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Cell Polarity: Spot-On Cdc42 Polarization Achieved on Demand

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A new study deploys optogenetics to induce the yeast bud on demand, at a site determined by a laser spot. The authors definitively prove that the initiation of cell polarization is driven by the Bem1-mediated positive feedback loop and reveal novel features of its regulation by the cell cycle.

Polarization of budding yeast cells provides arguably the best studied example of eukaryotic cell polarity [1]. As early as 1990, Pringle and colleagues discovered the budding yeast Rho GTPase Cdc42 and established its central role in orchestrating multiple processes that bring about the formation of a new bud or a mating protrusion. Thereafter, researchers recognized that Cdc42 is the master regulator of cell polarity across the entire eukaryotic kingdom. Introduction of reporters of Cdc42 activity into budding yeast revealed the existence of a dynamic Cdc42-GTP cluster that determines the nascent polarity site and then organizes all the downstream morphogenetic processes. Normally, cellular polarization is spatially controlled by either intracellular landmarks or gradients of extracellular signalling molecules. Genetic decoupling of the landmarks and the application of uniform mating pheromone revealed that the Cdc42 clusters emerge in these cells spontaneously, at random spatial locations, requiring only a permissive signal from the cell cycle. Furthermore, these clusters arise independently of the microtubule and actin cytoskeleton and are highly resilient to diverse genetic perturbations and environmental stresses. Thus, the budding yeast Cdc42 cluster provided a robust paradigm for the self-organized cellular structure that emerges in the initially unpolarized cell via a symmetry-breaking bifurcation.

As the importance of this paradigm transcends multiple fields of science, much experimental and theoretical effort has been invested into understanding the molecular mechanisms and biophysical principles of Cdc42 polarization. The discovery of a positive feedback loop that recruits the Cdc42 activator — the guanine nucleotide exchange factor (GEF) Cdc24 — into the Cdc42-GTP cluster via the scaffold effector protein Bem1 has been a pivotal point in defining these mechanisms [2,3]. The first biochemically detailed, mechanistic theoretical model

of symmetry-breaking Cdc42 polarization included this Bem1-mediated positive feedback loop as the source of autocatalytic generation of Cdc42-GTP on the membrane [4]. This model proposed that the Cdc42 cluster emerges via a Turing-like mechanism, which is a standard scenario for pattern formation in the out-of-equilibrium chemical and biological systems. Later proposed models explained the Cdc42 cluster formation also by resorting to positive feedback loops that auto-amplify the Cdc42-GTP cluster via the recruitment of either inactive Cdc42, or its GEF Cdc24, or both [5].

Direct testing of these models has been hampered by the absence of an experimental technique that would permit researchers to robustly recruit desired proteins to particular locations on the plasma membrane, at specific phases of the cell cycle. Now, in a new study, Witte *et al.* [6] have successfully employed an optogenetic strategy to address this issue. Optogenetic semi-synthetic constructs based on a set of natural proteins, the interactions between which are regulated by light, have recently become powerful tools of experimental manipulation and are used widely across cell biology [7]. The importance of these methods is likely to become as great as that of the fluorescent proteins introduced more than two decades ago. Glotzer and colleagues have already contributed to this technological revolution by developing tools known as tunable light-inducible dimerization tags (TULIPs), whose photo-activation is based on the LOV2 domain of *Avena sativa* phototropin [8]. In the initial proof-of-principle study they already demonstrated the potential to induce polarized mating projections by recruiting Cdc24 to the plasma membrane of pheromone-arrested cells [8]. In their new work, Glotzer and colleagues [6] now apply TULIPs to delve right into the nuts and bolts of the Cdc42 cluster formation machinery.

Witte *et al.* [6] used pulsed laser illumination to recruit either the Cdc24 or Bem1 optogenetic constructs to a sub-micron-sized spot on the yeast plasma membrane, starting from early in G1 phase until well into S phase when cells already have medium-sized buds. Remarkably, the authors found a time window in late G1 phase when the optogenetic recruitment

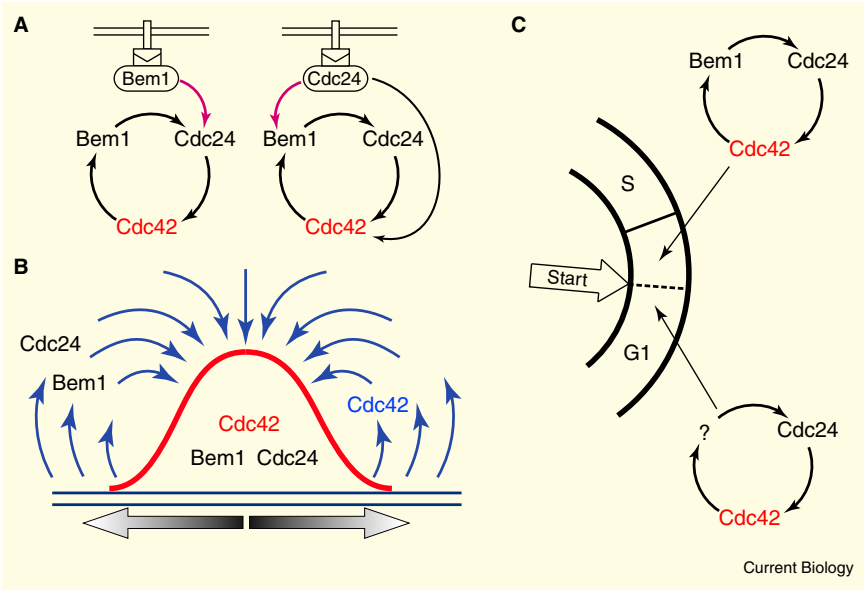

Figure 1. An optogenetic approach confirms the positive feedback mechanism of Cdc42 polarization in budding yeast and reveals novel features.
(A) Optogenetic constructs based on Bem1 and Cdc24 jump-start the Bem1-dependent positive feedback loop at the laser illumination spot on the yeast plasma membrane. Magenta arrows indicate Bem1–Cdc24 protein interaction between the optogenetic constructs and cytoplasmic proteins. (B) Cdc42 GTPase flux (thin arrows) continuously rebuilds the membrane-bound Cdc42 cluster from the cytoplasmic components and prevents its diffusive spreading on the membrane (gradient-filled arrows). Inactive Cdc42-GDP is shown in blue, and active Cdc42-GTP in red. (C) The Bem1-dependent positive feedback loop is enabled by Cdk1 signalling at Start. Prior to Start, a novel putative positive feedback loop independent of Bem1 is proposed to generate the Cdc42-GTP cluster via as yet unknown additional molecules (shown by the question mark).

of either the Bem1 or the Cdc24 construct induces robust Cdc42-GTP clusters that successfully proceed to complete bud formation. In wild-type cells, this window approximately corresponds to the time interval between the detectable emergence of the Cdc42 cluster and the beginning of bud protrusion [9]. Importantly, the fully competent Cdc42 clusters that were optogenetically induced within this time interval also contained the molecules from the fluorescently labelled cytoplasmic pools of Cdc24 and Bem1, regardless of which optogenetic construct was used to initiate cluster formation. Moreover, once optogenetically initiated, these clusters successfully maintained themselves even after the pulsed laser illumination was turned off. Taken together, these results unequivocally demonstrate the existence of the Bem1-mediated positive feedback loop that, once started, is fully capable of maintaining the Cdc42 cluster in a steady state.

Consistent with the wiring of molecular interactions constituting this positive feedback loop (Figure 1A), a Bem1 optogenetic construct that could not bind Cdc24 failed to induce the Cdc42 cluster, while a Cdc24 construct incapable of binding Bem1 was only partially defective. Indeed, since this Bem1-binding-defective Cdc24 construct retained full catalytic activity towards Cdc42, it activated Cdc42 locally on the membrane and then this localized pool of Cdc42-GTP was able to auto-amplify itself by recruiting functional Bem1 and Cdc24 from the cytoplasm. Furthermore, Witte *et al.* [6] provide direct evidence supporting the theoretical concept of the Cdc42 GTPase flux, which was introduced in the first modelling study [4]. In the model (Figure 1B), Cdc24, Bem1 and inactive Cdc42 are injected from the cytoplasm into the centre of the Cdc42 cluster, where the activity of the GTPase is maximal, and are recycled back to the cytoplasm from the membrane at the periphery of the cluster, where Cdc42 activity is waning. This circular diffusive flux of the cluster components, powered by the Cdc42-catalysed hydrolysis of GTP, was predicted by the model to counteract the diffusive spread of the cluster and proposed to explain the fast

recovery of the fluorescence of the cluster components that was invariably observed in the fluorescence recovery after photobleaching (FRAP) experiments.

While the direct demonstrations of the existence of positive feedback and Cdc42 GTPase flux are striking, given the existing body of published results they are not entirely unexpected. However, the ability to impose optogenetic control at the specific phases of the cell cycle enabled the authors to provide new insights into the regulation of Cdc42 polarization by the cell cycle. While the existence of such regulation has long been known, the exact nature of this signal is still poorly understood [10]. At least in part, the cell-cycle regulation of cell polarity was thought to arise from the inactivation of Cdc42 GTPase-activating proteins (GAPs) induced by cyclin-dependent kinase 1 (Cdk1). The current work from the Glotzer group now demonstrates that this effect is much more direct. As expected, acute optogenetic recruitment of Cdc24 produces localized activation of Cdc42 within all tested phases of the cell cycle. This, however, turned out not to be the case for Bem1. Indeed, Witte *et al.* [6] found that, surprisingly, the Bem1 optogenetic construct cannot induce Cdc42 cluster formation prior to the Start transition; reciprocally, the Cdc24 optogenetic construct cannot recruit cytoplasmic Bem1 before this transition. Start is the yeast-specific cell-cycle checkpoint during which the repressor Whi5 is exported out of the nucleus and activity of the cyclin–Cdk1 complexes rises rapidly (Figure 1C). Therefore, the results from Witte *et al.* [6] suggest that Cdk1-mediated phosphorylation is absolutely required to close the Bem1-mediated positive feedback loop by enabling the interaction between Bem1 and Cdc24. This proposal was further confirmed by using the analog-sensitive mutant Cdk1-as1, which can be rapidly inactivated by addition of a synthetic ATP analog.

Analysis of the stability of the Cdc42 clusters induced by the Cdc24 optogenetic construct prior to Start produced yet another surprise. Cessation of laser illumination reverses the recruitment of the optogenetic constructs within approximately the first 3 minutes [8]. Notwithstanding, the Cdc42 clusters induced by the Cdc24 construct remained 'alive' for as long as 30 minutes after the termination of laser illumination and were able to eventually induce bud formation. This suggests that the Cdc42 clusters induced in early G1 by Cdc24 could sustain themselves on the plasma membrane way beyond the time when the optogenetic construct that induced them had already left the membrane. Witte *et al.* [6] conclude that a novel, Bem1-independent positive feedback loop must exist to explain this puzzling observation.

The Cdc42 GTPase flux that first forms the Cdc42 cluster and then maintains it in the steady state, despite the dispersing influence of diffusion, produces rapid exchange of the cluster components between the plasma membrane and the cytoplasm (Figure 1B). Multiple Cdc42 clusters were predicted to compete for the common cytoplasmic pool of their components to the effect that only a single cluster would eventually survive and give rise to the only bud formed within a single cell cycle [4]. Several aspects of predicted competition between Cdc42 clusters were confirmed previously by combining cell rewiring, overexpression of Bem1, and fast time-lapse imaging [11]. The benefit of the optogenetic approach allowed Witte *et al.* [6] to directly induce multiple clusters and observe their competition during the distinct phases of the cell cycle. In early G1, prior to Start, additional clusters were induced by sequentially repositioning the target of illumination. The accumulation of Cdc42-GTP at the new site was concomitant with its dissipation at the old site, so that the two clusters co-existed for around 5 minutes. Taking into consideration that a single cluster can survive the cessation of laser illumination for up to 30 minutes, this result indicates that the sequentially induced clusters were in direct competition, with each subsequent cluster winning over its predecessor. Interestingly, after Start and, consequently, Cdk1-mediated activation of the 'classical' Bem1-dependent positive feedback loop, the competition between clusters became so fast that clusters, even if induced simultaneously by the optogenetic recruitment of either Bem1 or Cdc24, did not exhibit any detectable co-existence.

The results of Witte *et al.* [6] convincingly demonstrate the remarkable power of the optogenetic approach and show how this novel technology can be used together with conventional genetic manipulation and time-lapse fluorescence imaging to dissect a complex intracellular morphogenetic process. This approach also enabled the authors to determine novel cell-cycle-dependent regulation of Cdc42 cluster formation and revealed the existence of an additional putative feedback mechanism. Further development of this technology will undoubtedly be instrumental in untangling the wires of other important regulatory molecular networks that control cellular dynamics and morphogenesis.

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