowitz 1994). This phenomenon indicates that the polarity module can locally concentrate in the absence of Cdk1 activity, thereby locally activating Cdc42. Collectively, these data indicate that Cdk1 activation is not strictly required for polarization of Cdc42; thus, alternative mechanisms may exist to promote local concentration of Cdc42 prior to cell cycle entry.

Section 6: The use of optogenetics in probing biological processes

Section 6.1: The utility of optogenetics

Polarized domains often form rapidly in response to initiating cues, suggesting the existence of mechanisms that can locally direct and corral diffusive molecules. Current models of polarization schemes posit that positive feedback loops can harness diffusive polarity determinants and amplify their local concentration, thereby establishing polarity in response to changing cues. However, direct evidence for positive feedback loops is lacking as traditional genetic and cell biological analyses are not amenable to investigate the dynamic nature of polarization. Furthermore, a myriad of cellular processes are intricately regulated by precise spatial and temporal events; thus, tools that would permit the perturbation of signaling molecules have the potential to reveal critical mechanisms in a variety of signaling cascades. As such, a number of genetically-encoded tools have been developed that allow control of the spatial and temporal dynamics of diverse proteins using light (Tischer and Weiner 2014).

The initial genetically-encoded light-sensitive proteins were light-gated ion channels (Channelrhodopsin-2, ChR2) derived from algae (Nagel et al. 2003; Boyden et al. 2005). In response to blue light, hippocampal cells expressing ChR2 stimulated excitatory and inhibitory neuronal transmission with rapid precision (Boyden et al. 2005), thereby setting a new precedent in neuronal research. Moreover, the use of light-gated channels permitted interrogation into neuronal processes that had previously proved to be difficult or nearly impossible to investigate (Li et al. 2005; Nagel et al. 2005; Hegemann and Nagel 2013). While fruitful, light-gated ion channels can only exploit

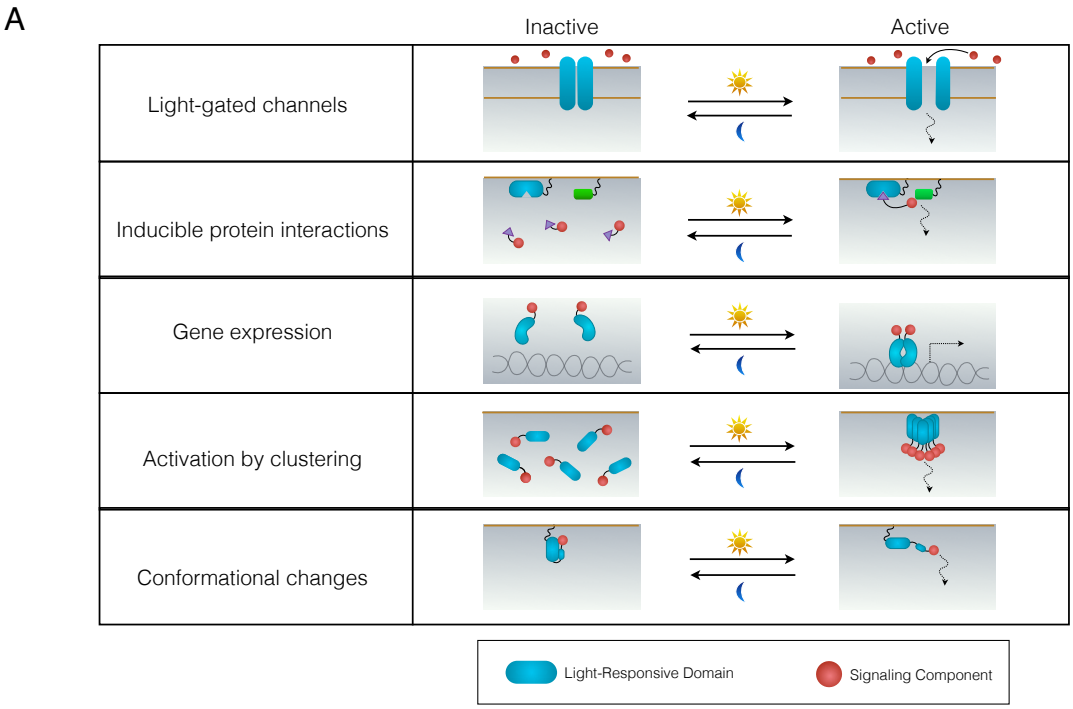


Figure 1.5: Diverse optogenetic tools allow regulation of varied cellular behaviors.
A) The diverse optogenetic toolkit available to experimentally control cellular behaviors. Light conversion induces cellular responses, whereas activation can be attenuated by removing light stimulation. In some instances, light stimulation can be used to inhibit signaling (not shown)
B) Molecular architecture of TULIPs. In the inactive state, the Jα helix sequesters the peptide from its cognate binding partner ePDZ. Blue light illumination releases the peptide and inducing high-affinity binding between ePDZ and LOVpep.
C) Schematic diagram of cellular TULIPs. GFP-LOVpep is localized to the plasma membrane via a transmembrane domain, while ePDZ-mCherry freely diffuses in the cytoplasm. Upon light exposure, ePDZ-mCherry rapidly translocates to the plasma membrane. Cessation of light exposure releases ePDZ-mCherry into the cytoplasm, with a half-life of ~80 seconds.

membrane potential. Thus, light-dependent interrogation of dynamic signaling processes must employ photo-responsive domains that are amenable to gating events like protein-protein interactions and signaling activation.

In one of the first studies that showed the potential of optoenetics in cell biology, the mammalian Rho GTPase Rac1 was fused to a light-responsive domain derived from *Avena sativa*, the LOV domain (Y. I. Wu et al. 2009; Christie et al. 1998). Light stimulation was sufficient to induce rapid cell crawling (Y. I. Wu et al. 2009), demonstrating that regulation of switch-like protein behavior could be readily controlled by an exogenous stimulus. While encouraging, not all proteins were amenable to conformational gating nor could the approach be generalized to signaling motifs that require the coupling of players. Several groups, including the Glotzer lab, focused instead on dimerization based tools. Conceptually similar to the FRB-FKBP system of dimerization (Banaszynski, Liu, and Wandless 2005; Haruki, Nishikawa, and Laemmli 2008), photo-responsive dimerization systems had the further benefits of reversibility and spatial control. Indeed, such dimerization systems have been used to investigate the properties of organellar positioning (van Bergeijk et al. 2015), to mediate G protein signaling in cell migration (O'Neill and Gautam 2014), and to regulate protein degradation (Renicke et al. 2013). Moreover, optogenetic tools have been customized to regulate signaling pathways like gene expression and clustering-based signaling platforms, highlighting the diversity in optogenetic toolkits available (Toettcher et al. 2010; Tischer and Weiner 2014). Furthermore, our own lab has developed a set of dimerization-based optogenetic tools that we have used to probe the yeast mating pathway in *Saccharomyces cerevisiae* (Strickland et al. 2012), to interrogate the

sufficiency of RhoA activation in mammalian cytokinesis (Wagner and Glotzer 2016), and to investigate Rho-family GTPase regulation in *Drosophila* (Rich and Glotzer, not published).

Section 6.2: The molecular details of optogenetics

The primary optogenetic tool that we employ is termed TULIPs, standing for **TU**nable-**L**ight-**I**nduced-**P**roteins. The optically-responsive protein is a derivative of the LOV domain from *Avena sativa* and has been used in an assortment of optogenetic tools (Tischer and Weiner 2014), as it undergoes a robust conformational change upon stimulation with blue light. The molecule itself contains an alpha helix, termed the Jα helix, that in the dark state is coiled and bound to the body of the protein (Harper, Christie, and Gardner 2004). Upon illumination with blue light, the Jα helix uncoils and releases to display an unstructured extension (Harper, Christie, and Gardner 2004; Halavaty and Moffat 2007). Our lab used protein engineering to append a small eight amino acid peptide to the end of the Jα helix (LOVpep) that is the high affinity binding partner for a highly engineered clamshell protein called enhanced PDZ (ePDZ) (J. Huang et al. 2008; J. Huang et al. 2009).

To translate this tool into a biological system, we tethered the optically-responsive protein (LOVpep) to the plasma membrane whereas its cognate binding partner (ePDZ) freely diffuses in the cytoplasm. Illumination uncages the peptide allowing it to interact with ePDZ and induce rapid (< 10 sec) relocation of ePDZ to the membrane. By fusing proteins of interest to ePDZ, we have shown that we can potently and dynamically control spatiotemporal localization of signaling molecules using light and induce

biological responses (Strickland et al. 2012; Wagner and Glotzer 2016). Critically, the dosage of light can be titrated such that a variety of perturbations can be assayed. For example, a small light dosage can engage the endogenous mechanisms regulating a signaling cascade. Alternatively, large doses of light can interrogate the effects of robust signaling activation on the system. In Wagner and Glotzer (Wagner and Glotzer 2016), our lab showed that optogenetic recruitment of a RhoA GEF was sufficient to induce RhoA activation that produced cytokinetic furrows irrespective of cell cycle state. Thus, our optogenetic system is a robust tool sufficient to investigate a myriad of cell signaling behaviors.

Section 7: What are the mechanisms of polarity establishment in yeast?

The tripartite complex of Cdc24-Bem1-Cla4 is known to play an essential role in symmetry breaking (See Chapter 1, Section 4) and it is suggested that symmetry breaking utilizes this complex to mediate a Cdc42-dependent positive feedback loop (Howell and Lew 2012)(See Chapter 1, Section 4.3.2). Computational models and in vivo analyses of symmetry breaking have uncovered behaviors consistent with positive feedback, including traveling waves and oscillatory accumulation of polarity proteins (Ozbudak, Becskei, and van Oudenaarden 2005; Howell et al. 2012)(See Chapter 1, Section 4.3.2). However, important assumptions of the models have not been directly tested. For instance, whenever Cdc42 is active it should induce accumulation of the polarity complex; nonetheless, the validity of this critical assumption has yet to be established. Similarly, although the events preceding bud emergence have been

dissected in considerable detail, the events that precede those have attracted relatively little attention. For example, in symmetry breaking polarization, do events in early G1 influence the site of symmetry breaking? To what extent are the functions of the polarity proteins influenced by the state of the cell cycle? How do cells ensure singularity? The central goal of this thesis aims to understand the requisite mechanisms needed to establish and maintain a single axis of polarity in *Saccharomyces cerevisiae.*

Historically, these questions have been difficult to interrogate due to the absence of tools that permit controlled perturbation of Cdc42 activation. Thus, we have employed optogenetic tools to spatially and temporally control critical proteins in polarity establishment, including Cdc24, Bem1, and Cla4. Additionally, we have utilized genetic and pharmacological perturbations in concert with optogenetics to query the cell cycle regulation of polarity establishment.

The results presented in this thesis provide direct evidence that positive feedback contributes to polarity establishment once Cdk1 is activated at Start. However, while the current model predicts that the polarity components invariably function together in a potent positive feedback loop, we find that they do not do so prior to Cdk1 activation and that Cdc42 activation does not invariably induce the canonical positive feedback loop. Rather, we demonstrate the existence of a second positive feedback loop involving Cdc24 that precedes the canonical loop and appears to function independently of Bem1. We uncover mechanisms that indicate robust competition that is mediated by a suite of Cdc42 auxiliary proteins and effectors functions to establish and reinforce a single axis of polarity.

Chapter 2: Optogenetic control of polarity establishment

[This chapter was adapted from Witte *et al.*, *eLIFE*, 2017]

Abstract

Crucial predictions of the positive feedback in polarity establishment demand that local accumulation of activated Cdc42 is sufficient to define the axis of polarity and that local activation of Cdc42 is sufficient to induce positive feedback. In this chapter, we optogenetically control Bem1 and Cdc24 localization to validate the former, but invalidate the latter. Our results indicate that mechanisms exist to restrain positive feedback activity.

Section 1: Introduction

At the onset of the cell cycle, budding yeast establish a single axis of polarity and retain that axis until the following cell cycle. Absolutely essential to establish polarity in budding yeast is the conserved polarity regulator, the RhoGTPase Cdc42 (Hartwell, Culotti, and Reid 1970). Cdc42 is a molecular switch (Vetter and Wittinghofer 2001). In its off state (GDP-bound) it is sequestered into the cytoplasm and therefore unable to initiate the signaling cascade(s) necessary for bud site selection. Conversely, its on-state (Cdc42-GTP) localizes to discrete cortical patches, wherein the downstream effectors necessary for bud emergence are recruited (Evangelista et al. 1997; Johnson 1999; Casamayor and Snyder 2002; Pruyne et al. 2004; Iwase et al. 2006). Critical to proper cell proliferation, is the retention of the Cdc42-GTP at a unique site on the cortex. In wildtype cells, landmark proteins facilitate Cdc42 recruitment to a pre-

determined position (Park et al. 1997; Howell and Lew 2012)(Figure 2.1A). However, cells lacking those intrinsic cues can robustly establish polarity in a process known as symmetry breaking (Figure 2.1B), suggesting a redundant pathway to establish polarity. In the absence of spatial cues, it is thought that a positive feedback loop amplifies sites of stochastic Cdc42 activation in such a manner that only a single axis of polarity is emerges. Specifically, this postulated positive feedback loop is mediated by a tripartite complex consisting of the Cdc42 GEF, Cdc24, the scaffold protein Bem1, and the Cdc42 binding protein Cla4 (Gulli et al. 2000; Bose et al. 2001; Butty et al. 1998). Indeed, the polarity complex is essential for Cdc42 activation and bud emergence (Bi and Park 2012). Despite exquisite genetic and cell biological interrogations of the polarity complex, direct evidence for a positive feedback is lacking. In theory, local accumulation of one or more members of the polarity complex would be sufficient to bias Cdc42 activation, thereby biasing positive feedback and polarity establishment. Critically, positive feedback necessitates that local concentration of a polarity component would be sufficient to amplify its concentration in a Cdc42-GTP-dependent fashion. In this chapter we use optogenetics to perturb the spatiotemporal dynamics of Bem1 and Cdc24 to directly test the positive feedback model (Figure 2.1C). In previous proof of principle experiments, we demonstrated that local recruitment of Cdc24 directs formation of a mating projection in α-factor treated cells (Strickland et al. 2012); thus, its application should be translatable to spatial regulation of bud site selection.

Section 2: Cells are constitutively responsive to optogenetic perturbations

Figure 2.1

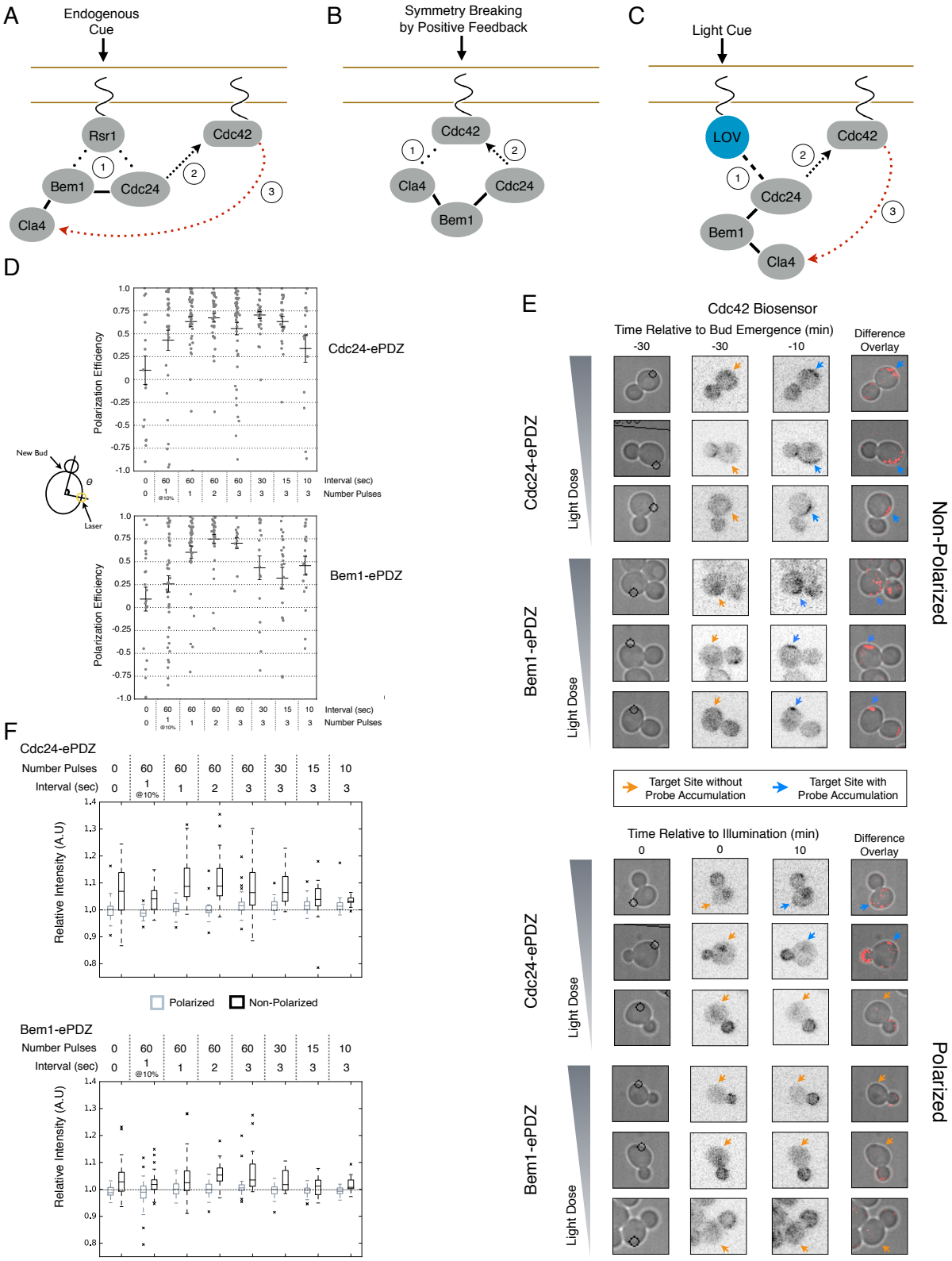

A
Endogenous Cue
Rsr1
Cdc42
Bem1
Cdc24
Cla4
B
Symmetry Breaking by Positive Feedback
Cdc42
Cla4
Cdc24
Bem1
C
Light Cue
LOV
Cdc42
Cdc24
Bem1
Cla4
D
Polarization Efficiency
Cdc24-ePDZ
Bem1-ePDZ
New Bud
Laser
Interval (sec)
Number Pulses
E
Cdc42 Biosensor
Time Relative to Bud Emergence (min)
Difference Overlay
Light Dose
Target Site without Probe Accumulation
Target Site with Probe Accumulation
Time Relative to Illumination (min)
Non-Polarized
Polarized
F
Relative Intensity (A.U)
Polarized
Non-Polarized

Figure 2.1 (continued): Cdc24 can activate Cdc42 in both polarized and unpolarized cells.
A) The endogenous cue is mediated by a system involving Rsr1 to yield patterned budding. Rsr1 directly interacts with Bem1 and Cdc24 to recruit and activate Cdc42 at an adjacent bud position. Rsr1 recruits Bem1 and/or Cdc24 **(1)** to activate Cdc42 **(2)** adjacent to the previous bud neck. Cdc42 undergoes positive feedback **(3)** by interacting with Cla4 to promote its own accumulation.
B) In the absence of Rsr1, cells undergo a symmetry breaking event mediated by positive feedback. The symmetry breaking event may involve a stochastic accumulation of Cdc42-GTP at a unique location that then recruits the Cla4-Bem1-Cdc24 complex **(1)**. Cdc24 activates additional molecules of Cdc42 **(2)** to promote positive feedback **(3)**.
C) Light-induced symmetry breaking by recruiting the GEF Cdc24 to activate Cdc42 at a prescribed position and induce positive feedback. Localized photo-activation of LOVpep recruits Cdc24-ePDZb **(1)** to activate Cdc42 **(2)**. Activated Cdc42 interacts with Cla4 to induce positive feedback.
D) Polarization efficiency of a population of cells where each point represents an individual cell. The angle ɵ is defined by the angle between the site of bud emergence and the laser position. Data are averages of all cells across multiple experiments (n experiments >= 2, N total cells > 15 for each group). Average and +/- SEM is indicated. Statistical analysis in Figure 1 - Supplemental Figure 1. Strains used: WYK8440 and WYK8301.
E) Representative phase and inverted fluorescent images depicting the activation of Cdc42 in response to either Cdc24 or Bem1 recruitment in polarized or non-polarized cells. Difference overlay images place the subtraction of the two fluorescent images overlaid onto the phase contrast image. An increase in fluorescent signal is depicted by red psuedo-coloring on the overlay. Cells were treated to increasing doses of light. Shown from bottom to top, representative images for each group:1 pulse/60 sec, 3 pulses/30 sec, and 3 pulses/15 sec. Each image is 16.2 μm x 16.2 μm. Strains used: WYK8440 and WYK8301.
F) Box-and-Whisker plots depicting the relative change in mean fluorescence intensity of the Cdc42 biosensor at targeted regions. The relative intensity of polarized cells is the mean of the intensity at 10 minutes post initial illumination compared to the intensity prior to illumination. The relative intensity for non-polarized cells compares activation of the biosensor 10 minutes before bud emergence relative to the intensity prior to illumination. Data is combined across multiple experiments (n experiments >= 2, N total cells > 15 for each group). Statistical analysis in Figure 1 - Supplemental Figure 3. Strains used: WYK8440 and WYK8301.

In order to probe the mechanism of cell polarization, we developed optogenetic tools to recruit yeast polarity proteins to defined sites on the cell cortex. Specifically, we co-expressed a membrane-tethered variant of LOVpep (Mid2-GFP-LOVpep) with either Bem1-ePDZ or Cdc24-ePDZ in diploid *rsr1Δ* cells. In all conditions, the TULIPs-tagged proteins were expressed under the control of a β-estradiol inducible promoter (Louvion, Havaux-Copf, and Picard 1993), and the ePDZ-tagged polarity component was expressed in addition to its endogenous counterpart. We perturbed cells by illuminating at a single site with a diffraction-limited laser. The response to this perturbation was

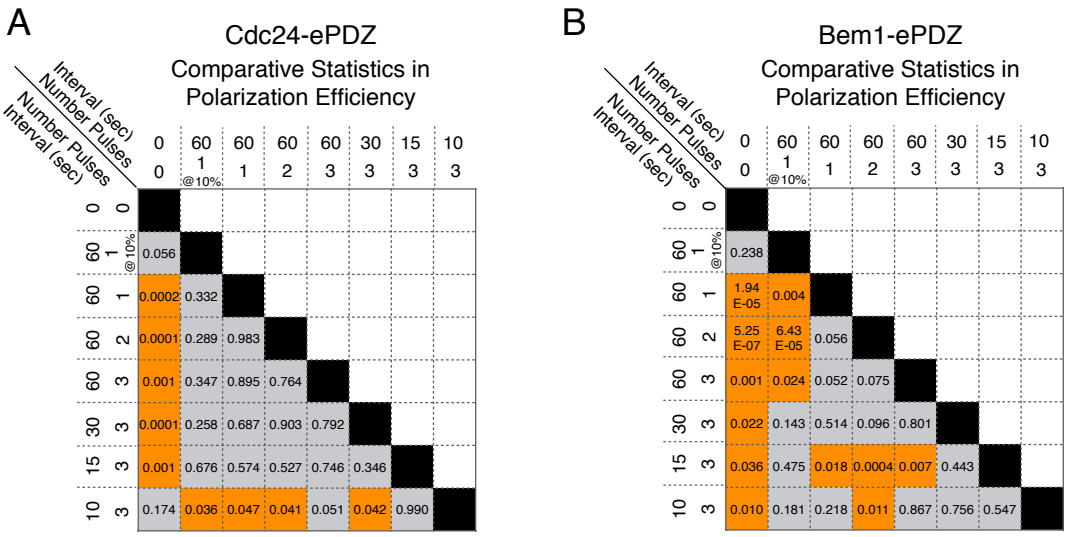
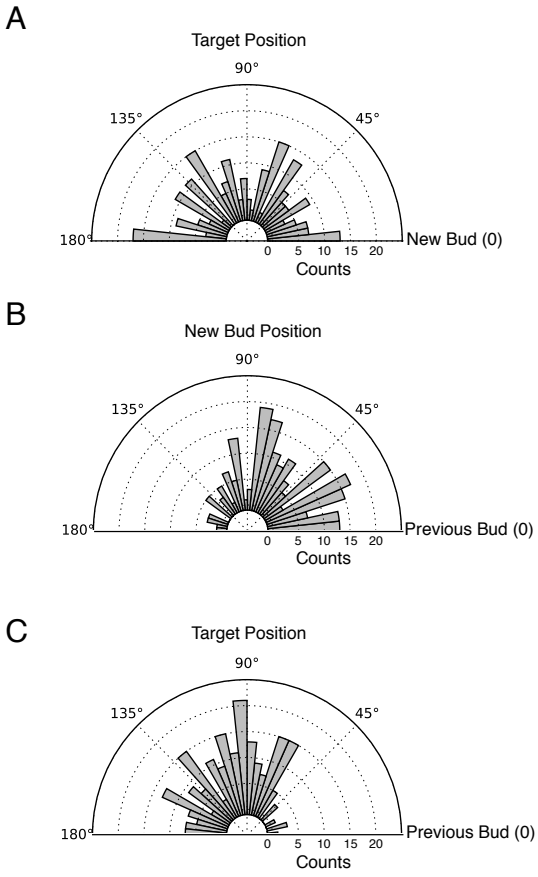


Figure 2.2: Statistical analysis of polarization efficiency as a function of light dose (Figure 2.1D).
A) Comparative statistical analysis for polarization efficiency in response to Cdc24-ePDZ recruitment and light dose. Gray box indicates populations not statistically different at p = 0.05, orange box denotes statistically significant at p < 0.05, Mann-Whitney *U* test.
B) Comparative statistics for Polarization efficiency in response to increasing light dose and Bem1 recruitment as in **A**.

examined by following the recruitment of a Cdc42 biosensor derived from the Cdc42 effector protein Gic2 (Tong et al. 2007) and Nomarski optics to observe bud emergence. We first examined the ability of Cdc24 and Bem1 to bias the site of polarization in unpolarized cells as a function of illumination frequency. We measured the angle, θ, between the site of illumination and the position of the nascent bud. These values were linearly scaled such that budding at the center of the laser target was assigned a score of 1 and budding opposite the target was assigned a score of -1; these scores were averaged for a population of cells (Polarization Efficiency = average(1-2θ/π)) (Figure 2.1D). Recruitment of Cdc24 or Bem1 recruitment was able to bias the bud site at very low light doses; Bem1 was slightly more efficient than Cdc24 (Figure 2.1D). Additionally, recruitment of either component induced robust accumulation of active Cdc42 at the site of illumination, without altering the timing of polarization (~95 minutes between bud



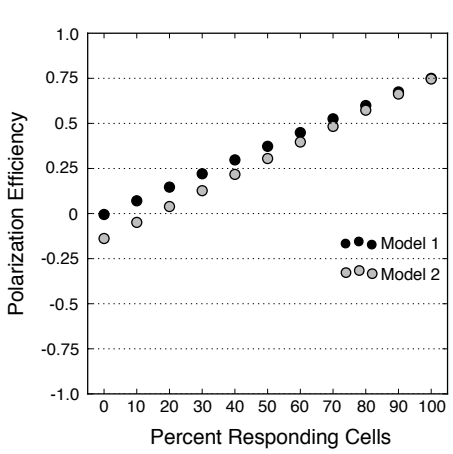


Figure 2.3: Bias in target position and new bud position relative to the previous bud (Figure 2.1D).
A) Distribution of targeting position relative to new bud formation in mock-illuminated cells (N cells > 120).
B) Distribution of new bud formation relative to the previous bud in mock-illuminated cells (N cells > 120).
C) Distribution of target position relative to the previous bud in mock-illuminated cells (N cells > 120).
D) Comparative polarization efficiency in two simulations. Model 1 assumes that there is no bias in target or new bud position. Model 2 approximates the biases the new bud and target positions as in **B** and **C**, respectively. Specifically, responding cells were simulated to respond with polarization efficiency = 0.75. In model 1, cells that do not respond were assumed to bud randomly in the range 46°-180°, with an average angle of 90°, corresponding to the angle expected if both targets and the new bud were random relative to the previous bud. In Model 2, cells that do not respond were assumed to bud randomly in the range 46°-180°, with an average angle of 102°. as an average difference of 102° approximates the aggregate bias resulting from the experimental bias in target position and the bias in bud site selection.

emergence events, regardless of photo-activation state or molecule recruited; data not shown). As the frequency of light increased, the ability of Cdc24 to bias the bud site remained roughly constant until the highest light dose, while the polarization efficiency of Bem1 dropped by ~50% once illumination increased to greater than 3 pulses per minute (Figure 2.1F, Figure 2.2). The reason for this drop is unclear. While symmetry breaking is predicted to be random relative to the previous bud site, the position of the new bud

was not completely random. Additionally, targets were not randomly positioned, as the area around the previous bud site was underrepresented. These biases would cause a slight underestimation of polarization efficiency (Figure 2.3).

We next assessed how the response varied as a function of both cell cycle position and the frequency with which cells were illuminated. At all doses of light, cells that were at the start of the cell cycle (~10 minutes before bud emergence) activated Cdc42 in response to both Cdc24 and Bem1 recruitment. Some non-illuminated cells also exhibited Cdc42 activation at the target site, as the bud site occasionally coincided with the target position (Figure 2.1D, F). To determine whether cells were constitutively responsive to optogenetic recruitment of Cdc24 or Bem1, we illuminated polarized cells with small to medium-sized buds. At infrequent light pulses, polarized cells did not activate Cdc42 in response to Cdc24 or Bem1 recruitment (1-3 pulses, Figure 2.1E, F, Figure 2.4). When cells were illuminated at a greater frequency (>2x per minute), those that expressed Cdc24-ePDZ activated detectable Cdc42 at all stages of the cell cycle. Conversely, Bem1 recruitment did not result in Cdc42 activation in polarized cells, even at higher illumination frequencies (Figure 2.1E, F, Figure 2.4). In summary, Cdc42 activation in unpolarized cells is readily attained in response to either Cdc24 or Bem1 recruitment; however, only high levels of Cdc24 recruitment can generate Cdc42-GTP in polarized cells, indicating that limitations to Cdc42 activation exist in polarized cells.

From these data, we conclude that local recruitment of either Cdc24 or Bem1 is able to efficiently bias the bud site. Because our primary interests lie in the endogenous regulation of Cdc42 activation, we chose to dissect polarity establishment using a light dose (3 pulses per minute) that efficiently biases the bud site, rather than the higher

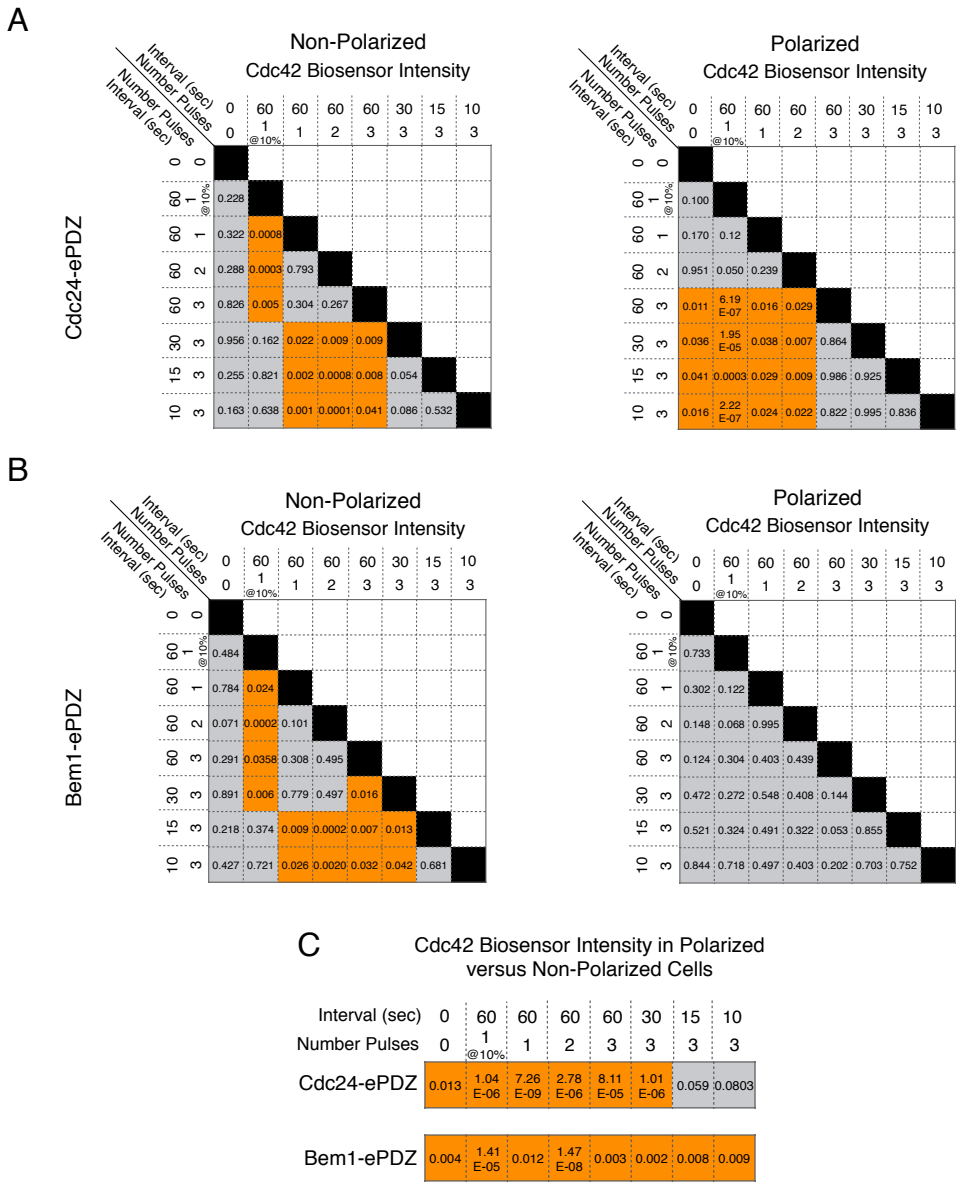


Figure 2.4: Statistical analysis of Cdc42 biosensor accumulation in Polarized and Non-Polarized cells as a function of light dose (Figure 2.1F).

A) Statistical analysis for Cdc42 biosensor accumulation in response to Cdc24-ePDZ recruitment in polarized and non-polarized cells. Gray box indicates populations not statistically different at p = 0.05, orange box denotes statistically significant at p < 0.05, Mann-Whitney *U* test.

B) Statistics for Cdc42 biosensor accumulation in response to Bem1 recruitment as in **A**.

C) Statistical comparison of Cdc42 biosensor accumulation in polarized vs. non-polarized cells at each light dose. Statistical analysis as in **A** and **B**.

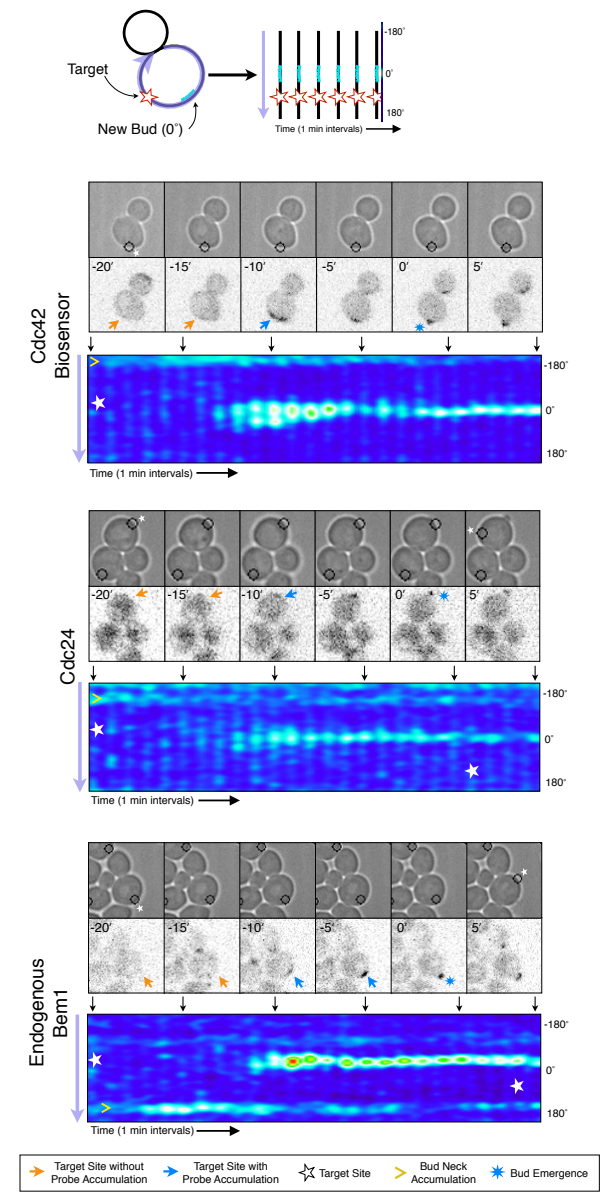
doses that might overcome the mechanisms that limit Cdc42 activation to one site in the cell.

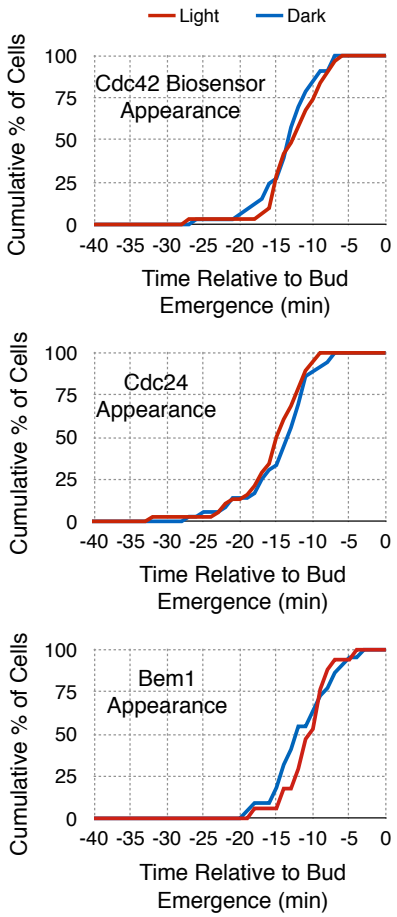
Section 3: Bem1 recruitment induces positive feedback

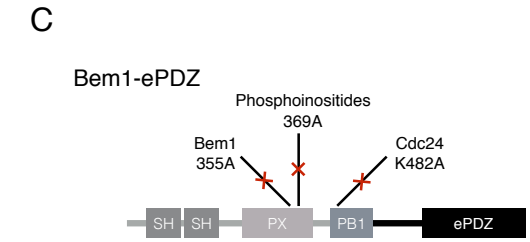
To understand the role of Bem1 in regulating polarization, we induced Bem1 recruitment in cells expressing either the Cdc42 biosensor, Cdc24-tdTomato expressed on a low-copy plasmid from its endogenous promoter, or cells in which one copy of Bem1 was tagged with tdTomato. Optogenetic recruitment of Bem1 was sufficient to promote activation of Cdc42, accumulation of Cdc24 (Figure 2.5A, B), and was able to efficiently position the bud site (Figure 2.5D). Critically, we found that recruitment of Bem1 was able to induce the accumulation of endogenous Bem1 at the prescribed site, directly showing that polarization in yeast proceeds via positive feedback (Figure 2.5A, B). These results raise the possibility that the observed activation of Cdc42 represents a combination of the direct activation of Cdc42 by the Cdc24 directly interacting with optogenetically-recruited Bem1 and amplification by endogenous mechanisms. Although Bem1 recruitment efficiently induced cell polarization, it did not induce precocious Cdc42 activation, or recruitment of Cdc24 or Bem1 as compared to control cells (Figure 2.5B, Figure 2.6). Thus, the activity of the Cdc24-Bem1-Cla4 feedback loop appears sensitive to cell cycle regulation.

If polarization induction by Bem1 proceeds via a positive feedback loop, Bem1 likely functions by directly interacting with the GEF Cdc24 (Kozubowski et al. 2008). To test this prediction, we introduced a mutation in the Bem1 PB1 domain that has previously been shown to abolish the Bem1-Cdc24 interaction (K482A) (Figure 2.5C) (Irazoqui,

Figure 2.5


A
Target
New Bud (0˚)
-180˚
0˚
180˚
Time (1 min intervals)
Cdc42 Biosensor
-20′
-15′
-10′
-5′
0′
5′
Cdc24
Endogenous Bem1
Target Site without Probe Accumulation
Target Site with Probe Accumulation
Target Site
Bud Neck Accumulation
Bud Emergence


B
Light
Dark
Cumulative % of Cells
Cdc42 Biosensor Appearance
Cdc24 Appearance
Bem1 Appearance
Time Relative to Bud Emergence (min)


C
Bem1-ePDZ
Phosphoinositides 369A
Bem1 355A
Cdc24 K482A
SH
SH
PX
PB1
ePDZ

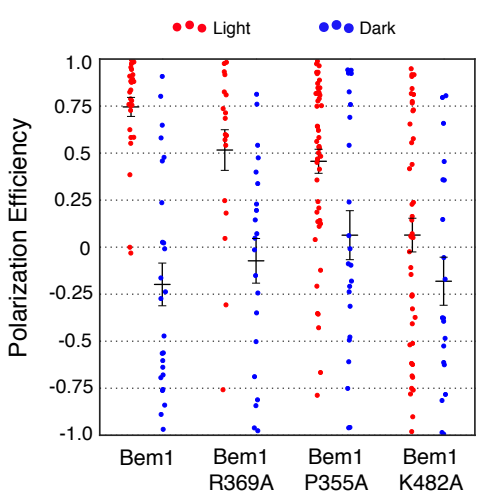

D
Light
Dark
Polarization Efficiency
Bem1
Bem1 R369A
Bem1 P355A
Bem1 K482A

Figure 2.5 (continued): Local accumulation of Bem1 is sufficient to bias the bud site.
A) Representative phase and fluorescence images and kymographs showing the position of the laser target and accumulation of the Cdc42 biosensor, Cdc24, and endogenous Bem1 (respectively). Each image is 16.2 μm x 16.2 μm. Kymographs are generated by iteratively linearizing the membrane at each time point (schematic). Bud emergence occurs at Y = 0° and at time = 0 minutes. Arrows between the kymograph and fluorescent images correlate to the time shown in the panels. Strains used: WYK8308, WYK8318, and WYK8576.
B) Accumulation kinetics for each component in response to Bem1 recruitment. Red line is photo-activated cells, blue line is mock-illuminated cells. Bud emergence is time = 0. Data combined across multiple experiments (n experiments >= 2, N total cells > 20 for each condition).
C) Domain schematic of Bem1-ePDZ, with mutation sites and interactions.
D) Polarization efficiency of a population of cells as in **1F.** Red dots are single photo-activated cells. Blue dots are single mock-illuminated cells. Data is combined across multiple experiments (n experiments > 2, N total cells > 20 for each group). Error bars S.E.M. polarization efficiency of Bem1, Bem1 R369A, and Bem1 P355A in the light was statistically significant relative to Bem1 K482A light and dark, and their respective dark-state controls. Bem1 K482A was not statistically significant relative to its dark state control. $p < 0.05$, Mann-Whitney *U* test. Strains used: WYK8308, WYK8434, WYK8435, and WYK8436.

Gladfelter, and Lew 2003) and tested its ability to induce cell polarization. Mutational inactivation of the Bem1 PB1 domain ablated its polarization efficiency (Figure 2.5D). In addition to its Cdc24-interacting domain, Bem1 contains a region that mediates homo-oligomerization as well as a region demonstrated to interact with phosphoinositides (PI) (Figure 2.5C). We introduced mutations known to abolish these activities (Irazoqui, Gladfelter, and Lew 2003) and assayed each for their ability to bias the bud site. While both functional domains were required for full activity, mutation of the oligomerization domain (P355A) was more detrimental than mutation of the PI-binding region (R369A) (Figure 2.5D). These results suggest that polarity induction by Bem1 requires direct interaction with Cdc24. Furthermore, they indicate that the most active form of Bem1 is likely a dimer or oligomer, at least in *rsr1Δ* cells (Irazoqui, Gladfelter, and Lew 2003). Although the PI-binding region is required for Bem1 function (Irazoqui, Gladfelter, and Lew 2003), light-mediated recruitment or indirect recruitment of endogenous Bem1 might compensate for this defect in this assay. We conclude that yeast polarization

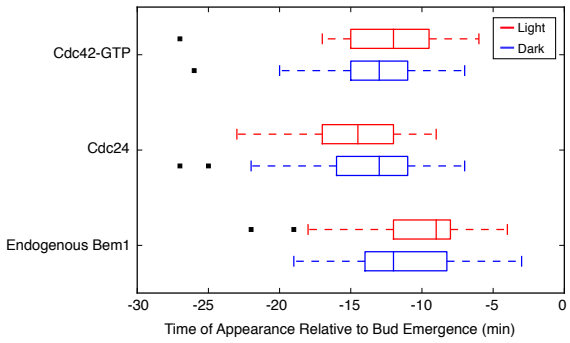


Figure 2.6: Photo-recruited Bem1 induces accumulation of Cdc42 biosensor, Cdc24, and Bem1.
Box-and-whisker plot of Cdc42-GTP and Cdc24 appearance in response to Bem1 recruitment in photo-activated (red) or mock-illuminated (blue) cells. Outliers are depicted by black squares. Data are as in **Figure 2.5B**.

polarization involves a positive feedback loop mediated by the Bem1-GEF complex. Furthermore, these data indicate that bud site selection requires activation of Cdc42.

Section 4: Cdc24 recruitment induces precocious activation of Cdc42

To gain a mechanistic understanding of light-induced symmetry breaking in response to recruitment of Cdc24 GEF, we determined which molecular features are required for induction of cell polarization (Figure 2.7A, B). In addition to GEF activity, Cdc24 also contains a PB1 domain that enables it to bind to a corresponding PB1 domain on Bem1 (Butty et al. 2002). Recently, it has been suggested that localization of Cdc24 is required for polarization via its ability to activate Cdc42 (Woods et al. 2015). As expected, we find that mutation of conserved surface residues in the GEF-GTPase interface (Rossman et al. 2002) rendered Cdc24 inactive for polarization activity (Figure 2.7A, B) and Cdc42 activation (data not shown). Deletion of the Bem1-binding domain of Cdc24 marginally reduced the polarization efficiency of the GEF (Figure 2.7A, B). These results indicate that local recruitment of Cdc24 polarizes yeast cell growth through its ability to activate Cdc42, and that the interaction of the recruited Cdc24 with

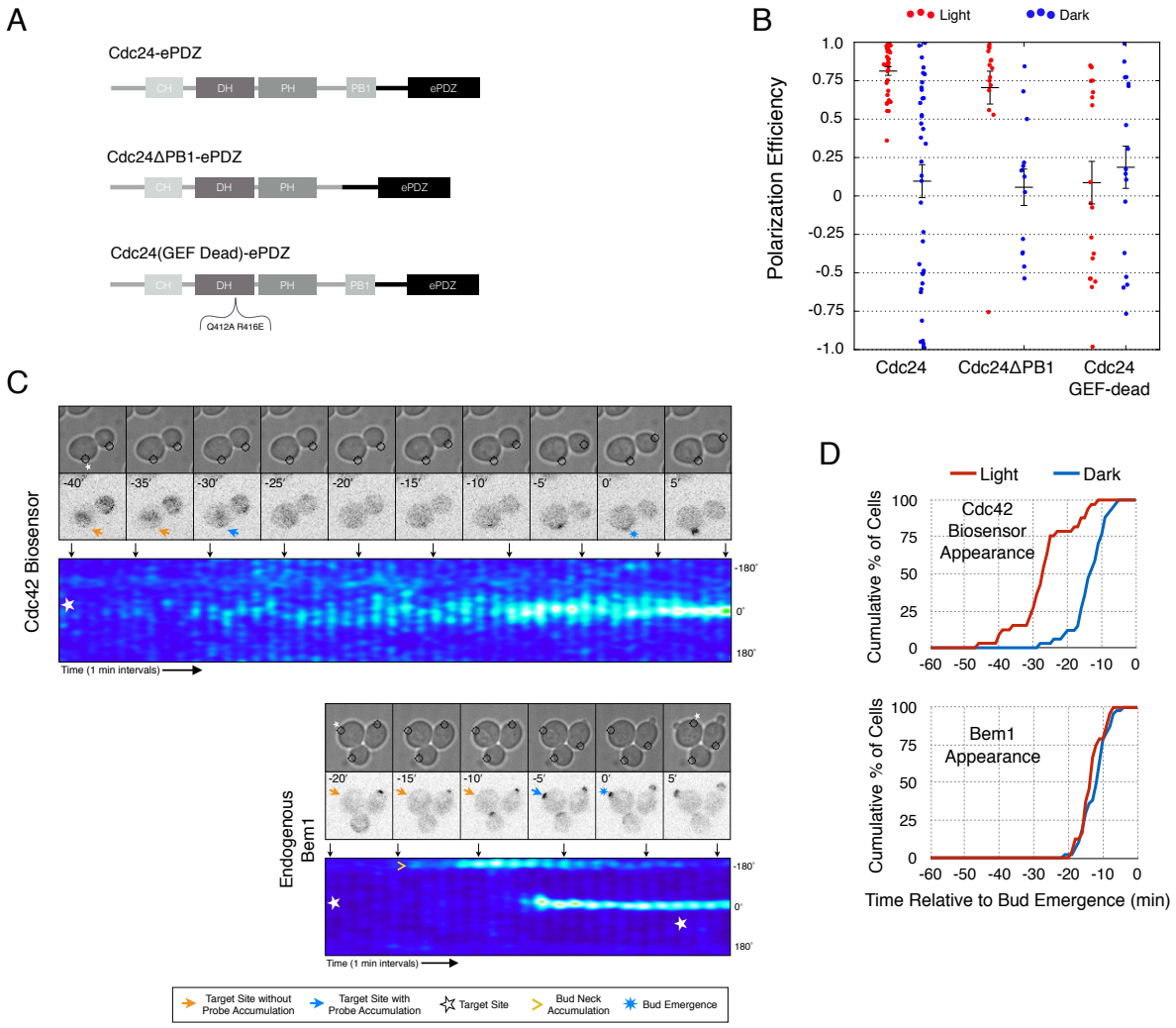


Figure 2.7: Light-mediated recruitment of Cdc24 is sufficient to induce symmetry breaking.
A) Domain schematic of Cdc24-ePDZ, with mutation sites and interactions.
B) Polarization efficiency of photo-illuminated (red) or mock-illuminated (blue) cells. Data are averaged across multiple experiments (n experiments > =2, N total cells > 15, for each group). Average and +/- S.E.M. indicated. polarization efficiency of Cdc24 and Cdc24ΔPB1 were statistically significant relative to Cdc24-GEF-dead light/dark, and statistically significant compared to their respective dark state controls. The difference between Cdc24 and Cdc24ΔPB1 were not statistically significant. The response to Cdc24-GEF dead was not statistically significant relative to its dark state control. $p < 0.05$, Mann-Whitney *U* test. Strains used: WYK8440, WYK8437, and WYK8439.
C) Panels of representative phase and fluorescence images and kymographs showing the position of the laser target and accumulation of a Cdc42 biosensor or endogenous Bem1 in response to Cdc24 recruitment. Each image is 16.2 μm x 16.2 μm. Strains used: WYK8440 and WYK8301.
D) Accumulation kinetics for the Cdc42 biosensor or endogenous Bem1 in response to Cdc24 recruitment. Data are combined across multiple experiments (n experiments >= 2, N total cells > 15 for each condition).

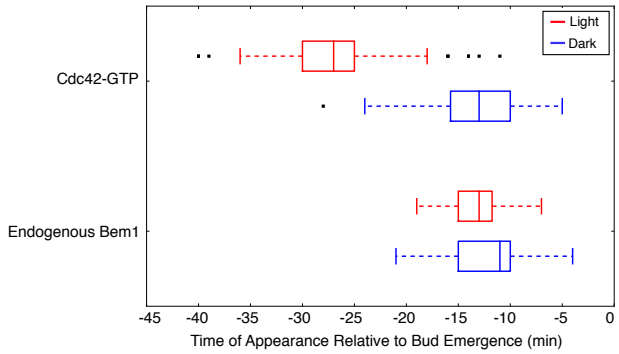


Figure 2.8: Light-induced recruitment of Cdc24 precociously activates Cdc42, but does not alter Bem1 kinetics. Box-and-whisker plot of Cdc42-GTP and Bem1 appearance in photo-activated (red) and mock-illuminated (blue) cells. Outliers are depicted by black squares. Bud emergence occurs at time = 0. Data are as in **Figure 2.7D**.

other cellular proteins - including endogenous wild-type Cdc24 - is not sufficient to induce polarization.

To observe the dynamics of Cdc42 activation in response to optogenetic Cdc24 recruitment, we used the biosensor of active Cdc42 to visualize GTPase activation (Figure 2.7C). In control cells, 50% of cells displayed Cdc42 activation 12 minutes prior to bud emergence (Figure 2.7D, Figure 2.8). In experiments in which Cdc24 was continuously recruited, we observed precocious Cdc42 activation; 50% of cells exhibited polarized accumulation of active Cdc42 27 minutes prior to bud emergence (Figure 2.7C, D). Precocious Cdc42 activation was less robust than that observed in the ~12 minutes prior to bud emergence. Thus, Cdc24 recruitment can induce Cdc42 activation at an earlier stage in the cell cycle than Bem1.

We next asked whether endogenous Bem1 precociously accumulated in a similar manner as activated Cdc42. Strikingly, Bem1 did not accumulate until ~13 minutes prior to bud emergence (Figure 2.7C, D). Indeed, despite accumulation of active Cdc42 as a consequence of Cdc24 recruitment, Bem1 accumulates at the same time relative to bud emergence in illuminated and mock-illuminated cells (-13 minutes and -12 minutes, respectively) (Figure 2.7D, Figure 2.8). Notably, the initial accumulation of Bem1 coincides with the time in which more robust Cdc42 activation is observed. These

results suggest that, unlike previous models for the activity of the polarity proteins, Cdc42 activation does not invariably lead to recruitment of the intact polarity complex.

Section 5: Both Bem1 and Cdc24 can override the endogenous pathway

As both Bem1 and Cdc24 recruitment proved to be effective at symmetry breaking, we assayed their respective capabilities in out-competing the endogenous landmark based signaling pathway. We generated heterozygous *RSR1/rsr1Δ* diploid cells and photo-recruited either Bem1 or Cdc24 to a random position on the cell cortex. Landmark-directed diploid cells bud in a bipolar fashion (Sloat, Adams, and Pringle 1981); thus, we oriented targets to avoid the cell poles. Strikingly, optogenetically recruited Cdc24 and Bem1 were both able to induce polarization (Figure 2.9A). Bem1 retained the equivalent polarization efficiency as Rsr1-deficient cells, whereas Cdc24 was somewhat less efficient at biasing the bud site. These data suggest that Bem1 accumulation at a presumptive bud site plays a critical role in establishing an axis of polarity.

Curious if Cdc24 recruitment was capable of inducing precocious activation of Cdc42 in *RSR1/rsr1Δ* cells as it does in cells lacking Rsr1, we quantified the rate of Cdc42 biosensor accumulation. In mock-illuminated cells, the Cdc42 biosensor appeared at the nascent bud site ~13 minutes prior to bud emergence, consistent with the kinetics observed in symmetry breaking. Conversely, photo-activated cells that budded toward the provided the cue, precociously activated Cdc42 (Figure 2.9B, C). However, in cells that oriented toward the landmark, Cdc24 was unable to induce Cdc42 activation. Furthermore, Bem1-induced activation of Cdc42 at the nascent bud site was limited to

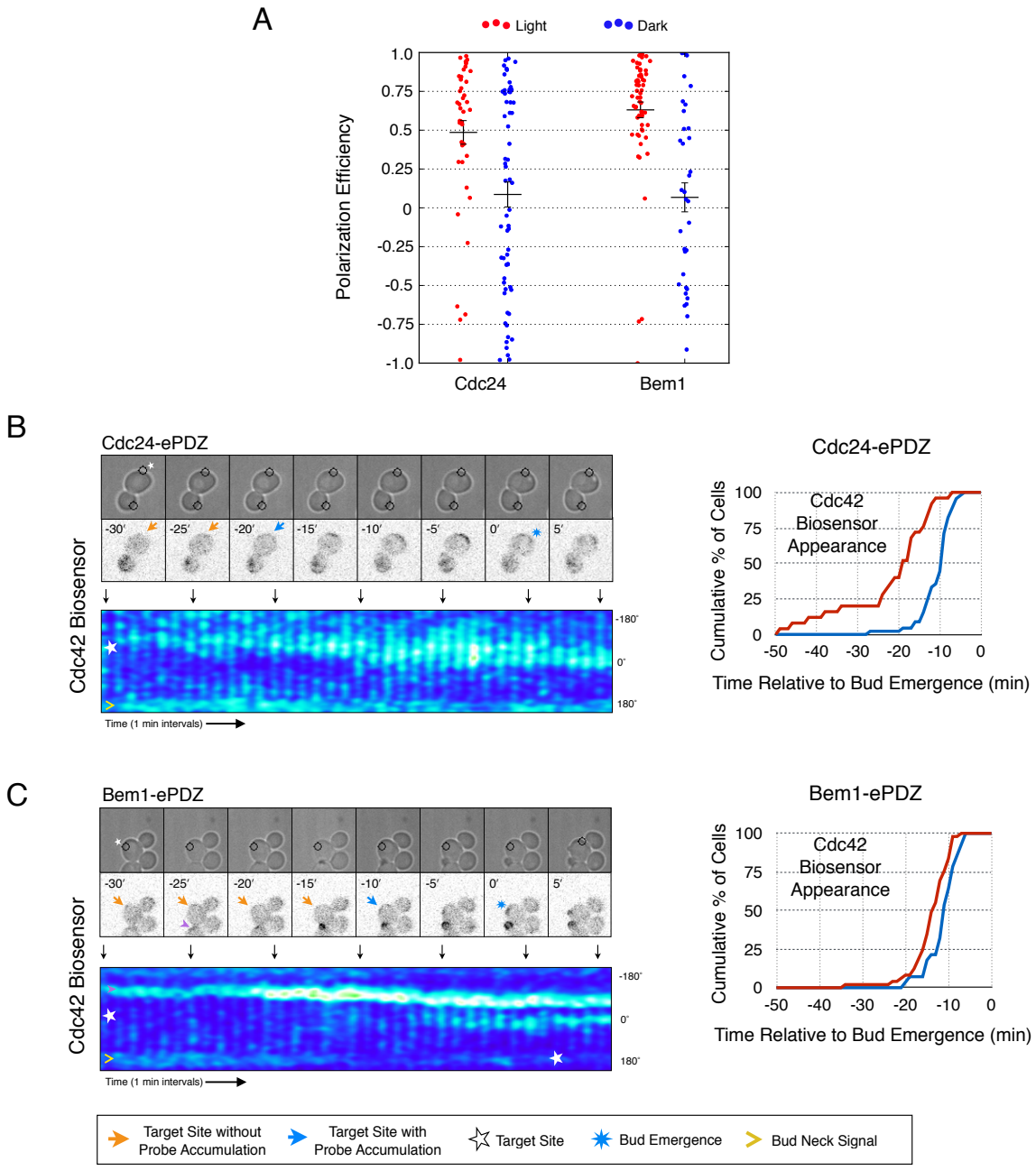


Figure 2.9: Local accumulation of either Cdc24 or Bem1 is sufficient to override the endogenous Rsr1 pathway.

A) Polarization efficiency of a population of cells heterozygous for Rsr1. Each point represents an individual cell. Average and +/- SEM is indicated. Both Cdc24 and Bem1 recruitment are statistically significant to their dark state controls. Strains used: WYK8598 and WYK8599.

B) Phase and fluorescent images and kymograph for a representative cell expressing the Cdc42 biosensor in response to Cdc24 recruitment. Each image is 16.2 μm x 16.2 μm. Accumulation kinetics relative to bud emergence for the Cdc42 biosensor in both dark and light conditions (n experiments >= 2, N total cells > 20 for each condition).

C) Representative phase and fluorescent images depicting the response of the Cdc42 biosensor response to Bem1 recruitment. Plots represent the accumulation kinetics for Cdc42-GTP in both photo- and mock-illumination conditions (n experiments >= 2, N total cells > 20 for each condition).

within ~15 minutes prior to bud emergence, consist with the kinetics observed in symmetry breaking (Figure 2.9B, C). These results further support the hypothesis that Cdc42 activation does not necessarily promote recruitment of the intact polarity complex.

Discussion

The current model of symmetry breaking polarization posits that a stochastic accumulation of Cdc42-GTP activates a positive feedback loop mediated by the Cla4-Bem1-Cdc24 polarity complex, thereby generating a local focus of activated Cdc42 (Howell and Lew 2012). A prediction of that model is that a seed of Cdc42-GTP would be sufficient to define the nascent bud site. We used low light doses to recruit limited amounts of either the GEF Cdc24 or the scaffold Bem1 to reproducibly induce cell polarization (Figure 2.1, Figure 2.5, and Figure 2.7); these conditions only resulted in Cdc42 activation at specific cell cycle stages. Rather than examining the direct consequences of experimentally induced GTPase activation (Wagner and Glotzer 2016), we sought to generate small perturbations and define the conditions in which those perturbations were amplified by the endogenous pathways. Using this approach, we have demonstrated the existence of a previously postulated positive feedback loop which promotes bud emergence.

We find that local, light-induced recruitment of either Cdc24 or Bem1 is sufficient to bias the presumptive bud site (Figure 2.1). Furthermore, the ability of these proteins to induce polarization requires the molecular features necessary to generate active Cdc42; specifically, GEF activity for Cdc24 and Cdc24-binding ability for Bem1 (Figure 2.7 and

Figure 2.5, respectively). These findings are consistent with the existing model for symmetry breaking, in which a positive feedback loop modulated by the polarity complex reinforces local activation of Cdc42 to promote bud emergence. We present direct evidence for positive feedback by optogenetically recruiting Bem1 and showing that endogenous Bem1 accumulates in response to that perturbation.

We gained further insight into polarity establishment by querying optogenetically-induced polarization efficiency in *RSR1/rsr1Δ* cells. Both Bem1 and Cdc24 were sufficient to out-compete the endogenous signal, though Cdc24 recruitment lost potency in the presence of Rsr1 (Figure 2.9). These data indicate that when challenged with an alternative nascent site, the landmark pathway does not invariably capture the requisite molecule(s) to establish an axis of polarity. As polarization efficiency of Bem1 is quite similar in landmark and symmetry breaking polarization, securing a threshold accumulation of Bem1 may be one mechanism by which an axis of polarity is established. Furthermore, the use of optogenetics allowed for unprecedented interrogation into polarity establishment by directly challenging landmark-direct ed polarization with an alternative site.

Although recruitment of either Bem1 or Cdc24 efficiently biases the site of polarization irrespective of landmark proteins, at certain stages of the cell cycle the molecular consequences of their recruitment are quite distinct. Approximately 15 minutes prior to bud emergence, light-induced recruitment of Bem1 promotes accumulation of active Cdc42, endogenous Bem1, and cytosolic Cdc24 (Figure 2.5), with kinetics similar to that observed in mock-illuminated cells. These results confirm prior models for polarization within 15 minutes of bud emergence. In contrast, optogenetic recruitment of the GEF

Cdc24 induces precocious activation of Cdc42, though it is not sufficient to induce precocious accumulation of endogenous Bem1 (Figure 2.7). These results indicate that discrete cortical activation of Cdc42 does not necessarily induce recruitment of the intact polarity complex. In *RSR1/rsr1Δ* cells, Bem1 or Cdc24 induced Cc42 activation displays kinetics that mirror those observed in the symmetry breaking regime (Figure 2.9). These observations indicate that precocious Cdc42 activation without concomitant recruitment of the polarity complex persists in the endogenous system.

Chapter 3: Cell cycle regulation

[This chapter was adapted from Witte *et al*., *eLIFE*, 2017]

Abstract

The current model for symmetry breaking indicates that Cdc42 activation invariably engages the tripartite polarity complex. However, the differential accumulation of Bem1 and Cdc42 in response to optogenetic recruitment of Cdc24 suggests that Cdc24 has the potential to activate Cdc42 independently of Bem1, and that activated Cdc42 does not inevitably induce recruitment of the polarity complex. We used high-intensity imaging of non-optogenetically perturbed cells to asses the localization of Cdc24, Bem1, and the Cdc42 biosensor throughout the cell cycle. Strikingly, we find that all three components do not invariably colocalize. Additionally, we use a pharmacologically sensitive allele of the master cell cycle regulator, Cdk1, to interrogate its function in polarity establishment. Our results reveal unprecedented cell cycle regulation of polarity establishment by symmetry breaking.

Section 1: Introduction

Budding yeast limit axis specification to the beginning of the cell cycle. However, how the cell cycle exerts control over polarity establishment is not well understood. Likely, the primary driver of cell cycle progression, Cdk1, plays a critical role in promoting the events necessary for polarity establishment. Indeed, Cdk1 is required for Cdc42-GTP, Bem1, and Cdc24 polarization (Gulli et al. 2000; McCusker et al. 2007). A myriad of Cdk1-dependent targets have been identified, including Cdc24 and the Cdc42 GAPs

(Ubersax et al. 2003), directly implicating Cdk1 in the cascade that regulates Cdc42 activation. What events necessitate Cdk1 activity? Potentially, Cdk1 licenses Cdc24 GEF activity to facilitate Cdc42 nucleotide exchange. Cdc24 has several Cdk1 consensus sites and displays Cln2-dependent increase in exchange activity towards Cdc42 (Moffat and Andrews, 2004) (Howell et al. 2009). However, ablation of all Cdc24 phosphorylation sites does not appear to affect polarity establishment (Kuo et al. 2014; Rapali et al. 2017). Thus, it is unlikely that altering Cdc24 activity is the sole mechanism by which Cdk1 induces polarization events. For example, Cdk1 may down-regulate GAP activity to promote stochastic Cdc42 activation on the cortex (Knaus et al. 2007; Sopko et al. 2007). Intriguingly, our data presented in the previous chapter suggest another mechanism: cell cycle regulation of positive feedback.

The molecular interactions amongst the polarity components are well-established (Howell and Lew 2012; Bi and Park 2012), and they are currently presumed to form a constitutive complex. Intriguingly, we show that early in G1 Cdc24 can induce Cdc42 activation without concomitant accumulation of Bem1, suggesting that neither Cdc24 nor activated Cdc42 are interacting with Bem1. Previous work has not revealed cell cycle regulation of complex assembly (Bose et al. 2001), however that analysis did not include early G1 cells, which exhibit this atypical behavior. Here we investigate the mechanisms by which Cdk1 regulates polarity establishment in budding yeast.

Section 2: The polarity components do not constitutively colocalize

The differential accumulation of Bem1 and Cdc42 in response to optogenetic recruitment of Cdc24 indicates that Cdc24 has the potential to activate Cdc42 independently of Bem1, and that activated Cdc42 does not inevitably induce recruitment of the polarity complex. If this is the case, then Cdc24, Bem1, and Cdc42-GTP may not constitutively colocalize in non-perturbed cells. To test this hypothesis, we performed a detailed colocalization analysis of the three pairwise combinations of active Cdc42, Bem1, and Cdc24. In all strains expressing fluorescent Bem1, one copy of endogenous Bem1 was tagged. The Cdc42 biosensor was used to assess the pool of active Cdc42. To visualize Cdc24, we transformed cells with a low-copy plasmid encoding an extra copy of Cdc24-GFP under its own promoter. Though it localizes to the expected sites and it can be readily detected, Cdc24-GFP was dimmer than Bem1-tdTomato, Bem1-GFP, and the Cdc42 biosensor.

Using imaging conditions optimized for detection of faint signals (see Materials and Methods), we acquired maximum intensity Z-projections of asynchronous cells. Using bud size and polarization as a guide, we sub-divided the cells into unbudded G1 cells, small-budded cells, and large-budded cells and characterized the localization pattern of each pair of probes in each cell cycle state. Unbudded cells were subdivided into two groups: early and late. Early cells were characterized by small distinct puncta of all three probes. Late cells featured a wide, cortically associated band in which all three probes localized. The categorization of these patterns as early and late is substantiated by previous studies (Ozbudak, Becskei, and van Oudenaarden 2005) and time-lapse imaging (see below, Figure 3.1C).

Figure 3.1

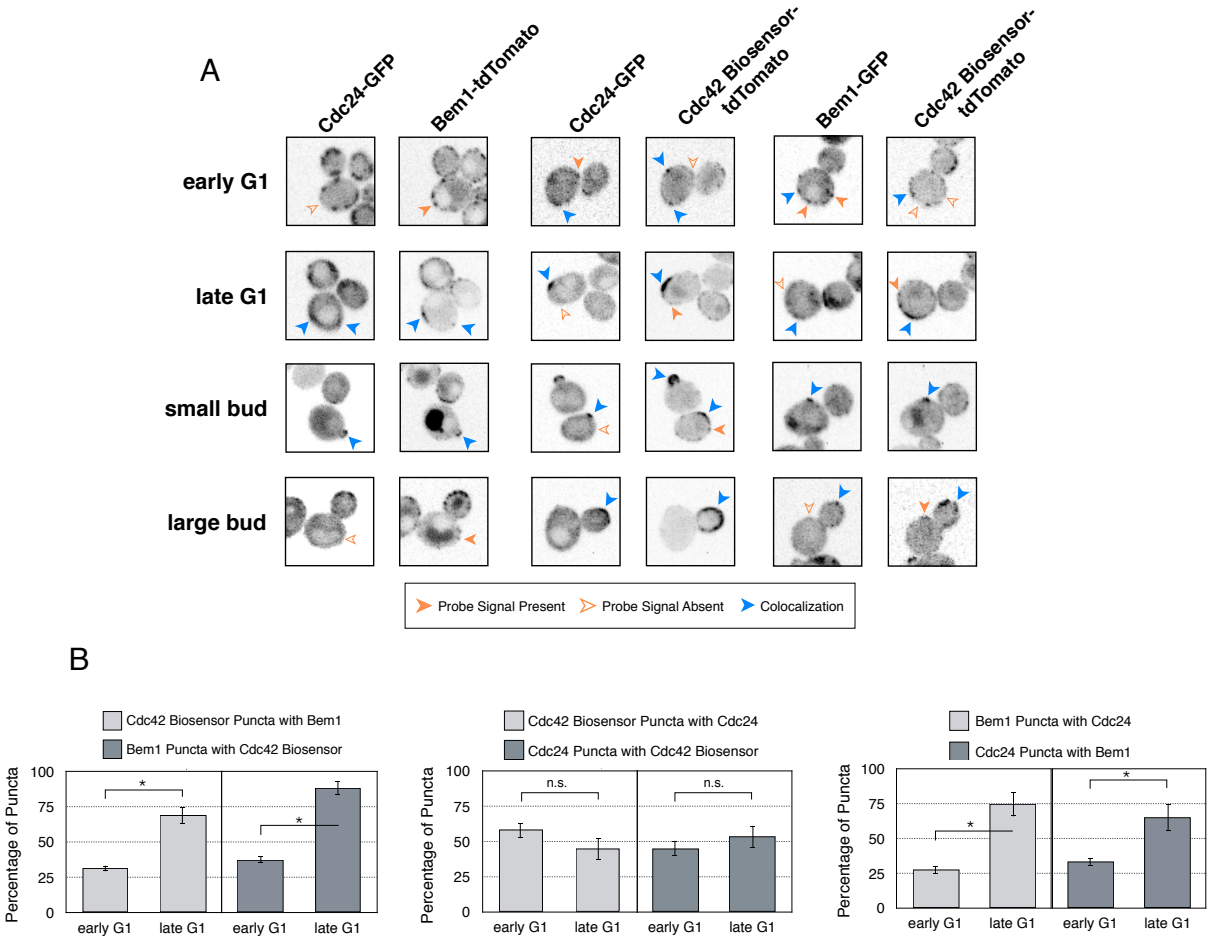

A
Cdc24-GFP
Bem1-tdTomato
Cdc24-GFP
Cdc42 Biosensor-tdTomato
Bem1-GFP
Cdc42 Biosensor-tdTomato
early G1
late G1
small bud
large bud
Probe Signal Present
Probe Signal Absent
Colocalization
B
Cdc42 Biosensor Puncta with Bem1
Bem1 Puncta with Cdc42 Biosensor
Cdc42 Biosensor Puncta with Cdc24
Cdc24 Puncta with Cdc42 Biosensor
Bem1 Puncta with Cdc24
Cdc24 Puncta with Bem1
Percentage of Puncta
100
75
50
25
0
n.s.
*
early G1
late G1
C
Bem1-tdTomato
Biosensor-tdTomato
Cdc24-tdTomato
0:00
1:00
2:00
3:00
3:30
4:00
4:30
5:00
5:30
6:30
7:00
8:00
9:00
10:00

Figure 3.1 (continued): Cdc42-GTP, Cdc24, and Bem1 do not constitutively colocalize and display rapid mobility prior to polarity establishment.
A) Inverted fluorescent images depicting the three pairwise combinations of Cdc42-GTP, Bem1, and Cdc24. All images are Z projections of 0.25 μm slices for the center 3 μm. Each image is 13.5 μm x 13.5 μm. Strains used: WYK8550, WYK8551, and WYK8552.
B) Percentage of colocalization amongst puncta in early G1 or late G1 cells. Plots are separated by pairs as in **A**. Data are averages of all cells across multiple experiments (n experiments = 2; N cells > 15 for each condition; N total cells > 100). Error bars S.E.M. n.s indicates populations not statistically different at $p >= 0.05$, * $p < 0.05$, Mann-Whitney *U* test.
C) Time-lapse images capturing polarization in non-perturbed cells expressing either the Cdc42 Biosensor-tdTomato, endogenous Bem1-tdTomato, or Cdc24-tdTomato. Images are single planes of the center of the cell. Blue arrows denote the time and position of polarity establishment. Time = 0 is the onset of imaging, and images were captured for ten minutes at either 60 sec or 30 sec intervals as denoted. Strains used: WYK8301, WYK8440, and WYK8575.

As expected, all three probes colocalize at the tips of small and nascent buds. However, at other stages of the cell cycle Bem1, Cdc24, and active Cdc42 do not colocalize (Figure 3.1A). In large-budded cells, activated Cdc42 and Bem1 localize throughout the growing bud, while the GEF is limited to the growing bud tip. Additionally, puncta of the Cdc42 biosensor are detected in the mother cell. In early G1 cells, Bem1 and Cdc24 each localize in cortically associated puncta, but surprisingly, these puncta do not overlap. Furthermore, both Bem1 puncta and Cdc24 puncta displayed limited overlap with the Cdc42 biosensor, with only 35% and ~50% of puncta colocalizing, respectively (Figure 3.1B, Figure 3.2). Non-overlapping puncta were also in seen single plane snapshots of each pairwise combination (Figure 3.3). Notably, the localization pattern shifted in late G1 cells as all three components localize to the wide band, and the amount of reciprocal overlap between Bem1-Cdc24 and Bem1-Cdc42 biosensor increased to greater than 70% (Figure 3.1B, Figure 3.2). This more extensive overlap amongst Cdc24, the Cd42 biosensor, and Bem1 in late G1 cells suggest that prior to the onset of polarization, Cdc24 has the potential to function independently of Bem1. Of note, the punctate patterns of Bem1 and Cdc42 seen in early G1 cells are below the

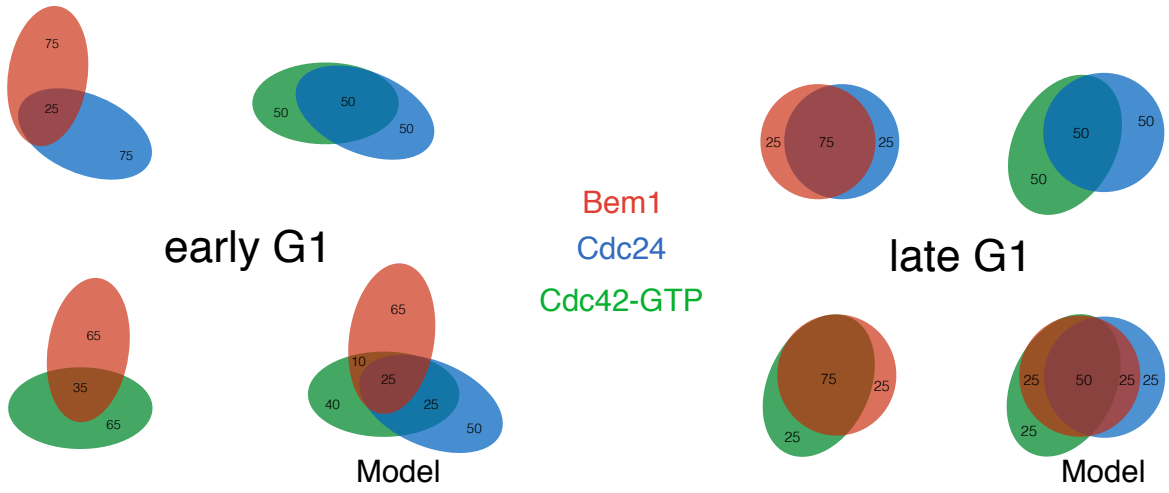


Figure 3.2: Pairwise analysis depicting the percent colocalization (related to Figure 3.1).
Venn diagrams for early G1 and late G1 depicting the extent of colocalization between each pair. The colocalization of all three components is presented in a model that is consistent with the pair-wise results. Data are as in **Figure 3.1B**.

limit of detection in the assays involving optogenetic recruitment. Exposure times in those experiments were far lower in order to limit photobleaching during long-term (>90 minutes) imaging.

To better understand the variation in protein localization at different cell cycle stages, we complemented the static imaging with time-lapse imaging. As proteins tagged with GFP tended to be dimmer than those tagged with tdTomato, we imaged cells expressing the tdTomato-tagged variants of the Cdc42 biosensor, Bem1, or Cdc24. Asynchronous populations of cells were imaged for a total of 10 minutes; images were acquired more frequently during the central four minutes. Each component was highly dynamic in the minutes leading up to the formation of a prominent wide band of accumulation, confirming that the punctate stage precedes polarization. While some puncta existed in the same spot for up to 3 minutes, appearance and disappearance of puncta were common (Figure 3.1C).

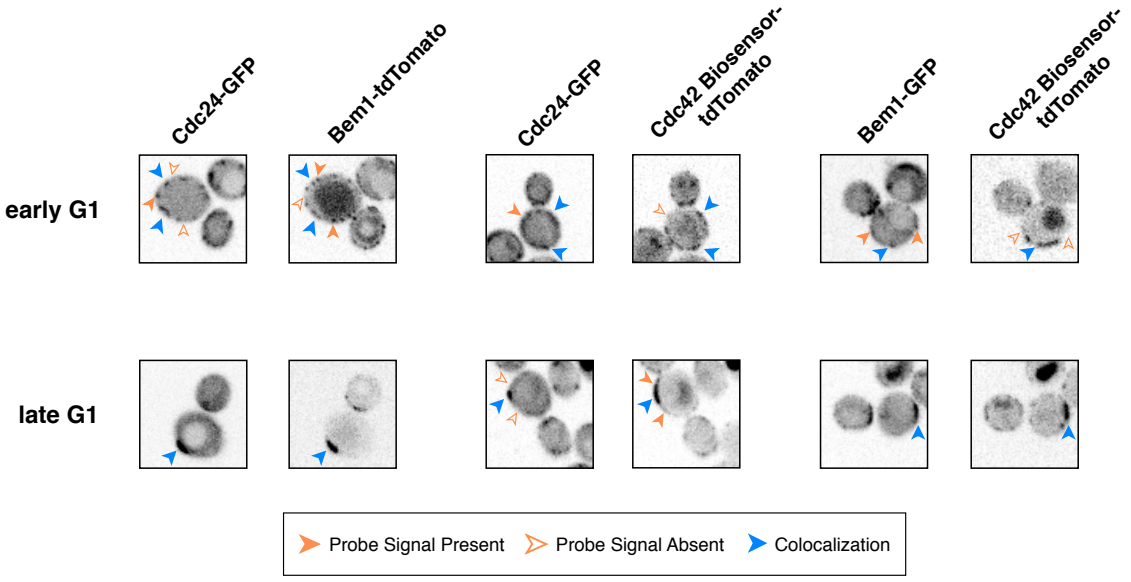


Figure 3.3: Single-plane images depict similar distributions as Z-stack projections.
Single plane snapshots feature similar distributions of Cdc24, Bem1, and Cdc42-GTP. Each image is 13.5 μm x 13.5 μm. Strains used: WYK8550, WYK8551, and WYK8552.

These results indicate that the majority of Cdc24 and Bem1 molecules are not in a constitutive complex during early G1, which is consistent with the finding that recruitment of Cdc24, but not Bem1, induces Cdc42 activation at this stage of the cell cycle. Cell cycle regulated assembly of the polarity complex could readily explain both results.

Section 3: Cdk1 activation is required for Bem1 accumulation

To gain insight into cell cycle regulation of the polarity complex, we performed Cdc24 recruitment studies in strains co-expressing a marker of cell cycle entry, Whi5-tdTomato, and either the Cdc42 biosensor or Bem1-tdTomato. Nuclear import of Whi5 is a marker of Cdk1 inactivation. Whi5 is concentrated in the nucleus during the interval between mitotic exit until Cdk1 activation at Start (Costanzo et al. 2004; Skotheim et al. 2008) (~25 minutes prior to bud emergence, Figure 3.4). Recruitment of Cdc24 can induce

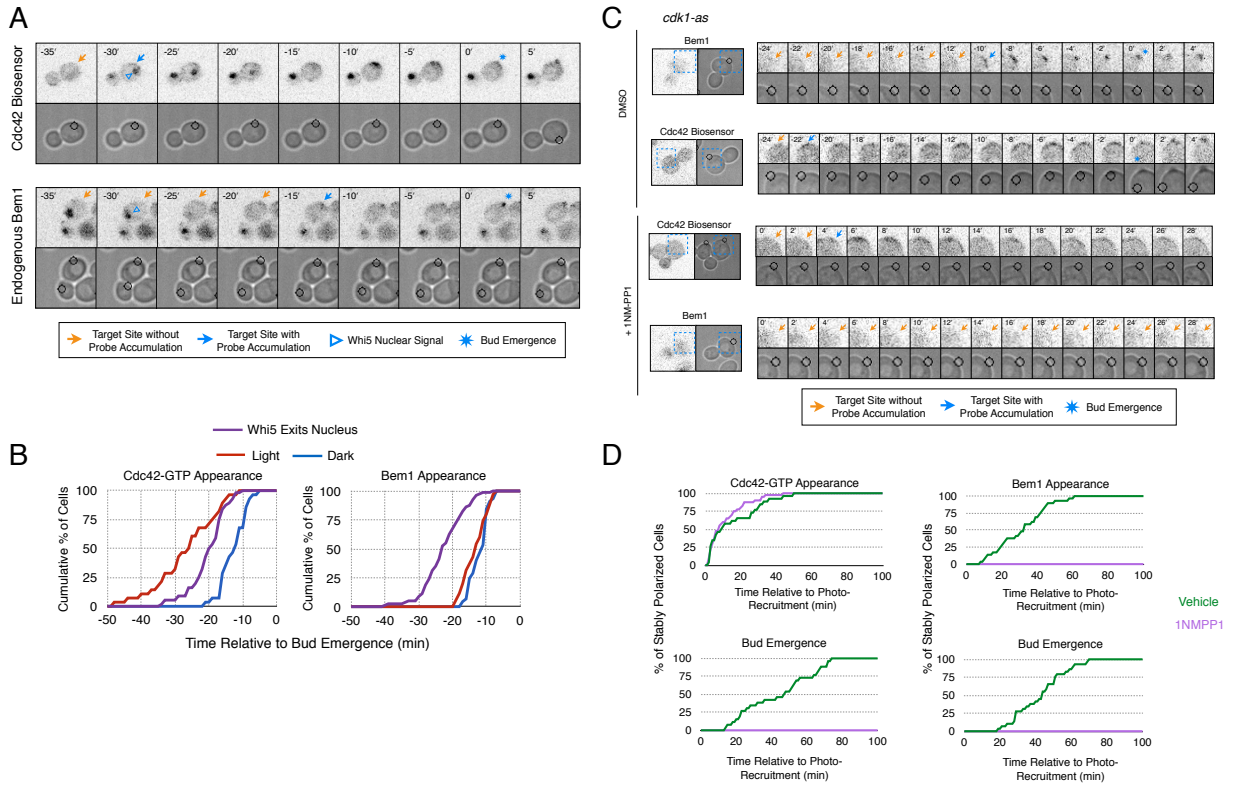


Figure 3.4: Cdk1 activation is required for Bem1 accumulation, but dispensable for Cdc42 activation.

A) Representative panel of time-course images from Cdc24 recruitment in cells co-expressing Whi5-tdTomato with either the Cdc42 biosensor or Bem1-tdTomato. Each image is 16.2 μm x 16.2 μm. Strains used: WYK8500 and WYK8502.

B) Whi5 nuclear exit kinetics and accumulation kinetics for either Cdc42 activation or Bem1 at Cdc24 recruitment sites. Purple line represents Whi5 exit, with data combined for both light- and dark-state conditions as they are not significantly different (Figure 5 - Supplemental Figure 1). Bud emergence occurs at time = 0. Data are combined across multiple experiments (n experiments > 2; N total cells > 25 for each condition).

C) Representative panels and sub-images of cells depicting the response to Cdc24 recruitment +/- Cdk1 activity. Each inset is 6.5 μm x 6.5 μm. Strains used: WYK8441 and WYK8442.

D) Accumulation plots indicating the response of Cdc42 biosensor or Bem1 in response to Cdc24 recruitment +/- Cdk1 activity. Purple lines represent cells treated with 75 μM 1NM-PP1. Green lines represent Vehicle-treated cells. Data are combined across multiple experiments (n experiments = 2; N total cells > 25 for each condition).

local Cdc42 activation in cells containing nuclear Whi5. Polarized accumulation of active Cdc42 precedes Whi5 nuclear export (Figure 3.5A, B). In contrast, cortical recruitment of Bem1-tdTomato did not occur until ~12 minutes after Whi5 nuclear exit, corresponding to ~13 min prior to budding (Figure 3.5A, B, Figure 3.6). These results

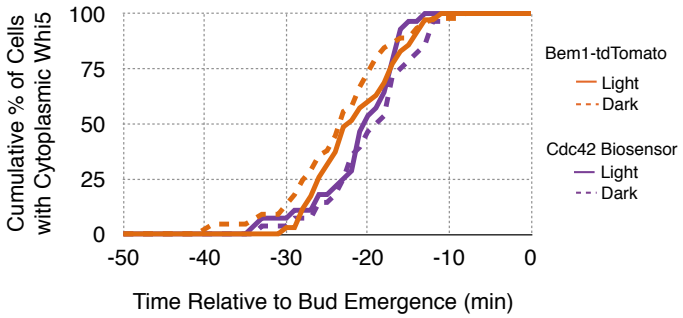


Figure 3.5: Expression of probe nor illumination affect the timing of Whi5 nuclear exit. Nuclear exit timing for Whi5 for each condition in **Figure 3.4B** plotted on a single plot.

are consistent with a model in which Cdk1 activation is required for the Bem1-GEF complex to engage active Cdc42.

To directly test whether Cdk1 activation is required for Bem1 recruitment in response to active Cdc42, we utilized an allele of Cdk1 that can be inhibited by an ATP-analog (cdk1-as1 and 1NM-PP1, respectively) (Bishop et al. 2000; McCusker et al. 2007). Asynchronous cells were pre-treated with 1NM-PP1 for 20 minutes and we subsequently monitored the ability of Cdc24 to induce Cdc42 activation or Bem1 accumulation in recruitment assays. We limited our analysis to mother cells with large-budded daughter cells, indicative of mother cells in early G1. Consistent with previous

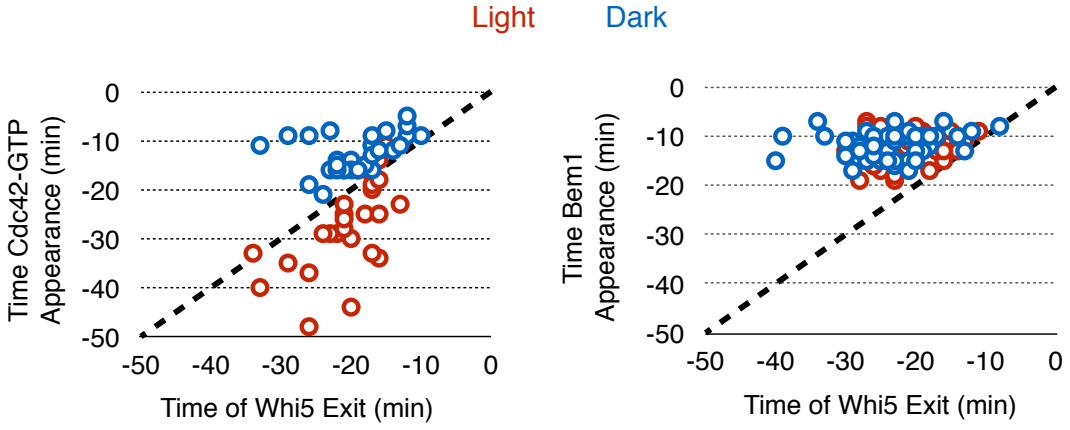


Figure 3.6: Photo-recruited Cdc24 activates Cdc42 prior to Whi5 nuclear exit. Correlation between Whi5 nuclear exit and either Cdc42 activation or Bem1 accumulation at Cdc24 recruitment sites. Dashed line indicates the event and Whi5 nuclear exit occurring simultaneously. Points below the dashed line indicate the event occurs before Whi5 nuclear exit. Points above the line indicate the event occurs after Whi5 nuclear exit. Data are as in **Figure 3.4B**.

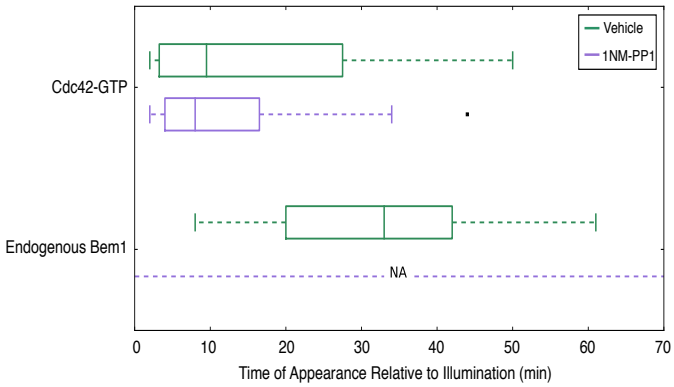


Figure 3.7: Bem1 accumulation requires Cdk1 activity in response to light-induced recruitment of Cdc24.
Box-and-whisker plot of Cdc42-GTP and Bem1 appearance in *cdk1-as* cells. Green boxes are vehicle-treated cells, while purple boxes are 1NM-PP1-treated cells. Outliers are depicted by black squares. Vehicle or 1NM-PP1 is added at time = -20 minutes; time = 0 is the start of live-cell imaging and photo-activation. Data are as in **Figure 3.4D**.

reports (McCusker et al. 2007), cells lacking Cdk1 activity could not undergo bud emergence nor could buds grow significantly (Figure 3.5C, D). Nevertheless, Cdc24 recruitment induced accumulation of Cdc42 in 67% of Cdk1-inhibited cells (data not shown). Indeed, Cdc42 was activated with the same kinetics irrespective of the presence or absence of the inhibitor (Figure 3.5D). Strikingly, when Cdk1 activity was suppressed, Bem1 failed to accumulate within the time frame of the experiment (greater

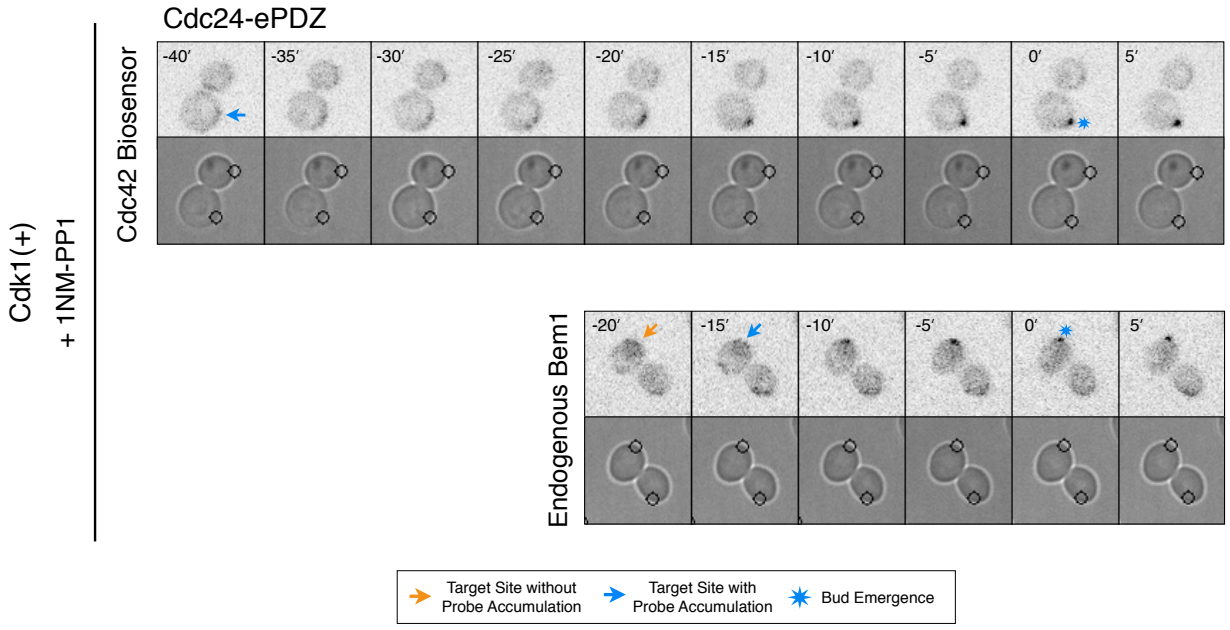


Figure 3.8: Addition of 1NM-PP1 to Cdk1(+) does not have adverse affects on Cdc42 biosensor or Bem1 accumulation or on bud emergence.
Fluorescent and phase images depicting representative Cdk1(+) treated with 1NM-PP1. Each image is 16.2 *u*m x 16.2 *u*m. Strains used: WYK8440 and WYK8301.

than 1.5 hours after addition of 1NM-PP1) (Figure 3.5C, D, Figure 3.7). Furthermore, when Bem1 was recruited, cells were unable to activate Cdc42 within the time frame of the experiment (data not shown). Cells expressing wild-type *cdk1* were unaffected by the addition of 1NM-PP1 (Figure 3.8). These results demonstrate that Cdk1 activation is required for both Bem1 accumulation and bud emergence even in cells with locally activated Cdc42.

Section 4: Precocious Cdc42 activation is not sufficient to recruit downstream effectors

Previous work has shown that Cdk1 activity is required for the accumulation of Cdc42 effectors, including those involved in actin assembly, vesicular trafficking, and septin collar formation (Evangelista et al. 1997; G. C. Chen, Kim, and Chan 1997; X. Zhang et al. 2001)}(Pruyne et al. 2004; Iwase et al. 2006). Strikingly, Cdk1 activity is dispensable for activation of Cdc42 by photo-induced Cdc24; yet, Cdk1 activation is required for accumulation of Bem1 in response to optogenetically recruited Cdc24 (Figure 3.5). Given that Bem1 is not strictly required for downstream signaling events - Bem1 is non-essential in Rsr1(+) cells (Irazoqui, Gladfelter, and Lew 2003) - we questioned whether precociously activated Cdc42 could engage other signaling pathways prior to Start. To that end, we assayed the accumulation kinetics of the polarisome component, Spa2, and the septin, Shs1, in mock-illuminated and photo-activated cells (Chenevert, Valtz, and Herskowitz 1994; Iwase et al. 2006). In both control and photo-activated cells, both Spa2-tdTomato and Shs1-tdTomato accumulated ~13 minutes before bud emergence (Figure 3.9A, B). These results suggest that precocious activation of Cdc42 is unable to

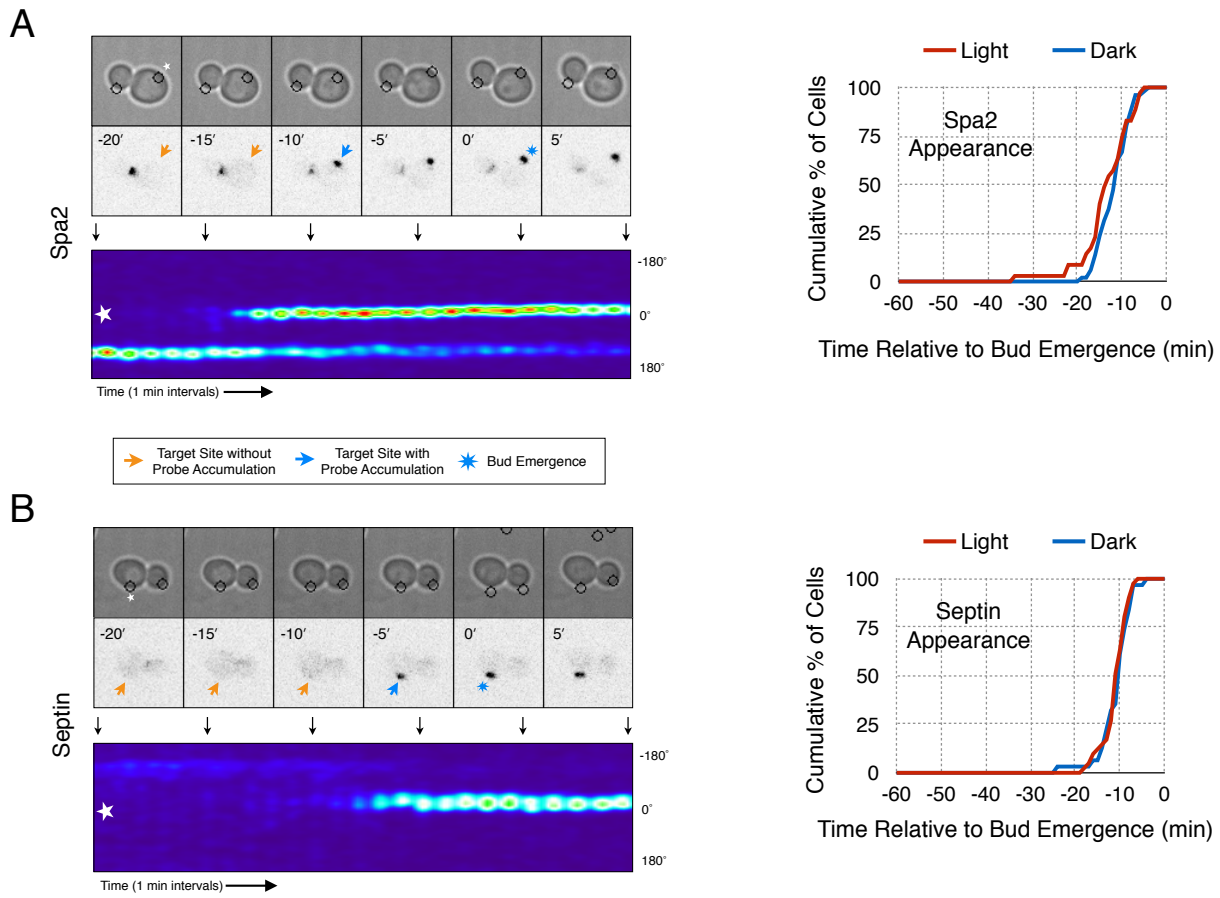


Figure 3.9: Precocious activation of Cdc42 is not sufficient to alter the kinetics of downstream effectors.
A) Representative panels depicting the accumulation of Spa2 in response to Cdc24 recruitment. Kymograph is below. Plots represent the accumulation kinetics for a population of cells in both photo- and mock-illuminated conditions (n experiments > 2, N total cells > 30 for each condition). Each image is 16.2 μm x 16.2 μm. Strain used: WYK8337.
B) Phase and fluorescence images and kymographs of a representative cell accumulating septins in response to Cdc24 recruitment. Accumulation kinetics for both dark and light conditions are to the right (n experiments > 2, N total cells > 30 for each condition). Each image is 16.2 μm x 16.2 μm. Strains used: WYK8335.

promote accumulation of downstream effectors involved in bud emergence, and thus, their accumulation to the nascent bud site requires Cdk1 activation.

Discussion

In an attempt to understand how the cell cycle exerts control over polarity establishment, we find that a critical mechanism appears to involve regulation of the

positive feedback-inducing polarity complex. We observe that Cdc24, Bem1, and Cdc42-GTP do not perpetually colocalize, with the lowest level of colocalization occurring prior to Cdk1 activation (Figure 3.1). The delay in Bem1-dependent positive feedback relative to Whi5 nuclear exit (Figure 3.5) suggests that the polarity complex is regulated by Cln1/2 (Skotheim et al. 2008). We propose that Cdk1 activity promotes assembly of the Bem1-Cdc24 complex, which is consistent with the accumulation of Bem1 at the bud neck in early G1 without accompanying Cdc42 activation (Atkins et al. 2013) (Figure 3.1), as well as a Cln2-dependent increase in GEF activity towards Cdc42 (Howell et al. 2009).

Cdk1 activity may regulate the association of both Cdc24 with Bem1 and Bem1 with Cla4. If the former were constitutive, then Bem1 recruitment during early G1 should also result in Cdc24 recruitment which would induce Cdc42 activation (Figure 10). However, Bem1 recruitment in early G1 had no detectable effect on Cdc42 activation (See Chapter 2, Section 3). Likewise, if the latter was constitutive, Cdc42 activation during G1 should induce Cla4 recruitment which would be predicted to induce Bem1 recruitment. However, Cdc24 recruitment in early G1 results in Cdc42 activation but does not result in Bem1 recruitment (See Chapter 2, Section 4). These results suggest that the canonical polarity complex is not assembled prior to Start. Additionally, G1 CDK activity is implicated in Rga2 and Bem3 regulation, GAPs that down-regulate Cdc42 activity (Knaus et al. 2007; Sopko et al. 2007). CDK activation at Start may therefore promote Cdc42 activation by at least two parallel pathways.

There also appears to be regulation of the positive feedback pathway after bud emergence. In polarized cells, Cdc24 recruitment at high light doses - but not Bem1

recruitment - activates Cdc42 (See Chapter 2, Section 2), raising the possibility that the well studied Cdc24-Bem1-Cla4 complex functions as a unit for a limited fraction of the cell cycle surrounding polarization. Further work focusing on the regulation of positive feedback activity after establishment would be informative in understanding singularity.

In spite of precocious activation of Cdc42 in early G1, we were unable to accelerate the accumulation of downstream effectors such as the polarisome and septins (Figure 3.9). It is unlikely that Bem1 is required for recruitment of downstream components, as it is not essential in *RSR1* cells (Irazoqui, Gladfelter, and Lew 2003). Alternatively, prior to Cdk1 activation, Cdc42 activity could be below the threshold required to recruit downstream effectors. Following Cdk1 activation (Figure 3.5), Cdc42 activity may cross this hypothetical threshold and recruit its downstream effectors. To test this possibility, we increased the light dose at the same position in a single cell, but did not observe precocious accumulation of downstream components (data not shown). The ability of the downstream effectors to functionally accumulate in response to Cdc42 activation appears to be cell cycle regulated, consistent with previous observations.

Chapter 4: Positive feedback in polarity establishment

[This chapter was adapted from Witte *et al*., *eLIFE*, 2017]

Abstract

Our data thus far indicates that prior to entry into the cell cycle, the canonical positive feedback loop is non-functional. Specifically, Bem1 and Cdc24 do not appear to be complexed prior to Cdk1 activation; yet, Cdc24 is fully capable of activating Cdc42 early in G1. These results suggest that Cdc24 can function independently of Bem1 prior to Start; though, the role of such functionality is unclear. In this chapter we investigate the characteristics of precocious Cdc42 activity in early G1. Strikingly, we reveal the existence of a second Cdc24-mediated positive feedback loop that functions in the apparent absence of Bem1. Furthermore, we show that this second positive feedback loop can be readily outcompeted by another site of Cdc42 activation, indicating that prior to Cdk1 activation cells are readily amenable to reorient their axis. Finally, we provide evidence that an actin-dependent positive feedback loop is not required for polarity establishment, further indicating that the role of actin is limited to polarity maintenance rather than establishment.

Section 1: Introduction

A variety of polarized cells invoke positive feedback as a primary mechanism in establishing and maintaining polarity (Thompson 2012). Positive feedback is robust as minute quantities of activators are sufficient to multiply rapidly and form a stable asymmetry that is resilient to diffusional dilution and active inhibitory mechanisms

(Turing 1952; Gierer and Meinhardt 1972). However, in many cellular contexts, positive feedback must only be functional at discrete times so as to coordinate the establishment of polarity with necessary cellular events (Johnson 1999; Das et al. 2012; Motegi and Seydoux 2013). In our previous chapter, we show that in budding yeast, positive feedback is not constitutively active throughout the cell cycle. Rather, positive feedback only becomes functional upon passage through Start. Previous models suggested that the critical event for initiating positive feedback was Cdk1-dependent Cdc42 activation. Nonetheless, we used optogenetic recruitment of the GEF, Cdc24, to show that cells were fully capable of activating Cdc42 in the absence of Cdk1 activity. What are the characteristics of Cdc42 activity in early G1? Does it play a role in promoting polarity establishment? By exploiting our optogenetic tool, we dynamically alter nascent sites of Cdc42 activation and assay the effects on polarity establishment. Additionally, we use pharmacological inhibition of actin assembly to query the requirement of an actin-mediated positive feedback loop in polarity establishment.

Section 2: Cdc42 activity can self-sustain prior to Cdk1 activation

A prominent model for positive feedback posits that the Bem1-GEF complex is constitutive (Bose et al. 2001). However, our data indicates that, prior to Cdk1 activation, optogenetically-recruited Cdc24 and Bem1 function differently. The ability of Cdc24 to activate Cdc42 in the apparent absence of Bem1 lacks precedent; therefore we studied the characteristics of these optogenetically-initiated sites. Specifically, we sought to determine the stability of these sites and whether these nascent sites of

polarization interact. To answer these questions, we exploited the ability of the optogenetic system to dynamically reposition the site of protein recruitment.

During the time window between the transition from isotropic growth and the time at which Cdc24 recruitment triggers Bem1 accumulation (i.e. ~13 minutes prior to bud

Figure 4.1

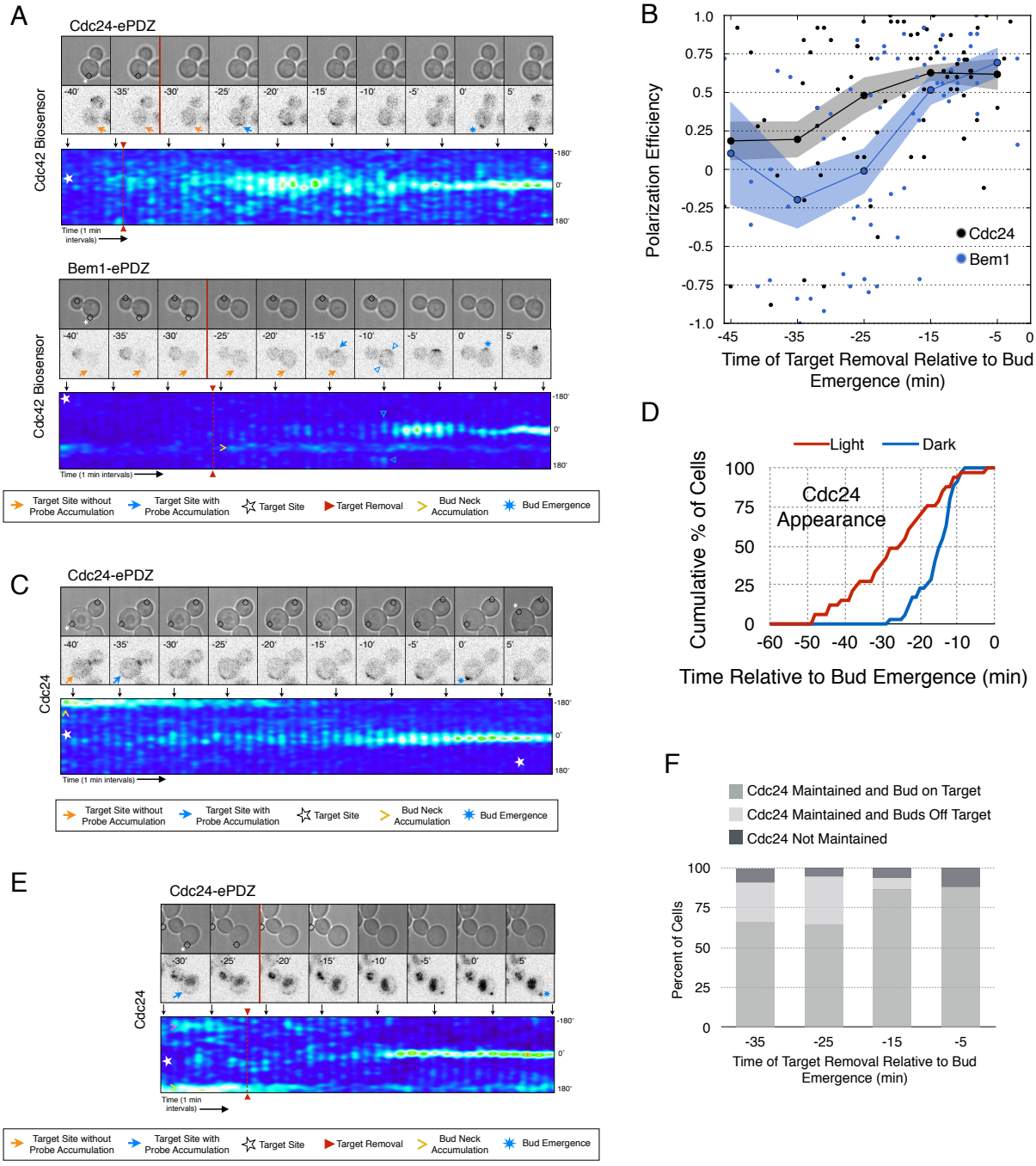


Figure 4.1 (continued): Cdc24 recruitment induces precocious Cdc42 activation and Cdc24-tdTomato accumulation that is self-sustaining.
A) Phase and fluorescence images and kymographs of representative cells depicting the response to the transient recruitment of Cdc24-ePDZ or Bem1-ePDZ. Initial target site denoted by white stars. Each image is 16.2 μm x 16.2 μm. Strains used: WYK8440 and WYK8308.
B) Polarization efficiency following transient recruitment of Cdc24 and Bem1. Time indicates when the target was removed relative to bud emergence (bud emergence occurs at time = 0). Black dots represent polarization of individual cells in response to transient Cdc24 recruitment. Blue dots represent polarization of individual cells in response to transient Bem1 recruitment. Lines represent averages (+/- SEM) of data binned in 10 min intervals, with the middle time point represented on the plot. Results are pooled across multiple experiments (n experiments >= 2; N cells > 10 for each time interval; N total cells > 75). Polarization Efficiencies of Cdc24 at -5, -15, and -25 min and Bem1 at -5 and -15 min were statistically significant relative to all earlier time points. Cdc24 and Bem1 Polarization Efficiencies were not statistically different at -45, -35, -15, and -5 min. $p < 0.05$, Mann-Whitney *U* test.
C) Representative phase and fluorescence images and kymographs showing the position of the laser target and accumulation of Cdc24-tdTomato in response to Cdc24-ePDZ recruitment. Strain used: WYK8575.
D) Accumulation kinetics for Cdc24-tdTomato. Data are combined across multiple experiments (n experiments > 2, N total cells > 30 for each condition).
E) Panels of representative phase and fluorescence images indicating the response of Cdc24-tdTomato to transient Cdc24-ePDZ recruitment. Orange arrows denote sites of illumination without Cdc24-tdTomato accumulation. Purple arrowhead indicates erroneous signal from vacuole. Strain used: WYK8575.
F) Stacked bar chart indicating the percentage of cells that maintain Cdc24-tdTomato accumulation in response to Cdc24 recruitment and whether they polarize to the prescribed site. Data is binned by 10 minute time intervals, with the middle time point represented on the plot. Data combined across multiple experiments (n experiments > 3, N total cells > 25)**.**

emergence), Cdc24 recruitment can induce Cdc42 activation. To test whether these sites are self sustaining, the targets were removed after Cdc42 activation was detected in early G1. Optogenetic recruitment of ePDZ-tagged proteins is largely reversed within 3 minutes after discontinuing illumination (Strickland et al. 2012), ensuring that any remaining signal is not due to Cdc24-ePDZ remaining at the target site due to the optogenetic tag. The region retained a faint but consistent signal of active Cdc42 for up to 30 minutes and ultimately resulted in budding from the specified site (Figure 4.1A, B). Conversely, Bem1 recruitment was only sufficient to bias the bud site if it was recruited within 15 minutes of bud emergence (Figure 4.1A, B). This observation shows that Cdc42 activity can be maintained at a unique site in the apparent absence of Bem1-

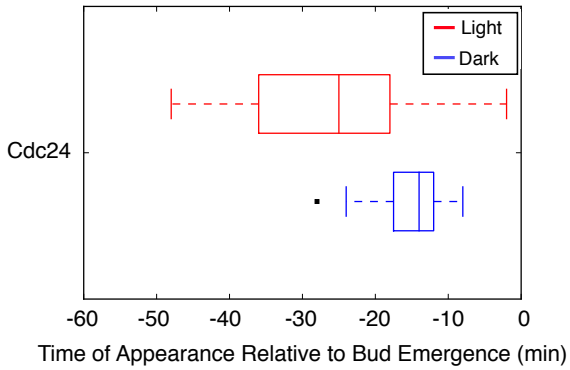


Figure 4.2: Optogenetic Cdc24 recruitment induces precocious accumulation of Cdc24-tdTomato. Box-and-whisker plot of Cdc24-tdTomato appearance in photo-activated (red) and mock-illuminated (blue) cells. Outliers are depicted by black squares. Bud emergence occurs at time = 0. Data as in **Figure 4.1D.**

mediated positive feedback. Thus optogenetic recruitment of Cdc24 can induce a self-sustaining pool of Cdc42 activation.

To determine how optogenetically induced Cdc42 activation could be maintained in the absence of the optogenetic cue, we tested whether recruited Cdc24 induces a change in the localization of cytosolic Cdc24. To that end, we recruited Cdc24 and visualized Cdc24-tdTomato. Indeed, optogenetically-recruited Cdc24-ePDZ altered the distribution of Cdc24-tdTomato in unpolarized cells (Figure 4.1C). Specifically, in 50% of cells in which Cdc24-ePDZ was recruited, Cdc24-tdTomato appeared on the cortex ~25 minutes before bud emergence, whereas in mock-illuminated cells, Cdc24-tdTomato appeared on the cortex ~15 minutes before bud emergence (Figure 4.1D, Figure 4.2). The kinetics of accumulation for Cdc24-tdTomato parallels that of activated Cdc42 in response to Cdc24 recruitment (See Chapter 2, Section 4, Figure 2.7). From these data we conclude that recruitment of Cdc24 can induce both Cdc42 activation and Cdc24 recruitment prior to Cdk1 activation, both of which appear to occur independently of Bem1. Collectively, these results reveal the existence of a second pathway for positive feedback. The observed activation of Cdc42 likely represents a combination of direct

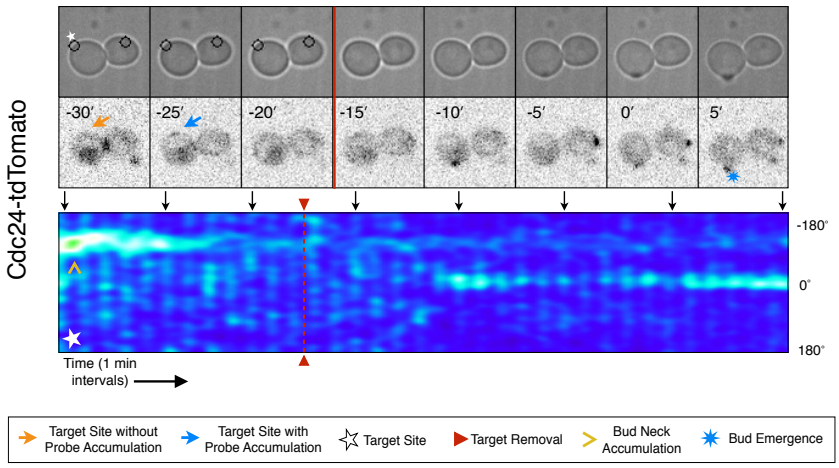


Figure 4.3: Transient Cdc24 recruitment can induce Cdc24-tdTomato accumulation, but cells can bud from an alternative position.
Representative fluorescence and phase time-course images for Cdc24-ePDZ transient recruitment when cells bud off target. Each image is 16.2 μm x 16.2 μm. Strain used: WYK8575.

Cdc42 activation by optogenetically recruited Cdc24 and its amplification by endogenous mechanisms.

Given that Cdc24-tdTomato accumulates at nascent sites, we hypothesized that it would remain at sites after the optogenetic cue was halted by removal of the target. Intriguingly, we observed Cdc24 was maintained at the site for ~6 minutes following cessation of optogenetic perturbation (Figure 4.1E). After this 6 minute time window, the Cdc24 signal became more punctate and these puncta dynamically associated with the previously targeted region. Greater than 65% of cells budded from the targeted region when target removal occurred ~30 minutes prior to bud emergence (Figure 4.1F). In cells that budded from sites outside the targeted region, Cdc24 was maintained at the targeted region until ~15 minutes before bud emergence when an intense focus of Cdc24 would appear at a new site that would ultimately form the bud (Figure 4.3). These data indicate that optogenetically-initiated sites of Cdc42 activation are stable

and are maintained, at least in part, by the accumulation of Cdc24, without accumulation of detectable Bem1.

Section 3: Nascent sites interact during early G1

To examine whether two sites of active Cdc42 in early G1 cells compete with one another, the target was repositioned within the same cell. Repositioning of the target caused Cdc42 accumulation at a new site and concomitant dissipation from the old site, with Cdc42 activity simultaneously detected at both sites for ~5 minutes (Figure 4.4A). However, the site of Cdc42 accumulation could not be continuously repositioned. A qualitative change in behavior occurs prior to bud emergence. When targets are repositioned within ~13 min of bud emergence, accumulation of Cdc42 at the initial site remains and Cdc42 accumulates weakly at the new site. The cell eventually buds from the site where Cdc42 was active at the ~13 minute transition point and Cdc42 signal from the alternate site dissipates upon bud emergence (Figure 4.4B). Our previous results demonstrate that Bem1 begins to accumulate at the targeted site ~13 minutes before bud emergence. To confirm that the basis for the qualitative switch relied on the ability of Cdc24 recruitment to induce Bem1 accumulation, we performed the same experiment in cells expressing Bem1-tdTomato. As previously shown, Bem1 only detectably accumulated at the target position defined at the ~13 minute transition point and it did not accumulate at new sites if the target was repositioned prior to bud emergence (Figure 4.4C, D). These results indicate that activation of the Bem1-dependent positive-feedback loop stabilizes sites of Cdc42 activation. Furthermore, these data suggest that prior to Cdk1 activation, nascent sites of polarization influence

Figure 4.4

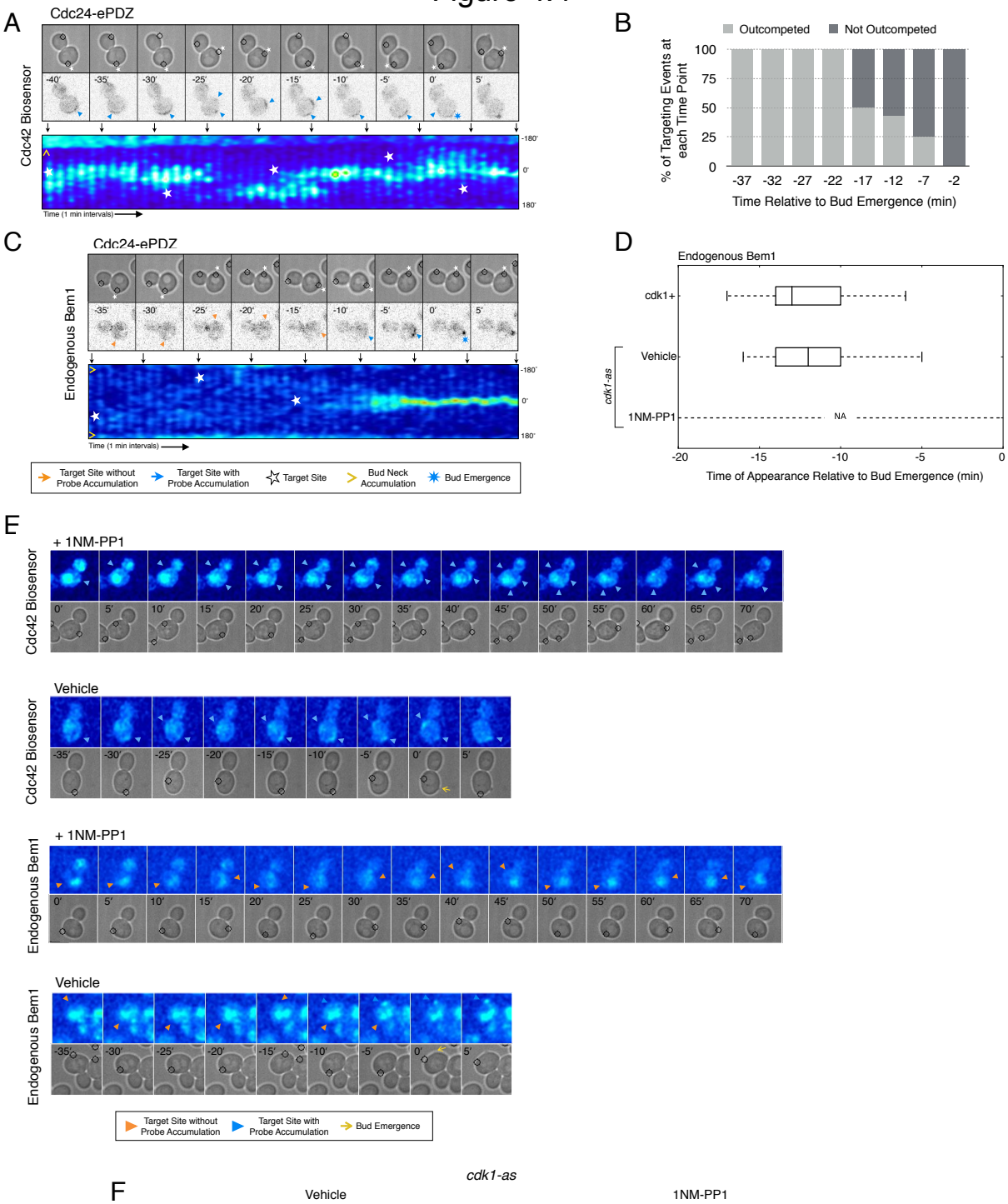

A
Cdc24-ePDZ
Cdc42 Biosensor
Time (1 min intervals)
B
Outcompeted
Not Outcompeted
% of Targeting Events at each Time Point
Time Relative to Bud Emergence (min)
C
Cdc24-ePDZ
Endogenous Bem1
Target Site without Probe Accumulation
Target Site with Probe Accumulation
Target Site
Bud Neck Accumulation
Bud Emergence
D
Endogenous Bem1
cdk1+
Vehicle
1NM-PP1
cdk1-as
NA
Time of Appearance Relative to Bud Emergence (min)
E
+ 1NM-PP1
Cdc42 Biosensor
Vehicle
Endogenous Bem1
F
cdk1-as
Vehicle
1NM-PP1
Time After Initial Cdc42 Activation (min)

Figure 4.4 (continued): Local Cdc24 recruitment induces precocious activation of Cdc42 that is dynamically maintained in the apparent absence of Bem1.
A) Panels of representative cells depicting the response to dynamically re-positioned Cdc24 recruitment. Upper panels consist of phase contrast and fluorescent images and the lower panel is a kymograph. The laser was moved every 10 +/- 2 minutes throughout the cell cycle, as denoted by the white stars. Each image is 16.2 μm x 16.2 μm. Strain used: WYK8440.
B) Percentage of targeting events "Outcompeted" or "Not Outcompeted" relative to bud emergence (Time = 0). Time indicates when the target was moved to a new position relative to bud emergence. Data is binned by 5 minute time intervals, with the middle time point represented on the plot. The event was scored as "outcompeted" if Cdc42 activity dissipated from the original position and accumulated at the new position. Conversely, the event was scored as "not outcompeted" if Cdc42 activity remained at the initial position upon target repositioning. Data are combined across multiple experiments (n total experiments > 2; n targeting events per time interval > 10; N total targeting events > 100; N total cells > 20).
C) Panels and a kymograph of a representative cell depicting the accumulation of Bem1 in response to dynamically re-positioned Cdc24 recruitment. Strain used: WYK8301.
D) Box-and-whisker plot denoting Bem1 accumulation in CDK1, *cdk1-as* + DMSO, and *cdk1-as* + 1NM-PP1 cells.
E) Time-course images of *cdk1-as* cells challenged with Cdc24 dynamic reorientation. Panels consist of phase contrast and either Cdc42-GTP or Bem1 accumulation psuedo-colored as a heat map. Cells were treated with either DMSO or 1NM-PP1 as denoted. Strains used: WYK8441 and WYK8442.
F) Quantification of dynamic reorientation in vehicle-treated or 1NM-PP1-treated *cdk1-as* cells. (n total experiments > 2; n targeting events per time interval > 10; N total targeting events > 100; N total cells > 20) Described in **B**.

Cdc42 activation at other sites.

Given that Bem1 accumulation at Cdc24-prescribed sites requires Cdk1 activation, we hypothesized that the dynamic properties of Cdc24-generated sites would persist in the absence of Cdk1 activation. To test this prediction, we repeated the experiment in cells expressing *cdk1-as* treated with 1NM-PP1 (Figure 4.4E). Again, limiting our analysis to mother cells with large-budded daughter cells, we found that Cdc42 activation could be dynamically repositioned for >70 minutes and that Bem1 was not detectably recruited within this time (Figure 4.4E, F). These results confirm that Cdk1 activation is required for Bem1-mediated positive feedback activity, which functions to establish the axis of polarity.

Combined, these results support three conclusions: (i) the ability of active Cdc42 to induce Bem1 accumulation is cell cycle regulated, (ii) once active, the canonical positive

feedback loop is highly stable and it prevents Cdc42 activation at competing sites, and (iii) maintenance of active Cdc42 and cytosolic Cdc24 before cell cycle entry appears independent of Bem1; therefore, the GTPase and the GEF participate in a positive feedback mechanism that functions earlier than the canonical Bem1-dependent positive feedback loop. This alternative positive feedback mechanism can be readily competed by a new site of Cdc24 recruitment; however, its existence suggests it could play a physiological role in establishing Cdc42 activity before Start in diploid cells.

Section 4: Actin is not required for polarity establishment

As F-actin has been proposed to play a role in polarity establishment (Wedlich-Soldner 2003; Jose et al. 2013), we examined whether actin depolymerization affects the response induced by local recruitment of Cdc24. Cells expressing light-recruitable Cdc24, Whi5-tdTomato and either the Cdc42 biosensor or Bem1-tdTomato were partially synchronized in G1 using a nocodazole block and release protocol and treated with Latrunculin A (LatA) to depolymerize F-actin. As previously shown, actin depolymerization partially inhibited cell polarization (Jose et al. 2013). While 88% of cells polarize Cdc42-GTP in the presence of f-actin, only 32% of cells polarize when actin is depolymerized (Figure 4.5). Actin depolymerization has a similar effect on the efficiency of Bem1-tdTomato polarization. Drug treatment also slows cell cycle entry as judged by the efficiency of Whi5 exit from the nucleus (Figure 4.6), indicating that actin depolymerization affects several cellular processes.

Filamentous actin has been suggested to facilitate delivery of Cdc42 to the cortex where it could be activated by Cdc24 (Wedlich-Soldner 2003). If true, Cdc24 recruitment would

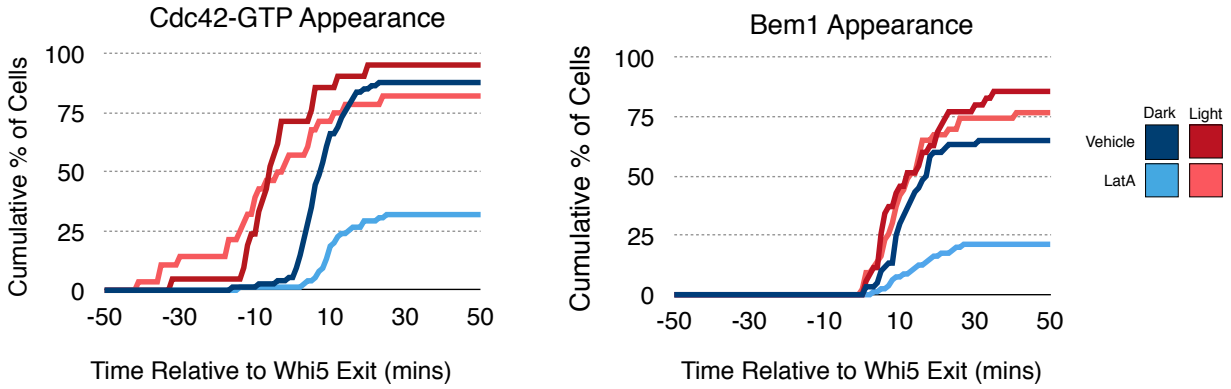


Figure 4.5: Localized recruitment of Cdc24 can overcome the polarity defect caused by actin depolymerization.
Accumulation kinetics for Cdc42-GTP or Bem1 in response to Cdc24 recruitment in the presence or absence of polymerized actin. Red lines represent cells exposed to light-induced Cdc24 recruitment. Blue lines represent mock-illuminated cells. Dark lines represent Vehicle-treated cells, while lighter-colored lines represent Latrunculin A-treated cells (see schematic). Whi5 nuclear exit was used as a cell cycle marker and accumulation of Cdc42-GTP or Bem1 was scored relative to Whi5 exit. Data was binned by 5 minute intervals and combined across multiple experiments (n experiments = 2; N total cells > 20). Stains used: WYK8500 and WYK8502.

not be predicted to correct the polarization defect. However, light-directed recruitment of Cdc24 dramatically increased the fraction of cells that locally accumulate Cdc42-GTP in the absence of actin. Similarly, Bem1 polarization was rescued by Cdc24 recruitment in cells treated with LatA (Figure 4.5). These results suggest that actin depolymerization does not appear to affect the availability of Cdc42, but rather it impacts the localization of the Bem1-Cdc24 complex, perhaps non-specifically.

Discussion

Discrete cortical localization of activated Cdc42 is required for symmetry breaking polarization (McCusker et al. 2007; Howell and Lew 2012; Bi and Park 2012). The formation and stabilization of the focus is thought to proceed via a positive feedback loop that employs the Cla4-Bem1-Cdc24 polarity complex, though direct evidence for its existence is lacking (Howell and Lew 2012). Utilizing two alternative optogenetic

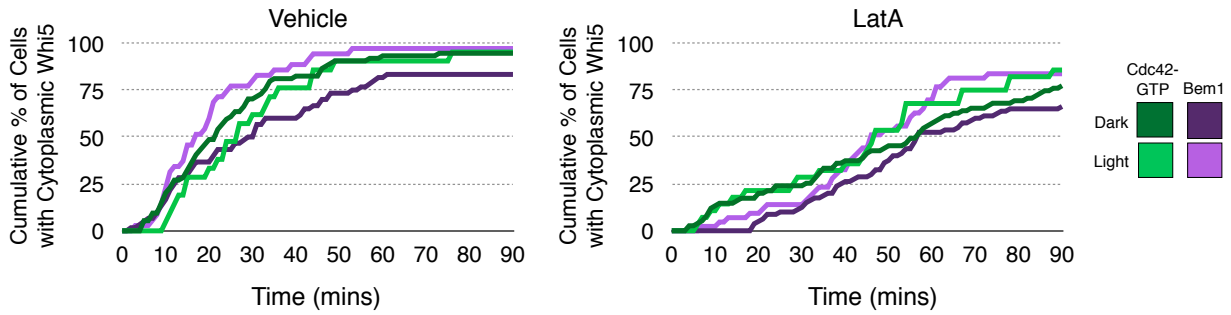


Figure 4.6: Loss of f-actin slows cell cycle entry.
Whi5 nuclear exit kinetics in Vehicle-treated (left) or LatrunculinA-treated (right) cells. Green lines refer to cells expressing the Cdc42 biosensor, while purple lines refer to cells expressing endogenous Bem1-tdTomato. Darker-colored lines represent mock-illuminated cells, while lighter-colored lines represent photo-activated cells (see schematic). Whi5 nuclear exit was slowed in LatA-treated cells. Data were binned by 5 minute intervals and combined across multiple experiments (n experiments = 2; N total cells > 20).

perturbations, we directly show that symmetry breaking engages positive feedback to establish polarity: 1) optogenetic recruitment of Bem1 induces accumulation of endogenous Bem1 (Figure 2.5) Cdc24 recruitment generates positive feedback by recruiting additional molecules of Cdc24 (Figure 4.1). Unexpectedly, we find that the GEF Cdc24 functions in two distinct positive feedback pathways; the Bem1-dependent loop that is active upon Cdk1 activation, and an earlier, positive feedback mechanism that appears to be Bem1-independent. The molecular mechanism of positive feedback during early G1 remains to be identified. It may involve Cdc24 oligomerization (Mionnet, Bogliolo, and Arkowitz 2008), however it is likely more complex as GEF-dead Cdc24 fails to induce Cdc42 activation or polarity establishment, suggesting Cdc42 also plays a role in this process.

By harnessing the spatiotemporal dynamics of optogenetically-controlled Cdc24, we revealed that engagement of the canonical Bem1-dependent positive feedback loop at a nascent bud site specifies the axis of polarity (Figure 4.4). If the canonical positive feedback loops commits a nascent site as the polarity axis, what could be the function

of the secondary positive feedback resulting in Cdc42 activity before Start? Prior models of symmetry breaking suggest that Cdc42 activation initiates upon Cdk1 activation (Howell and Lew 2012); though, we present unambiguous evidence to contradict this assumption (See Chapter 3, Section 3, Figure 3.5). Yet, according to this model, stochastic activation of Cdc42 recruits the Cla4-Bem1-Cdc24 complex to amplify this local inhomogeneity through positive feedback. Alternatively, Cdk1 activation may permit stochastic cortical association of Cla4-Bem1-Cdc24 via any of its myriad membrane association motifs. However, because Cdc24 can activate Cdc42 prior to Start, and because active Cdc42, Cdc24, and Bem1 are cortically detected prior to Start (See Chapter 3, Section 2, Figure 3.1), the site of polarization may not be dictated strictly by molecular noise or stochastic encounters of the Bem1-Cla4-Cdc24 complex with the membrane. Rather, a distinct mechanism(s) may exist that seeds the cortex with activated pools of Cdc42 during early G1 which pattern the “random” choice of bud site upon passage through Start (Figure 10). Indeed, Bud3, which is a validated Cdc42 GEF, induces an early wave of Cdc42 activation (Kang, Lee, and Park 2014). Though it is expressed in both haploid and diploid cells, Bud3 only impacts bud site selection in haploid cells (Chant et al. 1995). The linking of two mechanisms for Cdc42 activation could allow for more rapid axis specification than could be achieved by a reaction-diffusion based mechanism alone (Goryachev and Pokhilko 2008; Kang, Lee, and Park 2014). Indeed, such linked systems have been observed and modeled in a manner that can account for the speed of polarization events such as cell migration and the fixation of a single axis following an exploratory phase capable of reorientation (Brandman 2005).

Other models for symmetry breaking polarization invoke actin-dependent delivery of Cdc42 to the cortex (Wedlich-Soldner 2003; Jose et al. 2013). While depolymerization of actin does induce a polarity defect, we find that the defect can be readily suppressed by light-mediated recruitment of Cdc24 to the plasma membrane (Figure 4.5 and 4.6). This result indicates that in the absence of polymerized actin, Cdc42 still associates with the plasma membrane. Thus, actin filaments are more likely to facilitate, either directly or indirectly, the localization of the Cla4-Bem1-Cdc24 complex in a subset of cells.

Chapter 5: Ensuring singularity

[This chapter was adapted from Witte *et al.*, *eLIFE*, 2017 as well as unpublished data]

Abstract

Seemingly without fail, yeast cells iteratively form one and only one axis of polarity during each cell cycle. Moreover, our optogenetic investigations demonstrate that cells resist forming a new or second axis even when presented with the opportunity, indicating that cells robustly enforce singularity. In this chapter, we interrogate the mechanisms that ensure singularity. We uncover that the Bem1-dependent positive feedback loop is constrained to a single site in a manner that requires the RhoGDI, Rdi1. Building upon our prior actin results, we present evidence that actin filaments are one mechanism by which maintenance of an established axis occurs. Furthermore, we optogenetically probe the kinase Cla4, a seemingly multifaceted protein that is linked to both positive and negative regulation of polarization.

Section 1: Introduction

How do budding yeast ensure that only a single budding event occurs with each cell cycle? Strident effort has been made to understand this phenomenon, with long-standing models suggesting that one or more components of the polarity module were limiting such that only a single bud could emerge (Howell and Lew 2012; Howell et al. 2012). Yet, overexpression studies only marginally increase the rate of multi-buds (Howell et al. 2009), indicating that limiting components is not the sole singularity mechanism. Intriguingly, an allele of Cdc42 can induce multi-buds; however, it is

recessive, further supporting the existence of stringent mechanisms that ensure singularity (Caviston, Tcheperegine, and Bi 2002). Indeed, it is increasingly apparent that a coordinated effort amongst a wide variety of processes ensures singularity (Woods and Lew 2017).

For instance, recent work revealed that nascent sites of polarization can coexist, with each site undergoing oscillatory accumulations until an eventual “winner” emerged (Howell et al. 2012). Oscillations are necessarily a product of negative feedback; thus, this was the first evidence of a negative feedback loop in polarity establishment. Intriguingly, further work suggested that negative feedback was mediated through Cla4-dependent phosphorylation of the GEF, Cdc24; yet, ablation of Cdc24 phosphorylation sites did not adversely affect singularity (Kuo et al. 2014). Intriguingly, membrane-tethering this allele of Cdc24 and reducing the recycling of Cdc42 to the cytosol potently established multi-buds (C.-F. Wu et al. 2015), similar to that observed by the Cdc42 multi-budding allele (Caviston, Tcheperegine, and Bi 2002). These date support a model in which integration of negative feedback, cycling of the GEF on and off the membrane, and Cdc42 recycling promotes singularity.

The combinatorial properties of positive feedback and the mechanisms ascertained by Wu and colleagues suggest that singularity is maintained by an exquisite balance of polarity flux (C.-F. Wu et al. 2015; Woods et al. 2016). As such, one would predict that optogenetic recruitment of the GEF to a new axis may be sufficient to disrupt the flux and initiate a new axis. Our results unambiguously negate that prediction (See Chapter 2), suggesting that singularity is not fully understood. Here, we use optogenetics,

genetic perturbations, and a small molecule inhibitor to reveal the robust nature of the mechanisms that ensure singularity in budding yeast.

Section 2: Potent Bem1-dependent positive feedback only functions at a single site

In the previous experiments, only one Cdc24-targeted site was active at the moment when Cdk1 was activated resulting in activation of Bem1-mediated positive feedback loop. Cdc24 recruitment to multiple sites at this critical time might result in recruitment of Bem1 at multiple sites, leading to the formation of multiple buds. Alternatively, sites may compete with each other as they form resulting in only one site becoming fully established. Therefore, to investigate whether nascent sites also interact, we recruited Cdc24 to two sites simultaneously in unpolarized cells. Both sites generated activated Cdc42 (Figure 5.1A) and retained active Cdc42 until ~11 minutes before bud emergence. Subsequently, Cdc42 activity was limited to only one site (Figure 5.1B) and bud emergence occurred at that site. In a parallel experiment with recruited Cdc24, we monitored accumulation of Bem1 and observed that it accumulates at only one of the two sites, which invariably predicted the site of bud emergence (Figure 5.1A, B). Furthermore, despite recruitment of Bem1 to two sites simultaneously, in the overwhelming majority of cases (31/33 cells), Cdc42 activation and Bem1 accumulation occurred at one site, which ultimately defined the nascent bud (data not shown). These results indicate that multiple sites cannot coexist after activation of the Bem1-mediated positive feedback loop even under conditions in which they are simultaneously specified.

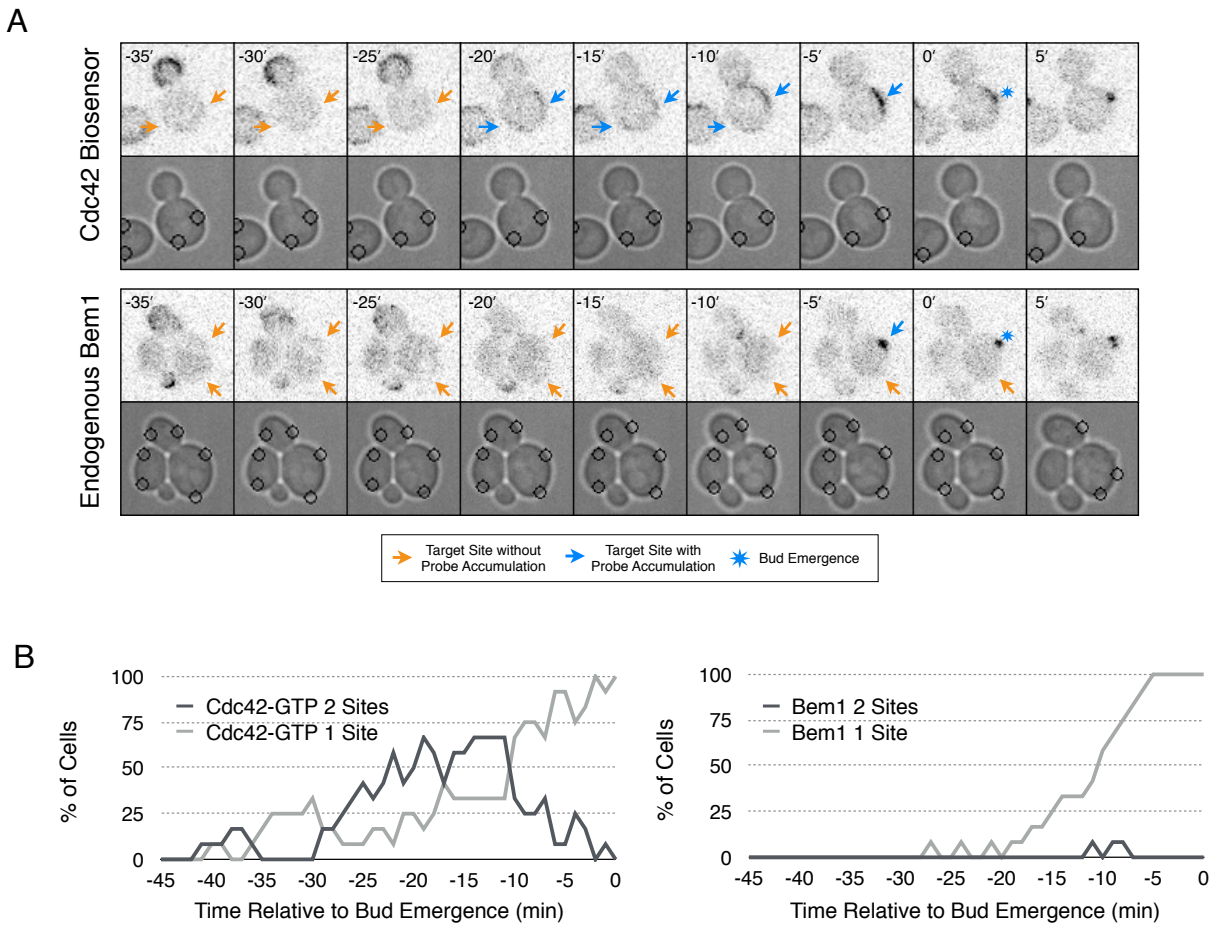


Figure 5.1: Nascent sites undergo competition to establish a single axis of polarity.
A) Representative fluorescence and phase images in response to recruitment of Cdc24 to two sites simultaneously. Top panel depicts Cdc42-GTP response. Bottom panel depicts Bem1 response. Each image is 16.2 μm x 16.2 μm. Strains used: WYK8440 and WYK8301.
B) Percentage of cells with signal at one or both sites at any given time relative to bud emergence. Dark gray lines depict percentage of cells with activation at two sites simultaneously. Light gray lines represent percentage of cells with accumulation at only a single site. Bud emergence occurs at time = 0. Data are combined across multiple experiments (n experiments >= 2; N total cells > 20 for each condition).

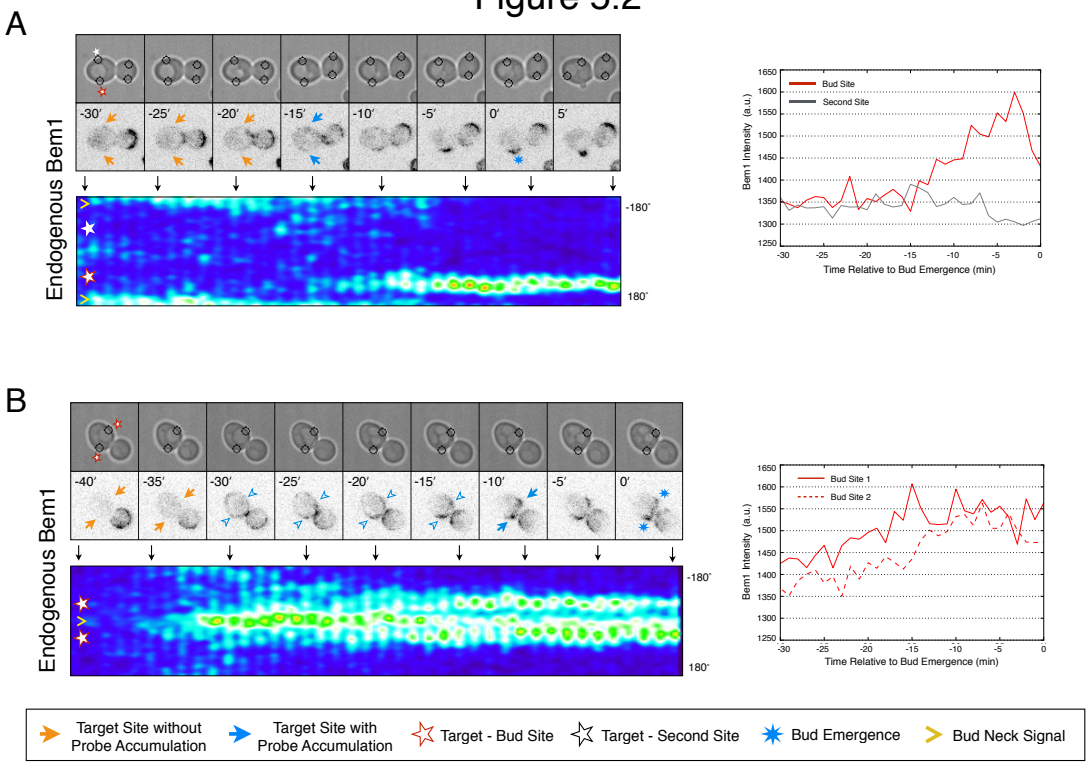
Section 3: Rdi1 deletion allows Bem1 at multiple sites

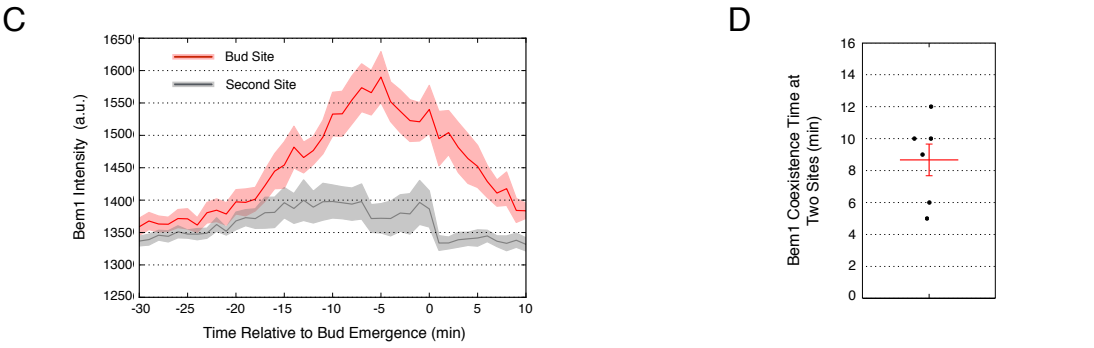
We wondered whether limitation of the canonical positive feedback loop to a single site was an inherent property of the polarity module. The Cdc42 auxiliary proteins, consisting of the GEF, Cdc24, the RhoGDI, Rdi1, and GAPs, Rga2/3, Bem2, and Bem3, coordinate their activities to balance Cdc42 activation at the cortex (Johnson 1999).

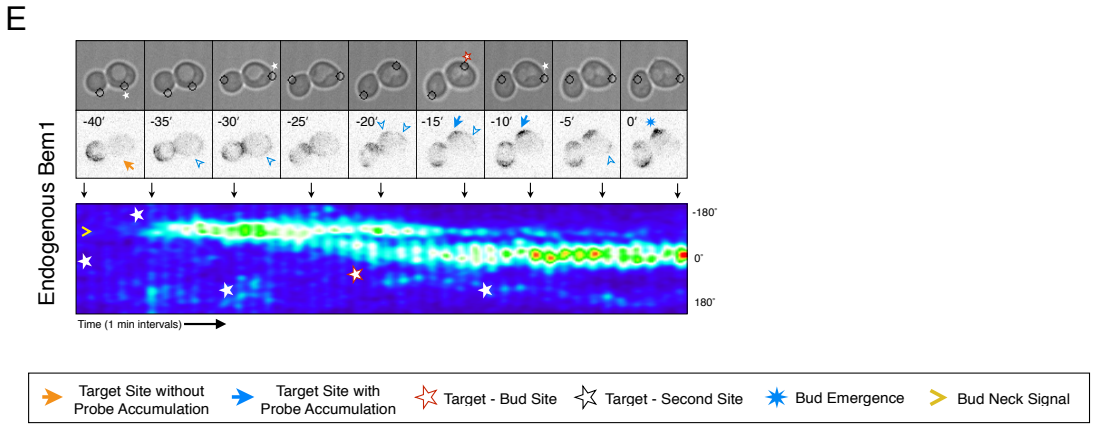
Given that Cdc24 is essential for Bem1-dependent positive feedback (Bose et al. 2001; Butty et al. 1998)(Howell, 2012), it is feasible that Rdi1 and/or GAPs may also modulate the positive feedback loop. In theory, Rdi1 has the capacity to modulate Cdc42 activity in two alternative ways: 1) it can extract inactive Cdc42 from the membrane, therefore limiting rapid reactivation and 2) it can "compete" with Cdc24 for inactive Cdc42, thereby sequestering Cdc42-GDP from its GEF. Collectively, this duality could both limit the growth of an incipient focus and inhibit the formation of a new focus. We generated diploid *rdi1Δ* strains and assayed the response of Bem1-tdTomato to multi-site recruitment of Cdc24.

Prior to any optogenetic perturbations, multi-budded cells were readily observed (2/12 cells). We illuminated unbudded cells at two sites simultaneously and observed accumulation of Bem1 at both sites. Intriguingly, multisite Bem1 accumulation occurred in the time period prior to Start, though only a small amount of Bem1 was detectable before Start (Figure 5.2A, B). If one site displayed increased Bem1 signal, that site consistently proved to be the "winner" and underwent bud emergence. In contrast, if both sites contained approximately the same amount of Bem1 ~15 minutes before bud emergence, Bem1 rapidly accumulated at both sites - presumably due to positive feedback activation (See Chapter 4, Section 3, Figure 4.4)(Figure 5.2C). The sites appeared to compete for Bem1, with a winner emerging within ~8 minutes (Figure 5.2D). In some instances, competition would not resolve prior to bud emergence and two buds would form (2/12 cells)(Figure 5.2B). Collectively, these results indicate that singularity is not inherent to the polarity module. Rather, additional factors, such as Rdi1, are required to ensure singularity.

Figure 5.2


A
Endogenous Bem1
-30′
-25′
-20′
-15′
-10′
-5′
0′
5′
-180°
180°
Bud Site
Second Site
Bem1 Intensity (a.u.)
Time Relative to Bud Emergence (min)
B
Endogenous Bem1
-40′
-35′
-30′
-25′
-20′
-15′
-10′
-5′
0′
-180°
180°
Bud Site 1
Bud Site 2
Bem1 Intensity (a.u.)
Time Relative to Bud Emergence (min)
Target Site without Probe Accumulation
Target Site with Probe Accumulation
Target - Bud Site
Target - Second Site
Bud Emergence
Bud Neck Signal


C
Bud Site
Second Site
Bem1 Intensity (a.u.)
Time Relative to Bud Emergence (min)
D
Bem1 Coexistence Time at Two Sites (min)


E
Endogenous Bem1
-40′
-35′
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-25′
-20′
-15′
-10′
-5′
0′
-180°
0°
180°
Time (1 min intervals)
Target Site without Probe Accumulation
Target Site with Probe Accumulation
Target - Bud Site
Target - Second Site
Bud Emergence
Bud Neck Signal

Figure 5.2 (continued): Rdi1 deletion facilitates Bem1 accumulation at multiple sites.
A) Panels of a representative cell depicting the response of endogenous Bem1 in *rdi1Δ* cells challenged with multi-site Cdc24 recruitment that buds from a single site. Upper panels consist of phase contrast and fluorescent images and the lower panel is a kymograph. Each image is 16.2 μm x 16.2 μm. Plot indicates Bem1 intensity in that particular cell at each targeted region relative to bud emergence. Red line indicates bud site trace. Gray line indicates second site trace. Strain used: WYK8410.
B) Panels of a representative cell depicting the response of endogenous Bem1 in *rdi1Δ* cells challenged with multi-site Cdc24 recruitment that buds from two sites. Upper panels consist of phase contrast and fluorescent images and the lower panel is a kymograph. Each image is 16.2 μm x 16.2 μm. Plot indicates Bem1 intensity in that particular cell at each targeted region relative to bud emergence.
C) Bem1 intensity profile for a population of cells that undergo budding from one position. Red line indicates bud site trace. Gray line indicates second site trace.
D) Coexistence time amongst nascent sites of Bem1.
E) Representative panel of *rdi1Δ* cells depicting the response to dynamically re-positioned Cdc24 recruitment. Upper panels consist of phase contrast and fluorescent images and the lower panel is a kymograph. Each image is 16.2 μm x 16.2 μm. Strain used: WYK8410.

These results are reminiscent of those observed by Wu and colleagues (C.-F. Wu et al. 2015)(See Introduction). The authors used *rdi1Δ* strains to slow the cycling of Cdc42 into the cytosol. In conjunction with membrane-tethered variants of either Bem1 or Cdc24, multiple buds readily formed (C.-F. Wu et al. 2015). The authors concluded that a greedy competition mechanism exists to resolve multiple nascent sites to a single axis of polarity. Our multisite interrogations are consistent with the greedy competition model. Moreover, we dynamically repositioned sites of Cdc24 recruitment in *rdi1Δ* diploids and observed Bem1 dynamics. Consistent with our multisite data, low levels of Bem1 accumulated at each nascent site and tended to remain cortical at previous sites until the onset of positive feedback (Figure 5.2E). Ultimately, a single site emerged to define the winner, in agreement with the greedy competition model. However, greedy competition does not appear to be the only mechanism of singularity. High doses of GEF recruitment are sufficient to activate Cdc42 in polarized cells (See Chapter 2, Section 2, Figure 2.1). One would predict the onset of competition between the growing bud and the newly initiated site (Jilkine and Edelstein-Keshet 2011; C.-F. Wu et al.

2015). Nevertheless, cells remain stably polarized toward the growing bud, indicating the existence of further mechanisms to reinforce singularity.

Section 4: Actin is required to maintain the established axis

While previous reports have suggested that actin filaments play a necessary role in polarity establishment (Wedlich-Soldner 2003)(Freisinger, 2013), we present data that indicates polarization defects caused by actin disassembly can be readily rescued by optogenetic recruitment of Cdc24 (See Chapter 4, Section 4, Figure 4.5). Moreover, such polarization defects may be attributed to deficiencies in cell cycle progression (See Chapter 4, Section 4, Figure 4.6). Consistent with these data, recent findings suggest that actin filaments do not participate in the initial process of polarity establishment, rather depolymerization of actin filaments likely induces cellular defects that obscure efficient polarization (Woods et al. 2016). Regardless, a large body evidence advocates that actin filaments are required for polarized growth (Wedlich-Soldner 2004; Slaughter et al. 2009; Jose et al. 2013). Therefore, we assessed the necessity of actin filaments in maintaining polarized growth toward the established axis.

Asynchronous populations of cells expressing photo-recruitable Cdc24 and either the Cdc42 biosensor or Bem1-tdTomato were treated with LatA to depolymerize F-actin. To directly compare the effects of optogenetic recruitment in stably polarized cells lacking F-actin, small-budded cells from the same coverslip were either challenged with optogenetic recruitment of Cdc24 or left unperturbed. Consistent with previous reports, loss of F-actin caused depolarization of both Cdc42-GTP and Bem1 (Wedlich-Soldner 2004). Strikingly, photo-recruitment of Cdc24 increased the rate of depolarization for

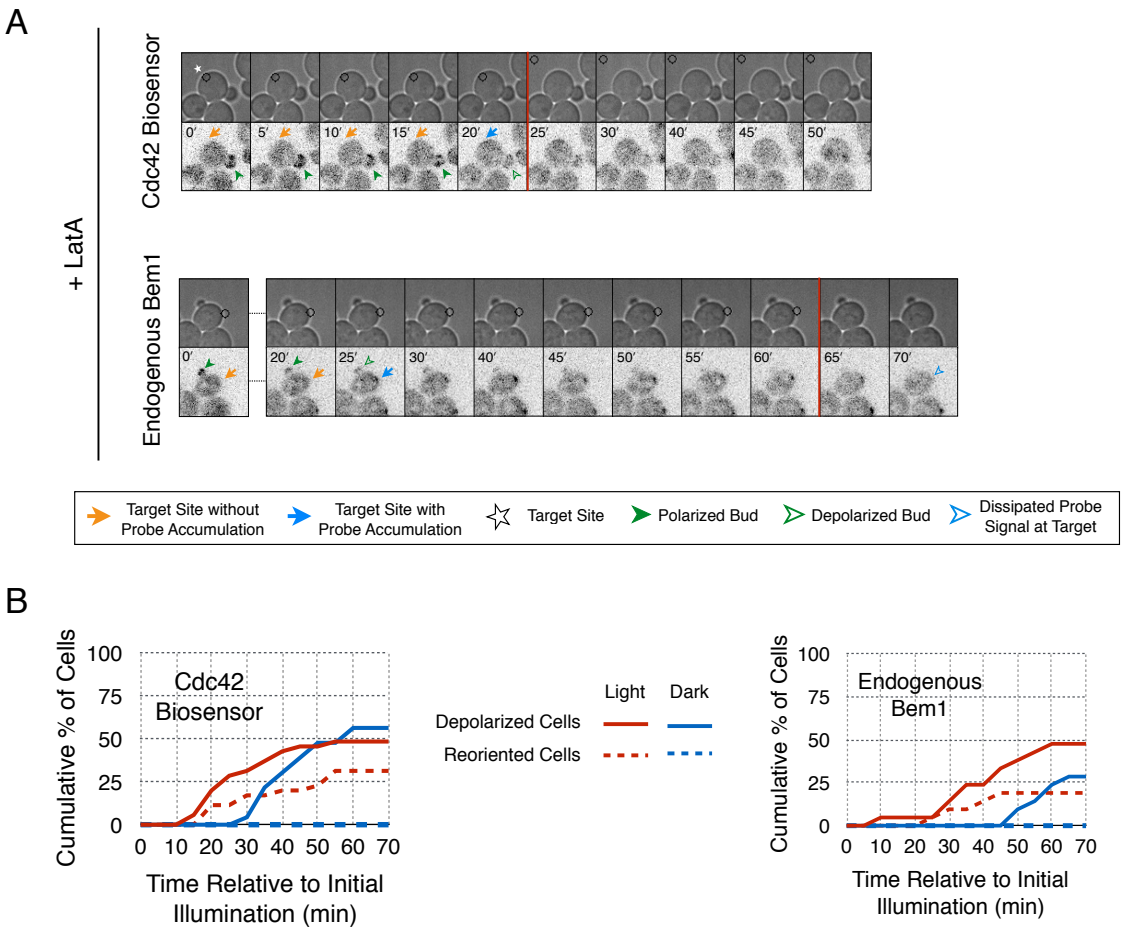


Figure 5.3: Actin is required to maintain the established axis in polarized cells.
A) Representative fluorescence and phase images in response to recruitment of Cdc24 in small-budded cells treated with LatrunculinA. Top panel depicts Cdc42 biosensor response. Bottom panel depicts Bem1 response. Each image is 16.2 μm x 16.2 μm. Strains used: WYK8440 and WYK8301.
B) Accumulation and reorientation kinetics for the Cdc42 biosensor or endogenous Bem1 in response to Cdc24 recruitment. Data are combined across multiple experiments (n experiments >= 4, N total cells > 20 for each condition).

both Cdc42-GTP and Bem1. Furthermore, depolarization of Bem1 was more prominent in photo-illuminated cells, whereas the percentage of cells featuring depolarized Cdc42-GTP was approximately proportionate under photo- and mock-illumination conditions (Figure 5.3A, B). These results suggest that in the absence of polymerized actin, initiating a new site of GEF activity can readily compete with polarity components in the growing bud.

Not only could optogenetic recruitment of Cdc24 increase the rate of depolarization, but a subset of depolarized cells were capable of reorienting Cdc42 activation and Bem1 accumulation toward the site of Cdc24 recruitment. Comparatively, reorientation of polarity components was not observed in mock-illuminated cells (Figure 5.3A, B), indicating that previously polarized cells are competent for initiating a new axis in the absence of F-actin. Collectively, these data suggest that actin filaments participate in reinforcing stable polarization of the polarity components; however, other mechanisms necessarily contribute to maintaining the axis. Upon first approximation, these data may reveal an actin-dependent mechanism for ensuring singularity. Further work will be required to validate and dissect this potential mechanism.

Discussion

Wild-type yeast cells rarely form multiple buds in a single cell cycle, suggesting the existence of mechanisms by which nascent bud sites compete in a winner take all competition. Recent work has shown that prior to bud emergence, cells are capable of forming multiple nascent foci. Yet, by employing mechanism(s) that appear to involve negative feedback, only one focus matures into the site of polarization (Howell et al. 2012), indicating competition between the nascent foci.

Cells have been genetically manipulated to generate multiple buds. For example, expression of activated alleles of Cdc42 can induce the formation of multiple buds (Caviston, Tcheperegine, and Bi 2002). Similarly, multiple buds result from membrane-tethering either Bem1 or a Cdc24 mutant that is resistant to negative feedback, while also hindering the membrane-cytoplasm exchange of Cdc42 by deleting the facilitating

chaperone, Rdi1. (C.-F. Wu et al. 2015). However, despite the fact that transient optogenetic membrane recruitment of Bem1 or Cdc24 can induce efficient polarization, neither proved robust enough to generate two buds, indicating that competition mechanism(s) are sufficiently strong to extinguish multiple nascent sites so that only a single axis emerges the winner. One such mechanism appears to utilize Rdi1.

Deletion of Rdi1 was sufficient to compromise singularity in a subset of cells as well as promote Bem1-dependent positive feedback at multiple sites (Figure 5.2). Rdi1 works in parallel with the GAP Bem2 to extract inactive Cdc42 from the focus, thereby counteracting the tendency to reactivate the GTPase that leads to cortical spreading of the patch (Woods et al. 2016). Mathematical modeling combined with genetic perturbations suggests that Rdi1 actively suppresses additional sites of Cdc42 activation by sequestering inactive Cdc42 into the cytosol (Klünder et al. 2013), a function that need not be limited to establishment. We were unable to induce new axes in polarized *rdi1Δ* cells (See Chapter 5, Section 5.3), despite the prediction that Cdc42 would be readily available (Klünder et al. 2013). In contrast, wildtype cells treated with the actin depolymerizing agent Latrunculin A, readily depolarized from growing buds and in some instances were able to reorient their axis. A model to account for these observations may invoke synergistic efforts between Rdi1 and actin, wherein Rdi1 limits the recruitment of cytosolic Cdc42 to multiple sites, while polymerized actin funnels vesicular-associated Cdc42 toward the established axis. Indeed, *rdi1Δ* cells can be encouraged to increase multi-bud formation by treatment with the moderate actin filament inhibitor Latrunculin B (Freisinger et al. 1AD). Further exploration utilizing

optogenetic recruitment of Cdc24 in *rdi1Δ* cells treated with an actin depolymerizing agent may reveal one mode by which cells ensure singularity.

A negative feedback loop extending from the polarity complex is slowly being elucidated as another mechanism of singularity (Gulli et al. 2000; Howell et al. 2012; Kuo et al. 2014; Rapali et al. 2017). Active Cdc42 likely recruits the polarity complex via interactions with Cla4, a kinase implicated in down-regulating GEF activity (Gulli et al. 2000; Howell et al. 2012; Kuo et al. 2014; Rapali et al. 2017). Specifically, Bem1 binding to Cdc24 appears to stimulate its GEF activity and such stimulation can be down regulated by Cla4-dependent phosphorylation. Strikingly, Cdc24 phosphorylation is exacerbated by Bem1, and phosphorylated Cdc24 is resistant to Bem1-induced stimulation. These observations indicate that Bem1 mediates the flux of Cdc42 activation at nascent sites (Rapali et al. 2017). The nature of this type of coordinated flux generally leads to pulsatile dynamics (Alon 2006), in which and initial expansion is followed by retraction or abatement of a cortical Cdc42-GTP patch. Upon initial decrease of GEF activity, Cdc42-GDP would be predicted to increase in nascent foci. However, Cdc42-GDP would be reactivated in the absence of mechanisms that recycle it to the cytoplasm, thereby broadening the zone of Cdc42 activation and encouraging the persistence of nascent sites that would normally be extinguished. Thus, Rdi1 and Cla4-dependent negative feedback may cooperate to limit erroneous Cdc42 activation, thereby promoting singularity.

What is quickly being revealed is that a myriad set of tightly-regulated mechanisms cooperate to ensure singularity. It is critical to understand how cells spatially and temporally integrate these components to maintain a single axis of polarity, as not all

mechanisms appear to be active nor do they appear to function similarly during both establishment and maintenance of polarized growth. Indeed, mechanisms that promote axis specification need not be the same mechanisms that reinforce the axis (Motegi and Seydoux 2013; Thompson 2012; C.-H. Huang et al. 2013)

Chapter 6: Conclusions

Section 1: Summary

In this work, we sought to understand the mechanisms with which yeast cells stably establish and maintain a single axis of polarity. Traditional genetic and cell biological analyses have identified the key components and revealed many behaviors associated with cell polarization, which have led to a set of models that invoke positive feedback (Wedlich-Soldner 2003; Irazoqui, Gladfelter, and Lew 2003; Goryachev and Pokhilko 2008; Howell et al. 2012; Howell and Lew 2012). However, the field has lacked the tools that can directly interrogate the spatio-temporal dynamics of signaling molecules and critically test these models. Here, we used optogenetics to probe the endogenous regulatory mechanisms that control Cdc42 activity. We have found that symmetry breaking engages a positive feedback loop mediated by Cdc24 and Bem1, presumably as part of the tripartite polarity complex, and that actin is not required to establish an axis of polarity. Further, this complex is sufficient to outcompete the landmark pathway. We have also found that the activity of this pathway is temporally confined by cell cycle regulation; therefore, stringent mechanisms exist to limit erroneous Cdc42 activity. For instance, our results indicate that polymerized actin functions to reinforce local concentration of polarity components toward the growing bud. In addition, we have demonstrated that potent mechanisms ensure that only a single site can undergo positive feedback at a given time. Finally, we have demonstrated the existence of a second, previously uncharacterized, positive feedback mechanism that can maintain a focus of Cdc42 activity prior to Cdk1 activation and which may play a role in symmetry breaking polarization (Figure 6.1).

Section 2: A mechanism to rapidly establish an axis of polarity

In symmetry breaking polarization, Cdk1-dependent stochastic accumulation of Cdc42 to unique cortical positions is thought to be the initial step in polarity establishment (Howell and Lew 2012; Bi and Park 2012). There are several mechanisms by which this could be achieved. For instance, one could imagine that the polarity complex may stochastically initiate many small “seeds” of Cdc42 activity that can be readily amplified by positive feedback. Alternatively, stochastic cortical activation of pre-Start Cdc42 may locally recruit the polarity complex to establish a single axis. The latter requires that sufficient Cdc42-GTP must be locally concentrated to engage the polarity complex. Intriguingly, mathematical models have suggested that as little as six molecules of activated Cdc42 may be sufficient (Goryachev and Pokhilko 2008; Howell et al. 2012; Kuo et al. 2014; Woods et al. 2016). Yet, GAP activity is relatively high in pre-Start conditions (Knaus et al. 2007; Sopko et al. 2007). As such, locally concentrated Cdc42 activity is unlikely to be dictated strictly by molecular noise (Altschuler et al. 2008; Chau et al. 2012). Accordingly, simulations of cluster formation in response to molecular noise proceed on time-scales much greater than that observed physiologically (Goryachev and Pokhilko 2008; Howell et al. 2012). Thus, how can an initial cluster form prior to Start? We have evidence of a previously uncharacterized mechanism in yeast symmetry breaking: a second positive feedback loop that functions prior to Start.

We provide multifaceted data that demonstrate Cdc42-GTP readily accumulates at distinct cortical patches prior to Cdk1 activation and that entry into Start initiates the

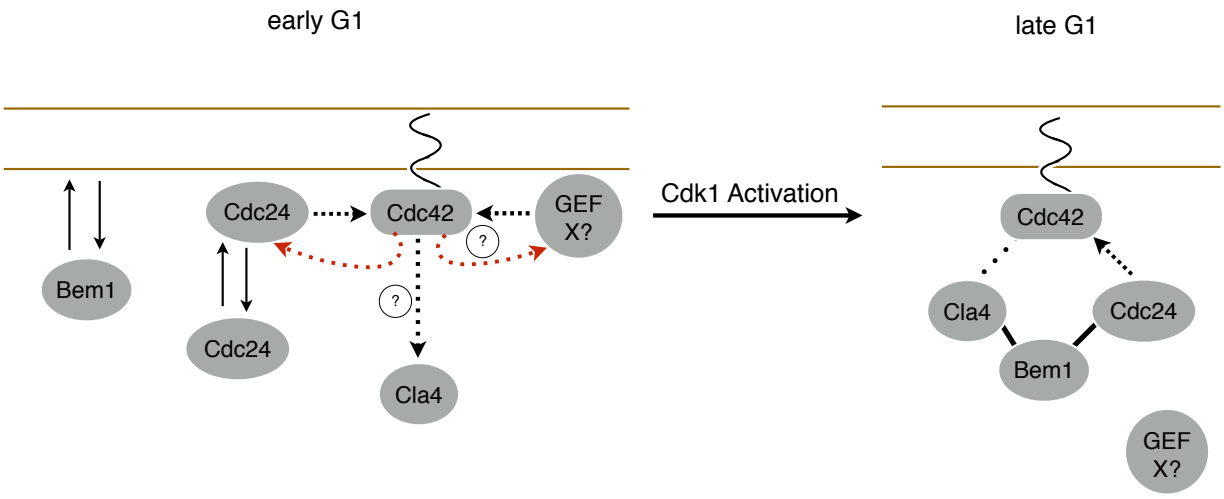


Figure 6.1: Model for polarity establishment.
In early G1, prior to Cdk1 activation, Cdc24, Bem1, and activated Cdc42 individually associate with the plasma membrane, but do not form stable complexes. With some frequency, Cdc24, and perhaps other Cdc42 GEFs, activate Cdc42 which can participate in a weak positive feedback loop with one or both GEFs. It is unclear whether Cdc42-GTP in early G1 interact with downstream effectors, such as Cla4, but if it does, it does not initiation Bem1 dependent positive feedback. Following Cdk1 activation, the Cla4-Bem1-Cdc24 complex assembles in late G1. This complex, may amplify the preexisting focus of Cdc42-GTP, and ultimately undergo strong positive feedback to generate a single focus of Cdc42-GTP that subsequently triggers bud emergence.

Bem1-dependent positive feedback loop to fully commit a nascent site to bud emergence. Prior to Start both Bem1 and Cdc24 are dynamically associated with the cortex, yet they do not appreciably colocalize. Further, pre-Start cells do not respond to Bem1 recruitment, yet they potently activate Cdc42 in response to GEF recruitment. Strikingly, pre-Start Cdc42 activity is metastable and is sustained by a positive feedback loop involving Cdc24. Collectively, these results provide a potential mechanism by which pre-Start positive feedback can “seed” the cortex with many sites of Cdc42 activation, acting as a multi-cluster intermediate stage that is primed for amplification upon entry into Start when the Bem1-containing complex is activated. Consistent with this model, multi-cluster intermediates have been observed in both symmetry breaking and landmark-directed cells, suggesting that this stage is critical in promoting yeast polarization (Howell et al. 2012; C. F. Wu, Savage, and Lew 2013).

To form this multi-cluster intermediate stage despite high GAP activity, a positive feedback loop capable of forming multi-fronts would likely be sufficient to counteract rapid GAP inhibition (Otsuji et al. 2007; Altschuler et al. 2008). A mathematical model that well-describes these behaviors is a wave-pinning system, a model which features a fast-diffusing activator that promotes its own accumulation (Jilkine and Edelstein-Keshet 2011). Fast-diffusion allows for multiple fronts as the activator is not locally limited to its initial source of production. Intriguingly, this model is consistent with the reorientation behaviors observed in mating cells responding to a pheromone gradient, a program that also proceeds during the pre-Start phase of the cell cycle (Dyer et al. 2013). However, fast diffusion implies that generation of a relatively stable site of activation requires overcoming a high threshold (Mori, Jilkine, and Edelstein-Keshet 2008). In the yeast system prior to Cdk1 activation, the high threshold would be set by global GAP activity. Following entry into the cell cycle, GAP activity decreases (Knaus et al. 2007; Sopko et al. 2007); thus, continued employment of a wave-pinning type of polarization would rapidly evolve into either rampant depolarization or multi-buds (Jilkine and Edelstein-Keshet 2011; Mori, Jilkine, and Edelstein-Keshet 2008). As such, a new mechanism must engage - likely, Bem1-dependent positive feedback.

Consistent with this hypothesis, hair-trigger Turing-type models are most often used to describe yeast polarization. Under this type of polarization scheme, there is a low threshold (i.e. reduced GAP activity) to noise (i.e. nascent pre-Start clusters) that allows for rapid formation of a stable focus in response to small fluctuations (i.e. Bem1-dependent positive feedback). Accordingly, the first mathematical model of yeast polarization utilized a Turing-type reaction-diffusion system that necessitated an initial

cluster of Cdc42 to engage the Bem1-GEF complex and induce a stable asymmetry, else polarization required extended time periods (60 minutes) to stabilize (Goryachev and Pokhilko 2008). However, in order to limit the formation of a focus to a discrete position on the cell cortex, a fast-acting inhibitor must also be present (Turing 1952; Gierer and Meinhardt 1972). Likely, a set of mechanisms synergize to ensure that only a single axis of polarity emerges. This will be discussed in more detail in the following section.

We propose a model in which two linked positive feedback loops promote the rapid evolution of polarity establishment in symmetry breaking (Figure 6.1). The first is highly dynamic and capable of initiating many small clusters of Cdc42 activity at distinct cortical patches. The second is robust, functioning to specify the axis as well as squelch the formation of incipient sites. Further work involving mathematical modeling, genetics, and optogenetic perturbations will be required to both validate and dissect the molecular mechanisms by which these positive feedback loops cooperate to establish polarity.

Section 3: Ensuring singularity

Critical to the proliferation of budding yeast is the establishment of a single axis of polarity. Multi-budding leads to DNA segregation defects and ultimately aberrant cell growth (Freisinger et al. 1AD; Caviston et al. 2003); thus, robust mechanisms must be in place to ensure singularity (Howell and Lew 2012; Bi and Park 2012). Current data implicate mechanisms involving negative feedback, Cdc42 inactivation and recycling, and global inhibition as means to ensure singularity. Accordingly, we provide evidence to

suggest that several mechanisms are temporally and spatially coordinated to ensure that only a single bud emerges (See Chapter 5).

3.1: Global inhibition

One mechanism is likely executed by the engagement of delayed negative feedback to hinder the growth of a nascent bud. This mode of negative feedback appears mediated by Cla4-dependent phosphorylation of Cdc24 (Gulli et al. 2000; Kuo et al. 2014; Rapali et al. 2017). A recent study found that Bem1 gates Cdc42 activity by stimulating the GEF, Cdc24, and then subsequently promoting down-regulation of Cdc24 by Cla4-dependent phosphorylation. Ultimately, the down-regulated Cdc24 was no longer responsive to Bem1-dependent stimulation (Rapali et al. 2017). This mechanism could provide not only local negative feedback, but it could also act as a long-range inhibitor. Down-regulation of the GEF by phosphorylation appears to persist (Gulli et al. 2000; Rapali et al. 2017), suggesting a mechanism to decrease the rate of Cdc42 activation after an initial pulse induced by Cdk1 activation (Ozbudak, Becskei, and van Oudenaarden 2005; Howell et al. 2012; Kuo et al. 2014; C.-F. Wu et al. 2015)(See Chapter 3, Section 3.3). As cytoplasmic diffusion readily occurs (Valdez-Taubas and Pelham 2003), the down-regulated complex can effectively act as a long-range "inhibitor" of secondary incipient sites in that association of the complex with these opposing site would not be able to amplify cortical Cdc42 at a greater rate than the initial patch (Meinhardt and Gierer 1974; Alon 2006). In this manner, a distinct cluster of cortical Cdc42 activity would both dampen its own growth and limit the growth of other nascent sites. Consistent with such a model, the initial detection of Cdc42-GTP or Bem1

at nascent sites overshoots the final steady state levels observed upon bud emergence (Ozbudak, Becskei, and van Oudenaarden 2005; Howell et al. 2012; Kuo et al. 2014; C.-F. Wu et al. 2015), an observation consistent with inhibition. Moreover, this mechanism of global inhibition would favor the evolution of Cdc42 larger clusters into the axis of polarity, as smaller clusters would be unable to compete. Indeed, resolution of multiple nascent sites tends to favor larger clusters (Howell et al. 2009; Howell et al. 2012)(Chapter 5, Section 5.1 and 5.2).

A permutation of this type of long-range inhibition is observed in the filamentous fungi *Ustilago maydis*, which engages a similar tripartite complex to polarize the Rho-family GTPase Rac1 and promote filamentous growth (Leveleki et al. 2004; Mahlert et al. 2006; Alvarez-Tabarés and Pérez-Martín 2008). Cla4-dependent phosphorylation of Cdc24 in *U. maydis* directs Cdc24 degradation, thereby decreasing the rate of Rac1 activation. Moreover, stabilization of Cdc24 abrogates filamentous growth, indicating that GEF inhibition is require for polarization (Frieser et al. 2011). Thus, long-range inhibition is achieved not by GEF down-regulation, but rather, degradation of the GEF. These results underscore the critical nature of coupling positive feedback and long-range inhibition and suggest a core circuit that ensures polarity emergence (Gierer and Meinhardt 1972; Chau et al. 2012).

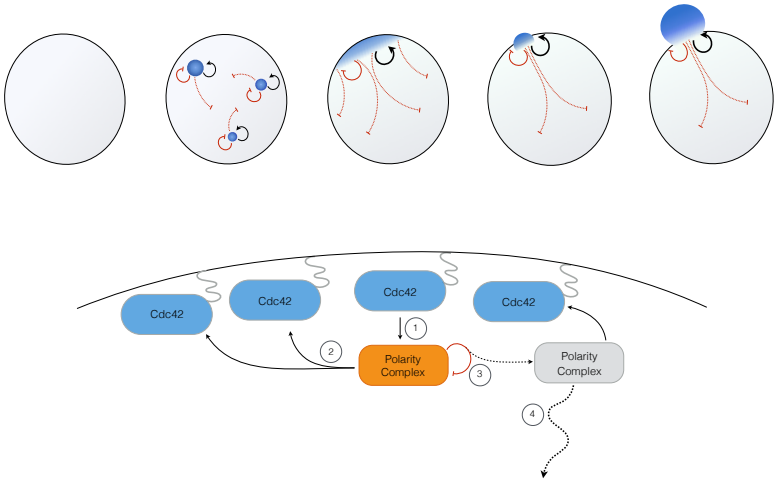
3.2: Manipulating diffusive flux

While it is evident that a series of mechanisms ensure singularity in budding yeast, fission yeast must form two stable zones of polarized growth. Furthermore, both yeasts appear to employ a similar molecular architecture to reinforce their polarized domains

Figure 6.2

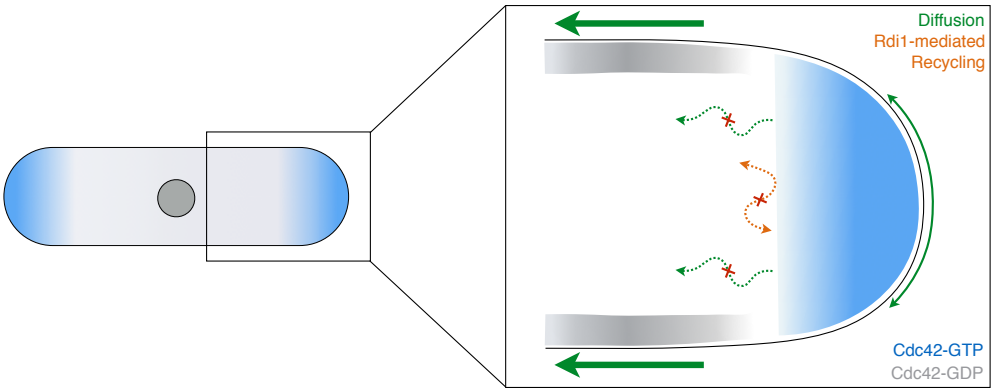
A

Global inhibition as a contributor to singularity



B

Bipolarity emergence from membrane-biased flux



C

Singularity emergence from cytoplasmic flux

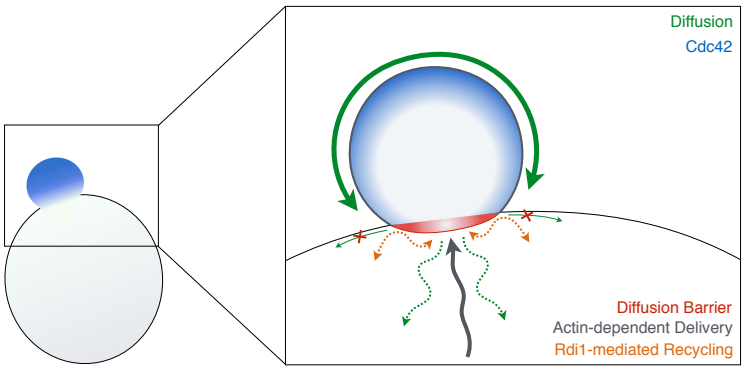


Figure 6.2: Mechanisms to ensure singularity.
A) A multi-cluster intermediate stage may precede polarity establishment, wherein each cluster competes with and inhibits the others until a single site emerges the winner. 1) Cdc42-GTP recruits an initially hyperactive polarity complex (orange). 2) The hyperactive complex generates an initial pulse of Cdc42 activity. 3) Cla4 phosphorylation-induced down-regulation of the polarity complex yields negative feedback. 4) The down-regulated complex (gray), attenuates the rate of Cdc42 activation and ultimately diffuses from its source of productions, acting as a global down-regulator of Cdc42 activation.
B) In fission yeast, spatial flux of Cdc42 primarily occurs through lateral membrane diffusion. By restricting Cdc42 to the plane in which it polarizes, bipolarity may emerge via acquisition of shared pool of Cdc42-GDP.
C) Lateral membrane diffusion is limited in budding yeast, with Cdc42 spatial flux mediated through membrane-cytoplasmic exchange. Singularity may emerge by sequestering Cdc42 from alternative membrane positions, thereby spatially restricting Cdc42 to the established axis.

(F. Chang and Peter 2003; Martin and Arkowitz 2014), suggesting that the activity of the polarity module can be tuned to allow for multi-axis growth. Potentially, such tuning could be achieved through altering spatial Cdc42 flux.

3.2.1: Membrane flux as a mechanism to promote bipolarity

In fission yeast, the primary mechanism for spatial Cdc42 flux from the polarized cell tips is through lateral membrane diffusion, as constitutively tethering Cdc42 to the plasma membrane or deleting Rdi1 has no affect on polarization (Bendezú et al. 2015). Indeed, Cdc42-GTP concentrates at the cell tips whereas Cdc42-GDP accumulates at the cell sides (Bendezú et al. 2015), suggesting that each pole draws from a membrane-associated pool of Cdc42-GDP. Using positive feedback, cell poles could capture membrane-associated Cdc42-GDP while corralling Cdc42-GTP, which displays a much slower membrane diffusion rate than the inactive molecule (Bendezú et al. 2015). Therefore, reinforcement of the polarized domain is limited by the dynamics of membrane-associated Cdc42-GDP, rather than a combinatorial flux that also engages Rdi-mediated extraction.

These properties can also account for the oscillatory accumulations observed in fission yeast (Das et al. 2012). Locally acting negative feedback could generate fast-diffusing Cdc42-GDP that will at some rate escape and rapidly diffuse to the opposite pole (Das et al. 2012; Bendezú et al. 2015). As microtubule-dependent delivery of landmark-proteins appears to direct the Shk1-Scd2-Scd1 complex toward each pole (F. Chang and Peter 2003; F. Chang and Martin 2009), the complex could locally activate escaped Cdc42-GDP, thereby establishing bipolar growth. In this model, bipolarity emerges from the retention of Cdc42 in the membrane, thereby limiting its spatial flux to the same sub-cellular space in which polarization occurs.

3.2.2: Cytoplasmic flux as a mechanism to ensure singularity

While it is unclear whether the nucleotide state of Cdc42 in budding yeast also endows the molecule with differential membrane diffusion rates, reduced membrane diffusion appears to play a critical role in promoting polarization. Non-uniform diffusion of Cdc42 is observed in polarizing cells, with diffusion drastically reduced in cortical patches of Cdc42 (Slaughter et al. 1AD). Additionally, membrane diffusion between the polarized bud and the mother cell is potently inhibited soon after bud emergence as daughter cell identity is established upon septal ring assembly (Okada et al. 2013). These observations suggest that leakage of polarized Cdc42 through membrane-based diffusion is hindered relative to that in fission yeast. Consistent with this hypothesis, we observe dynamic mobility of Cdc42-GTP, Cdc24, and Bem1 within the growing bud that is absent within the mother cell (data not shown).

In further contrast to fission yeast, membrane-cytoplasmic exchange of polarized Cdc42 is prominent (Wedlich-Soldner 2004; Slaughter et al. 2009; Freisinger et al. 1AD).

Surprisingly, both Cdc42-GDP and Cdc42-GTP undergo exchange; though, exchange of the inactive molecule is greater (Woods et al. 2016). In this context, cytoplasmic leakage of Cdc42-GTP out of the polarized domain would tend to dissipate polarity. However, this exchange appears to be counteracted by actin-dependent delivery of Cdc42 toward the growing bud (See Chapter 5, Section 4) (Wedlich-Soldner 2003; Wedlich-Soldner 2004), endocytic corralling (Jose et al. 2013), and Rdi1-mediated delivery of Cdc42-GDP (Woods et al. 2016). Collectively, these data support a model in which spatial Cdc42 flux is biased toward membrane-cytoplasmic exchange, thereby limiting the association of Cdc42 with non-polarized membrane. Under this regime, singularity is reinforced by sequestering Cdc42 from alternative membrane positions that appear amenable for polarization (See Chapter, Section 4). Therefore, while the molecular architecture of polarization in budding and fission yeast is similar, tuning of properties such as membrane diffusion and recycling may account for the generation of bipolarity or singularity. Indeed, the beauty of simple Turing-type reaction-diffusion mechanisms is in the vast parameter space that can be almost infinitely diversified to construct complex morphogenetic patterns (Kondo and Miura 2010).

3.2.3: The exploitation of diffusive properties in other polarity systems

The exploitation of diffusive properties may have applications in higher organisms as well. Differential diffusion rates are employed in the polarized *C. elegans* embryo. Both fluorescence recovery after photobleaching (FRAP) and single-molecule studies have shown that the PAR proteins are highly dynamic, rapidly diffusing within and dissociating from the membrane (Cheeks et al. 2004; Goehring et al. 2011; Sailer et al. 2015). However, the diffusional properties of both anterior and posterior PARs are reduced

upon the formation of dynamic clusters (Sailer et al. 2015). The anterior PAR, PAR-3 appears to form high order oligomers, acting as a docking site for other anterior PARs. Additionally, a component of the posterior PAR module, CHIN-1, which is a GAP for Cdc42, forms clusters within the posterior domain. The formation of these complementary clusters appears necessary to limit the spread of the opposing domain. Likely, the rapid diffusion and cycling dynamics of individual molecules initiates ultrasensitive cluster growth (Sailer et al. 2015), thereby establishing a dynamic cross-inhibitory pathway to maintain the polarized asymmetry.

Chemotaxing cells may also exploit diffusional properties. Comparatively, the membrane diffusion rate of Cdc42-GTP in mammalian cells is much greater than that observed in yeast (Valdez-Taubas and Pelham 2003). As chemotaxing cells must necessarily reorient their axis in response to changing cues (Van Haastert and Devreotes 2004; King and Insall 2009), harnessing rapid diffusion could be one mechanism by which such cells can retain their plasticity. For instance, upon neutrophil polarization, tension in the plasma membrane decreases diffusion from the polarized pseudopod. Relinquishing such tension expands the polarized domain, consistent with the idea that rapidly diffusing components can dynamically shape zones of polarization in response to changing cues (Houk et al. 2012).

Section 4: Integrating optogenetics

Bud site selection in budding yeast is a simple, yet intricate system amenable for uncovering the underlying mechanisms that promote polarization in a wide variety of cell types. By using optogenetic perturbations to control the dynamic signaling molecules

that promote polarity establishment in budding yeast, we have revealed unexpected cell cycle regulation of the yeast polarity module. In addition, we present evidence that polarity establishment integrates dual positive feedback loops to rapidly specify an axis of polarity. Finally, our perturbations reveal robust and synergistic mechanisms that ensure singularity. Our work exemplifies the utilization of optogenetic tools in analyzing the intricate dynamics of signaling molecules in polarization schemes.

Not only can optogenetics be used to interrogate the sub-cellular dynamics that promote polarization, similar manipulations can be employed to interrogate a wide variety of cellular programs. For instance, developing organisms intricately coordinate a myriad of behaviors - including migration, signaling, polarization, and contraction - that are executed at the tissue, cellular, and sub-cellular level. Strikingly, each of these processes are exquisitely timed so as to ensure robust development (Leptin 2005; Weijer 2009; Basson 2012). Yet, it is unclear how these phenomena are temporally executed. Evidence from single cells suggests that cellular machinery may be “primed” for activity, rapidly engaging in response to a temporally-constrained signaling cue (C.-H. Huang et al. 2013; Yang, Collins, and Meyer 2016). Optogenetic control of such signaling molecules alleviates this temporal constraint, thereby providing a means to direct assess whether output behaviors are primed for activity. Indeed, work from our own lab in developing *Drosophila* epithelia indicate that tissue-wide morphogenetic processes can be accelerated in response to an optogenetic cue (Rich and Glotzer, unpublished), suggesting the existence of underlying organization that is poised at the response boundary.

Cellular processes that reside at such boundaries necessarily employ robust noise filtering mechanisms to limit erroneous activity (Cardelli et al. 2016; Zechner et al. 2016). Most often a multitude of parallel pathways cooperate to ensure such a robust filter (Kitano 2004; Xu and Lan 2015); thus, it is difficult to ascertain the nature of these mechanisms as parallel pathways can compensate for the hinderance of one arm in response to endogenous cues (Rao, Wolf, and Arkin 2002; Kitano 2004; Alon 2006). By optogenetically stimulating a sensitized system, it may be possible to identify mutants that would not necessarily give a phenotype in response to the endogenous signal.

Not only can optogenetic perturbations uncover noise filtering mechanisms, these perturbations can quantitatively investigate the transformation from noise-filtering to signal response. In some instances, activity is a consequence of linear exposure, either by passage through distinct steps or by crossing a specified threshold (Kholodenko 2006). In a proof of principle experiment, optogenetic analysis of the Ras-Erk pathway has revealed exquisite information processing that promotes analog sensitivity rather than digital cellular responses. (Toettcher, Weiner, and Lim 2013). Alternatively, some systems appear to exhibit ultrasensitivity, in which bistable states emerge from a sharp boundary transition (Flory 1953).

Similar investigations could also be used to understand bistable switches as a consequence of ultrasensitivity. In the context of anterior-posterior polarity in the *C. elegans* embryos, CHIN-1 clustering is requisite to maintain the axis and formation of these clusters appears dependent on ultrasensitivity (Sailer et al. 2015). Sub-cellular optogenetic control of CHIN-1 could directly test the ultrasensitivity model for cluster formation, and identify the critical transition point. Moreover, control of cluster formation

would provide direct interrogation of polarity robustness in response to optogenetically-induced CHIN-1 cluster formation in the anterior. Indeed, sub-cellular control of signaling molecules is a powerful tool, yet the understanding of how positional information regulates signaling output is only just being uncovered.

In Ras GTPase signaling, sub-cellular microenvironments promote alternative kinase cascades by activating distinct scaffolding proteins (Casar et al. 2009). Current models suggest that local concentration of specific factors, like scaffolding components, may be sufficient to define a microenvironment (Casar et al. 2009; Langeberg and Scott 2015). Optogenetic-induced clustering with sub-cellular precision would be sufficient to form such microdomains *de novo* (Tischer and Weiner 2014); however, concomitant spatiotemporal regulation of signaling molecules is requisite to fully ascertain the sufficiency of microdomains to instruct signaling cascades. As such, concerted effort is being put forth to identify light activated proteins that respond to wavelengths outside of the canonical blue range (Tischer and Weiner 2014). Development of alternatively responsive light systems would allow the integration of multiple optogenetic systems within a single cell, thereby allowing exquisite interrogation of the mechanisms that control position-specific signaling events. By taking cues from the evolution of modularity in signaling components, these advances will promote the construction of customizable cellular circuits that can intricately control multi-platform signaling cascades that execute diverse and complex biological processes, thereby granting unprecedented ability to both predict and control emerging biological behaviors.

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Appendix

Materials and Methods

Plasmid and strain construction

DNA manipulations were simulated with SnapGene (GSL Biotech). Plasmids were generated using a combination of conventional ligation, homologous recombination (SLICE) in bacteria (Y. Zhang, Werling, and Edelmann 2012), and Gibson Assembly (Gibson et al. 2009). All plasmids were verified by DNA sequencing.

All strains (Supplementary File 1A) were constructed in the W303 background (leu2- 3 112 ura3-52 can1-100 ade2-1 his3-11 trp1-1). Haploid a and α cells were mated by incubating overnight in 500 μL YPD and then plating on selective media. All integrating plasmids were of the YIplac series. All low copy plasmids were of the pRS series (Supplementary File 1B). Gene deletions were generated by one-step gene disruptions using standard procedure. Yeast were transformed using lithium acetate, single-stranded carrier DNA and polyethylene glycol. All strains were verified by colony PCR. Endogenous genes were epitope tagged by one-step PCR (Longtine et al. 1998). PCR products for C-terminal td-Tomato fusions were amplified from a plasmid containing a tdTomato::HIS3MX cassette (DLB3299, a generous gift from Danny Lew). The endogenous Cdc24 promoter sequence consisted of 600bp upstream of the *cdc24* locus that was amplified from genomic DNA and inserted by Gibson Assembly into Cen plasmids that encoded for either Cdc24-GFP or Cdc24-tdTomato.

The *cdc28-as1* allele was inserted by linearizing a hygromycin-resistant plasmid containing the *cdc28-as1* coding sequence with an AflII restriction enzyme site adjacent to the F88G point mutation (pKW50); the plasmid encoding the *cdc28-as1* allele was generously provided by Eric Weiss (pELW886).

Treatment of cells for live cell imaging and drug treatment

Cells were grown in the dark at room temperature overnight in SC -His-Leu-Ura-Trp+Ade and diluted to OD600 = 0.1-0.2. For optogenetic experiments, cells were treated with 50 nM of ß-estradiol after 2 hours of growth to induce expression of the optogenetic components. After 2 hours of induction, cells were concentrated 10-fold to 20-fold in fresh media + ß-estradiol and prepped for imaging. Cells co-expressing Cdc24-tdTomato and Cdc24-ePDZ, were induced for 90 minutes to limit deleterious overexpression of the GEF. For experiments less than 2 hours, cells were imaged on a 2.5% agar pad soaked in minimal media + ß-estradiol + drug (where applicable) for > 20 minutes. For experiments longer than 2 hours, cells were imaged in a CellASIC Onix microfluidic perfusion chamber (EMD Millipore Corporation) to provide continuous nutrients. For non-optogenetic experiments, cells were concentrated 10-fold to 20-fold after 2 hours of growth and imaged on 2.5% agar pad soaked in minimal media.

After 1.5 hours of induction with 50 nM ß-estradiol, *cdk1-as* cells were treated with 75 μM 1NM-PP1 or solvent (1% DMSO) at room temperature, in the dark, and without shaking for 20 minutes. Subsequently, cells were imaged for > 90 minutes, for a total time in 1NM-PP1 of approximately 2 hours.

To synchronize diploid cells in early G1, exponentially growing cells were treated with 15 μg/ml nocodazole for 2 hours. Cells were induced with 50 nM ß-estradiol and treated with a second dose of nocodazole at 7.5 μg/ml. After 1 hour, cells were washed three times with fresh media and released into minimal media + 50nM ß-estradiol for 30 minutes. Cells were treated with 100 μM Latrunculin A (Molecular Probes) or solvent (1% ethanol) for 20 minutes and then imaged for 90 minutes. Asynchronous populations of cells treated with LatrunculinA were induced with 50 nM ß-estradiol for 2 hours, followed by 100 μM Latrunculin A (Molecular Probes) for 20 minutes prior to imaging.

Optogenetic manipulations

Cells were imaged on an Axiovert 200M microscope (Zeiss) equipped with a spinning disk confocal (CSU10, Yokogawa), a 20- mW, 561-nm laser (Cobolt), and an electron-multiplying charge-coupled device (EMCCD) camera (Cascade 512B, Photometrics) using a 63×, 1.4 numerical aperture objective (Zeiss). The microscope was controlled using MetaMorph (Molecular Devices). A 550-nm long-pass filter (Edmund Optics) was placed in the transmitted light path to avoid photoexciting the LOV domain when using Nomarski optics. A galvanometer-steerable 440-nm dye laser (Micropoint, Photonics Instruments) for local photo excitation of Mid2-localized LOVpep. Illumination intensity was controlled by using an adjustable internal attenuator plate and an external optical density of 1.0 absorptive neutral density filter (ThorLabs) was placed in the beam path.
For polarization experiments, cells were photo-excited using the Micropoint laser. The coordinates of targeted sites (x, y, t) were recorded with each photo-excitation. Following illumination, a confocal tdTomato image and phase contrast image were acquired. For control (dark state) experiments, the experiment was performed identically except that the Micropoint laser was off. Exposure times were 500 msec for tdTomato and 100 msec for phase contrast.

Live cell imaging of unperturbed cells

For non-optogenetic experiments, cells were imaged with a Zeiss Axioimager M1 equipped with a Yokogawa CSU-X1 spinning disk unit (Solamere) and illuminated with 50-mW, 488-nm and 50-mW, 561-nm lasers (Coherent). Images were captured on a Cascade 1K electron microscope (EM) CCD camera or a Cascade 512BT (Photometrics) controlled by MetaMorph (Molecular Devices).
For maximum intensity Z-projection snapshots, cells were imaged through the center 3μm at 0.25μm slices. The GFP and tdTomato images were acquired sequentially, followed by a phase contrast image. Max intensity projections were generated using Metamorph. Single plane snapshots were acquired at the mid-plane of the cell, with GFP and tdTomato images acquired sequentially. Exposure times were 900 msec for tdTomato, 900 msec for GFP, and 66 msec for phase contrast.
Time-lapse imaging acquired single plane snapshots of a confocal tdTomato image and phase contrast image at the mid-plane of the cell. Cells were imaged at a rate of once per minute for 3 minutes, followed by a rate of once per 30 seconds for 4 minutes, and finally imaged at a rate of once per minute for 3 minutes. Exposure times were 700 msec for tdTomato and 66 msec for phase contrast.

Image analysis

All images were analyzed in ImageJ (Schneider, Rasband, and Eliceiri 2012), with custom-written macros. To determine the angle between the laser position and the nascent bud site, we generated kymographs displaying the laser position, the site of bud emergence, and the intensity of the probe. To generate the kymographs, the cell outline was tracked using the plugin JFilament (Smith et al. 2010), which created a series of XY-coordinates to be converted into an ROI for each image in the stack . The ROI was then overlaid on its cognate image, linearized, and normalized to 100 pixels in length by 6 pixels in width. This was repeated iteratively through each slice of the stack to form a kymograph. The kymographs were annotated with the center coordinate of each target in each frame of the time-lapse, the time and position of bud emergence, and the position of the previous bud site.

Polarization Efficiency was defined as $(1-2\theta/\pi)$ where θ is the angle between the site of illumination and the position of the nascent bud. A population measure of polarization efficiency was found by taking the average of the polarization efficiency value for all cells.

To quantify the appearance time of a polarity component, the time of bud emergence, and the polarization efficiency, we analyzed time-lapse images as follows: cells were only scored if they underwent both polarization and bud emergence within the time-course of the movie. The analysis was limited to mother cells and cells that budded within the first 20 minutes were excluded. In cells in which Whi5 nuclear exit was scored, nuclear exit was defined as when nuclear Whi5 signal equaled that of the cytoplasm. To quantify the appearance of weak fluorescent signals, accumulation was scored by blinding the fluorescent image and scoring manually, by eye, accumulation of the probe.

To analyze colocalization, cells were separated into cell cycle stages depending on both the bud size and the distribution of the probes. Data were blinded and cells were pulled at random from each cell cycle subset. The number of puncta with colocalization (GFP with tdTomato and tdTomato with GP) was manually counted.

For *cdk1-as* cells, we limited our analysis to large-budded mother cells that accumulated Bem1 or the Cdc42 biosensor to the bud neck, indicative of early G1 cells. A “large-bud” was considered to have an area more than 4.5 μm^2. As *cdk1-as* cells treated with 1NM-PP1 do not undergo bud emergence, to be scored as polarized, they needed to maintain polarization for >15 minutes.

To quantify competition in dynamic reorientation experiments, a targeting event was scored as “Outcompeted” if the Cdc42 biosensor signal disappeared from the initial position and accumulated at the new position within the time that the target was maintained at the new position. The event was deemed “Not Outcompeted” if the Cdc42 biosensor neither disappeared from the initial position nor accumulated at the new position.

Supplementary Tables

Table 1: Strain List

Strain	Genotype
WYK8301	TRP1Δ::Gal4-rMR1/+; pGal-pGal-Mid2-GFP-LOVpep::LEU/+; Bem1-tdTomato::HIS3MX/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8308	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Bem1-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8318	TRP1Δ::Gal4-rMR1/+; Bem1-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Bem1-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/ rsr1Δ::KanMX
WYK8335	TRP1Δ::Gal4-rMR1/+; pGal-pGal-Mid2-GFP-LOVpep::LEU/+; Shs1-tdTomato::HIS3MX/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8337	TRP1Δ::Gal4-rMR1/+; pGal-pGal-Mid2-GFP-LOVpep::LEU/+; Spa2-tdTomato::HIS3MX/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8410	rdi1Δ::KanMX/rdi1Δ::HygR; rsr1Δ::TRP1/ rsr1Δ::TRP1; Bem1-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA;
WYK8434	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Bem1(R369A)-ePDZb1::URA/ pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/ rsr1Δ::KanMX
WYK8435	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Bem1(P355A)-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/ rsr1Δ::KanMX
WYK8436	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Bem1(K482A)-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8437	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Cdc24ΔPB1-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX

WYK8439	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Cdc24(Q412A R416E)-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8440	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8441	TRP1Δ::Gal4-rMR1/+; Bem1-tdTomato::HIS3MX/+; cdc28as::HygR/cdc28as::HygR; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8442	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; cdc28as:HygR/cdc28as::HygR; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8500	TRP1Δ::Gal4-rMR1/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; Whi5-tdTomato:HIS3MX/+; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8502	TRP1Δ::Gal4-rMR1/+; pGal-Mid2-GFP-LOVpep::LEU/+; Bem1-tdTomato::HIS3MX/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; Whi5-tdTomato:HIS3MX/+; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8550	Bem1-GFP::HIS3MX/+; pADH1-Gal4-VP16-ER::URA/+; Gic2(1-208)-tdTomato::HIS3MX/+; rsr1Δ::TRP1/rsr1Δ::KanMX
WYK8551	TRP1Δ::Gal4-rMR1/+; Bem1-tdTomato::HIS3MX/+; pADH1-Gal4-VP16-ER::URA/+; pCdc24-Cdc24-GFP::LEU (pKW102); rsr1Δ::TRP/rsr1Δ::KanMX
WYK8552	pADH1-Gal4-VP16-ER::URA/+; Gic2(1-208)-tdTomato::HIS3MX/+; rsr1Δ::TRP/rsr1Δ::KanMX; pCdc24-Cdc24-GFP::LEU (pKW102)
WYK8575	TRP1Δ::Gal4-rMR1/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX; pCdc24-Cdc24-tdTomato::HIS3 (pKW101)
WYK8576	TRP1Δ::Gal4-rMR1/+; pGal-Mid2-GFP-LOVpep::LEU/+; pGal-Bem1-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::TRP1/rsr1Δ::KanMX; pCdc24-Cdc24-tdTomato::HIS3 (pKW101)
WYK8598	TRP1Δ::Gal4-rMR1/+; pGal-Mid2-GFP-LOVpep::LEU/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Cdc24-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::KanMX/+

WYK8599	TRP1Δ::Gal4-rMR1/+; pGal-Mid2-GFP-LOVpep::LEU/+; Gic2(1-208)-tdTomato::HIS3MX/+; pGal-Bem1-ePDZb1::URA/pADH1-Gal4-VP16-ER::URA; rsr1Δ::KanMX/+
All strains are *MATa/MATα* diploids constructed in the W303 background. They have the following additional markers: *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	

Table 2: Plasmid List

Plasmid Name	Type	Contents	Marker	Source
DLB3299	pFA6a	tdTomato	HIS3MX	Longtine *et al* 1998
pDS300	YIplac	pGal-Cdc24-ePDZb1	URA	Strickland *et al* 2012
pDS311	YIplac	pGal-Cdc24ΔPB1-ePDZb1	URA	This Study
pDS343	YIplac	pGal-Mid2-GFP-LOVpep250	LEU	This Study
pDS357	YIplac	pGal-Bem1-ePDZb1	URA	This Study
pDS377	YIplac	pGal-Bem1(R369A)-ePDZb1	URA	This Study
pDS381	YIplac	pGal-Bem1(P355A)-ePDZb1	URA	This Study
pDS382	YIplac	pGal-Bem1(K482A)-ePDZb1	URA	This Study
pKW34	YIplac	pGal-Cdc24(Q412A R416E)-ePDZb1	URA	This Study
pKW50	pFA6a	cdc28-as1	HygR	This Study
pKW101	pRS	pCdc24-Cdc24-tdTomato	HIS	This Study
pKW102	pRS	pCdc24-Cdc24-GFP	LEU	This Study
pELW886	pRS	cdc28-as1	URA	Bishop *et al* 2000
pELW909	YIplac	pADH1-Gal4-VP16-ER	URA	Louvion *et al* 1993

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