Membrane curvature directs the localization of Cdc42p to novel foci required for cell–cell fusion

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Cell fusion is ubiquitous in eukaryotic fertilization and development. The highly conserved Rho–GTPase Cdc42p promotes yeast fusion through interaction with Fus2p, a pheromone-induced amphiphysin-like protein. We show that in prezygotes, Cdc42p forms a novel Fus2p-dependent focus at the center of the zone of cell fusion (ZCF) and remains associated with remnant cell walls after initial fusion. At the ZCF and during fusion, Cdc42p and Fus2p colocalized. In contrast, in shmoos, both proteins were near the cortex but spatially separate. Cdc42p focus formation depends on ZCF membrane curvature: mutant analysis showed that Cdc42p localization is negatively affected by shmoost-like positive ZCF curvature, consistent with the flattening of the ZCF during fusion. BAR-domain proteins such as the fusion proteins Fus2p and Rvs161p are known to recognize membrane curvature. We find that mutations that disrupt binding of the Fus2p/Rvs161p heterodimer to membranes affect Cdc42p ZCF localization. We propose that Fus2p localizes Cdc42p to the flat ZCF to promote cell wall degradation.

Introduction

During sexual reproduction, the combination of haploid gamete genomes requires fusion (Gadella and Evans, 2011; Shinn-Thomas and Mohler, 2011). In multicellular organisms, cell fusion is also required for muscle, vertebrate eye, and placenta development (Shi et al., 2009; Huppertz and Gauster, 2011; Shinn-Thomas and Mohler, 2011; Kim et al., 2015). Cell fusion has also recently been implicated in cancer development and progression (Parris, 2013; Bastida-Ruiz et al., 2016). Despite the significance of cell fusion, relatively little is known about the molecular mechanisms and regulation.

Cell fusion occurs during mating of the budding yeast Saccharomyces cerevisiae to form a diploid zygote. Haploid yeast cells of mating types a and α secrete pheromones that are detected by the other mating type. The pheromone response results in induction of mating-specific genes, cell-cycle arrest, and polarized growth (Merlini et al., 2013; Alvaro and Thorner, 2016). Altered morphogenesis results in pear-shaped cells known as “shmoos.” After the shmoos come into contact, the cell walls and membranes flatten out, forming a synapse-like region called the zone of cell fusion (ZCF). The ZCF marks the site where the cell wall will be removed and the plasma membranes fuse (Gammie et al., 1998; Ydenberg and Rose, 2008; Merlini et al., 2013).

Several genes have been identified that are thought to be direct regulators of cell fusion. FUS1, FUS2, and PRM1 are expressed only after pheromone induction (Trueheart et al., 1987; Elion et al., 1995; Heiman and Walter, 2000). Kel1p, a kelch protein, and Rvs161p, an amphiphysin, promote fusion but have alternative roles in regulating mitotic exit and endocytosis, respectively (Crouzet et al., 1991; Brizio et al., 1998; Philips and Herskowitz, 1998; Höfken and Schiebel, 2002; Se-shan et al., 2002; Knaus et al., 2005; Friesen et al., 2006; Smith and Rose, 2016). Fus1p is a heavily glycosylated type I membrane protein thought to act as a scaffold for cell fusion proteins (Trueheart et al., 1987; Trueheart and Fink, 1989). Fus2p is a BAR-domain protein (Stein et al., 2015) that is initially sequestered in the nucleus but then enters the cytoplasm after cell-cycle arrest (Ydenberg and Rose, 2009; Kim and Rose, 2012) where it interacts with Rvs161p (Brizio et al., 1998; Stein et al., 2015). The heterodimer is transported to the shmoo tip dependent on Kel1p (Palmer et al., 2008; Sheltzer and Rose, 2009), where it is anchored at the cortex dependent upon Fus1p, actin, and Kel1p (Palmer et al., 2008; Smith and Rose, 2016). Mutations in FUS1, FUS2, RVS161, and KEL1 block the removal of the cell wall between the mating cells (Trueheart et al., 1987; Trueheart and Fink, 1989; Gammie et al., 1998; Smith and Rose, 2016). Prm1p is a transmembrane protein that promotes plasma membrane fusion after cell wall removal (Heiman and Walter, 2000).

Cdc42p is a highly conserved essential Rho-like GTPase with several roles during growth and morphogenesis, in-
including establishment of polarity, reorganization of the actin cytoskeleton, polarized secretion, budding, and activation of p21-activated kinases (Madden and Snyder, 1998; Johnson, 1999; Richman et al., 1999; Kozminski et al., 2000; Adamo et al., 2001; Perez and Rincón, 2010). During yeast mating, Cdc42p functions in three pathways: pheromone signaling (Simon et al., 1995; Zhao et al., 1995), morphogenesis (Nern and Arkowitz, 1998, 1999), and cell fusion (Barale et al., 2006; Ydenberg et al., 2012). Although the functions of Cdc42p early in the mating pathway (signaling and morphogenesis) are understood, the function of Cdc42p in cell fusion has remained unclear. Cdc42p interacts with Fus2p, and cell fusion–specific alleles of cdc42 defective for Fus2p binding exhibit a cell fusion defect (Ydenberg et al., 2012), without having a significant effect on signaling or morphogenesis. Fus2p preferentially binds GTP-bound Cdc42p, suggesting that it recruits activated Cdc42p (Nelson et al., 2004; Ydenberg et al., 2012).

Proteins homologous to yeast Cdc42p are implicated in Drosophila melanogaster, mouse, and zebrafish myoblast fusion, mostly by promoting actin polymerization and cell polarization (Vasyutina et al., 2009; Abmayr and Pavlath, 2012). In the fission yeast Schizosaccharomyces pombe, early in cell fusion, active Cdc42p forms dynamic zones that explore the periphery of the cell and are required for pheromone secretion, polarization, and signaling before cell pairing (Bendezi and Martin, 2013; Merlino et al., 2016). However, little is known about cell wall degradation and plasma membrane fusion in fission yeast or whether Cdc42p plays a specific role in these later steps.

In budding yeast, mating-specific vesicles cluster at the ZCF; dependent on Fus1p and cell polarization proteins (e.g., Spa2p), and remain closely associated with the remnant cell walls after fusion (Baba et al., 1989; Gammie et al., 1998). Exocytosis is thought to release hydrolases into the periplasmic space between the cells, breaking down the cell wall to allow plasma membrane fusion. In support, active secretion is required for fusion (Grote, 2010), and deletion of two putative glucanase genes, SCW4 and SCW10, causes a synthetic mating defect (Cappellaro et al., 1998).

We hypothesize that Fus2p functions in cell fusion to localize active Cdc42p to the center of the ZCF to promote cell wall degradation. However, because Cdc42p has multiple functions, it is already broadly localized at the shmoo tip (Ziman et al., 1993), raising the question of how localization could play a specific function in cell fusion. We find that Cdc42p forms a novel focus at the center of the ZCF that is required for fusion. The focus is dependent upon Fus2p and other known fusion proteins for formation and sensitive to the curvature of the ZCF. Because of the conservation of Cdc42p throughout eukaryotes and its roles in mouse and Drosophila myoblast fusion, these results have broad implications about the regulation of cell fusion.

Results and discussion

Cdc42p forms a focus at the center of the ZCF

On the basis of the requirement of the Cdc42pGTP–Fus2p interaction for fusion, it was hypothesized that Fus2p localizes Cdc42p to the shmoo tip/ZCF to promote fusion (Ydenberg et al., 2012; Smith and Rose, 2016). However, Cdc42p was already known to localize to regions of active growth, like the shmoo tip, obscuring the need for specific localization during cell fusion. We therefore examined Cdc42p localization in prezygotes, using an integrating plasmid containing CDC42 N-terminally tagged with 3xGFP (Dyer et al., 2013). Prezygotes are mating pairs that have not initiated cell wall removal. Because the tagged version is partially functional, a WT copy of CDC42 remains at the endogenous locus.

In crosses to WT, GFP–Cdc42p–positive cells showed a discrete focus forming at the center of the ZCF (Fig. 1 A and Video 1). In the lower prezygote, GFP–Cdc42p was initially distributed across the ZCF but became tightly localized between the 3- and 6-min time points. Cell fusion occurred between the 9- and 12-min time points, after which Cdc42–GFP was more broadly localized. The upper prezygote shows a similar pattern but further along in cell fusion.

Because of the fleeting nature of the ZCF, we used a compromised (fus1Δ/fus2Δ) mating partner to slow fusion to provide sufficient prezygotes for analysis. Chemical fixation of the prezygotes allowed acquisition of image stacks; deconvolution and optical slicing provided a clearer image of the ZCF. When crosses of GFP–CDC42 x fus1Δ/fus2Δ were analyzed, Cdc42p formed a bright focus at the center of the ZCF, similar to what was observed in the live-cell imaging (Fig. 1 B). In zygotes after fusion had begun, Cdc42p was concentrated at the edges of the remnant cell walls (Fig. 1 B). To quantify localization, we measured fluorescence intensity along a line through the ZCF (as denoted by the arrows in Fig. 1 B) and plotted this as a function of the distance (Fig. 1 C). A representative individual prezygote showed a strong plateau of fluorescence intensity, roughly 1 µm wide, in the center of the ZCF, five- to sixfold brighter than the surrounding cortex. This region corresponds to the initial site of cell wall degradation previously observed by electron microscopy (Gammie et al., 1998). The partially fused zygote showed two peaks corresponding to the edges of the remnant cell walls. To determine if the localization in prezygotes was simply due to polarized growth, we examined localization of GFP–Cdc42p in shmoo. Cdc42p localized broadly over the shmoo tip but was not significantly brighter in the center of the shmoo tip (Fig. 1 D), as confirmed by quantification of fluorescence intensity (Fig. 1 E). Therefore, we have identified a novel localization for Cdc42p, specific to prezygotes, at the center of the ZCF.

To quantify focus formation for a population of WT prezygotes, we measured fluorescence intensity as a function of distance across the ZCF for >120 prezygotes. The mean full width at half maximum of the focus in the WT prezygotes was 0.84 ± 0.02 µm. In shmoo and mutant cells, the distribution of Cdc42p was too irregular to allow measurement of a full width at half maximum. Instead, to compare the mean intensity and localization of Cdc42p among different strains, we normalized the GFP intensities are reported as the ratios to the mean values of the two edges (12% on each side). After normalization, prezygotes with a Cdc42p focus showed a strong central peak of fluorescence (Fig. 1 F). The peak was broader than for a single prezygote, because of heterogeneity in the width of the ZCF. In Fig. 1 F, the error bars indicate SEM. For clarity, error bars are suppressed in later figures.

Cdc42p and Fus2p colocalize in prezygotes

Fus2p localizes to the center of the ZCF and forms an expanding ring as the remnant cell walls are degraded (Paterson et al.,
Given the similarity of the Cdc42p focus to Fus2p localization, and their physical interaction, we examined their co-localization. To visualize GFP–Cdc42p and Fus2p in the same cell, we used a Fus2p construct tagged with mCherry at residue 104, where the functional Fus2p–GFP construct is tagged. Fluorescence microscopy of prezygotes showed colocalization of GFP–Cdc42p and Fus2p–mCherry at the center of the ZCF, which persisted on the remnant cell walls in postfusion zygotes (Fig. 2 A). When shmoos were imaged, colocalization was considerably more variable: some shmoos showed clear separation between the markers (Fig. 2 B, top), but partial colocalization was observed in others (Fig. 2 B, bottom). To quantify colocalization, we determined the peaks of fluorescence for GFP and mCherry along an axis orthogonal to the shmoo tip/ZCF and measured the distance between them. In prezygotes, the mean distance between the peaks was <1 pixel (1 pixel = 0.0648 µm); the distance in shmoos was almost threefold greater (P = 0.0001). Therefore, Fus2p and Cdc42p colocalization is specific to prezygotes.

Cdc42p focus formation is dependent upon Fus2p and other cell fusion proteins

Colocalization of Cdc42p and Fus2p at the ZCF could be independent, perhaps because it is a specialized location, or dependent, if one protein recruits the other. Imaging of Cdc42p in a fus2Δ strain showed Cdc42p localized broadly across the ZCF, rather than to a central focus (Fig. 3 A). Quantification of Cdc42p localization across all fus2Δ prezygotes showed a significantly flatter curve than WT (Fig. 3 B; P = 2 × 10^{-5}). On the basis of this analysis (Fig. 3 B), Cdc42p focus formation is dependent upon Fus2p. Mutation of Kel1p also caused a localization defect for Cdc42p (Fig. S1). As Kel1p is a known interaction partner for Fus2p (Smith and Rose, 2016), these data support our model of Fus2p regulating Cdc42p localization.

Fus1p, a pheromone-induced transmembrane protein (Trueheart et al., 1987; Trueheart and Fink, 1989), has multiple roles in cell fusion, including stabilizing Fus2p localization at the cortex (Paterson et al., 2008; Smith and Rose, 2016). Imaging of Cdc42p in a fus1Δ strain showed broad Cdc42p localization (Fig. 3 A) significantly flatter than WT (Fig. 3 B). Fus1p and Fus2p play partially independent roles in cell wall degradation; a synthetic mating defect is seen in double-mutant strains (Gammie et al., 1998). To determine if Fus1p affects Cdc42p localization to the ZCF entirely through Fus2p localization, we analyzed a fus1Δ fus2Δ strain. Cdc42p was even more poorly localized at the ZCF in this strain (Fig. 3, A and B). When the phenotype was quantified, Cdc42p localization in fus1Δ fus2Δ was significantly worse than either fus1Δ (P = 0.008) or fus2Δ (P = 0.0001). Because Fus1p itself is localized broadly across the shmoo tip (Trueheart et al., 1987; Nelson et al., 2004;...
Barfield et al., 2009), we hypothesize that Fus2p is the main regulator of Cdc42p focus formation, whereas Fus1p plays an additional minor role in Cdc42p localization (Fig. 3 B).

To confirm that the phenotypic difference between the mutants is specific to the ZCF, we analyzed Cdc42p localization in WT, fus1Δ, fus2Δ, and fus1Δ fus2Δ shmoos. As expected, Cdc42p localized broadly across the shmoo tip in all of these strains, with no obvious focus at the shmoo tip (Fig. 3 C). Quantification showed only a modest peak at the shmoo tip (Fig. 3 D), rather than the large peak seen at the ZCF (Fig. 3 B), consistent with the lack of a focus in shmoos. There was no significant difference between WT versus fus1Δ (P = 0.3) or fus1Δ fus2Δ (P = 0.5). Deletion of FUS2 caused a slight reduction relative to WT (P = 0.015), which was not significantly different from either fus1Δ (P = 0.4) or fus1Δ fus2Δ (P = 0.4). We therefore conclude that both Fus1p and Fus2p are required for Cdc42p focus formation at the ZCF and that the localization of Cdc42p in shmoos is largely independent of both proteins.

The cdc42–138 allele (D122A), in the rho-insert domain, eliminates the interaction between Cdc42p and Fus2p and causes a cell fusion defect (Ydenberg et al., 2012). When the cdc42–138 mutation was introduced into GFP-tagged CDC42, GFP–Cdc42p138 localized broadly over the ZCF, similar to the fus1Δ fus2Δ phenotype (Fig. 3, E and F). Previous evidence showed that the cortical localization of Fus2p was not affected by cdc42–138 in shmoos (Ydenberg et al., 2012). However, because the ZCF behaves differently from the shmoo tip, we examined localization of Fus2p in cdc42–138 prezygotes. GFP–tagged Fus2p localized to the center of the ZCF in cdc42–138 prezygotes, with a fluorescence intensity of 70% of Fus2p in WT prezygotes (Fig. 3, G and H). We conclude that loss of the Cdc42p–Fus2p interaction does not have a large effect on Fus2p localization but greatly affects Cdc42p localization to the ZCF. The determinants for Fus2p localization are not altered by the cdc42–138 mutation, but reduced Cdc42p interaction may affect the stability of Fus2p at the ZCF.

Cdc42p localizes to flat interfaces

Given that Fus1p and Fus2p are both expressed and localized to the shmoo tip, but are not required for Cdc42p localization in shmoos, the question arises as to what is different in prezygotes that leads to focus formation at the ZCF. Although there are several possibilities, we note that one prominent difference between the shmoo tip and the ZCF is the curvature of the plasma membrane. As cells polarize toward each other and shmoo, an area of strong positive curvature is formed (Fig. 4 A). However, after cells come into contact and grow together, the cell walls and plasma membranes flatten, reducing the previous positive curvature at the shmoo tip to zero (Fig. 4 A). Given that the BAR-domain proteins, which include Fus2p/Rvs161p, are known to recognize membrane curvature (Rao and Haucke, 2011), we hypothesized that the change in curvature may comprise a key signal for Cdc42p focus formation. To test this hypothesis, we sought to identify conditions in which the ZCF in prezygotes would have positive, negative, or zero curvature.

Deletion of FPS1, which encodes a glycerol efflux pump, causes increased cellular osmotic pressure and a cell fusion defect (Philips and Herskowitz, 1997). However, unlike all other fusion proteins in which deletion of the gene in both mating partners exacerbates the defect, deletion of FPS1 in both mating partners actually suppresses the cell fusion defect. The authors suggested that imbalanced osmotic pressure between two mating partners might signal to stop cell fusion. We hypothesized that the imbalanced osmotic pressure might be manifest by changes in the curvature at the ZCF. To determine this, we analyzed crosses of FPS1 or fis1Δ mated to either fus1Δ fus2Δ or fis1Δ-
fus2Δ/fus1Δ. We used fus1Δfus2Δ to allow imaging of the ZCF, because fus1Δ × fus1Δ zygotes do not have a fusion defect, and the ZCF is therefore fleeting. When mating partners were imaged with a membrane-specific lipophilic dye, FM4-64, the ZCF appeared curved if either partner lacked FPS1 (Fig. 4 B). However, curvature of the ZCF was suppressed when both mating partners lacked FPS1, consistent with the hypothesis that imbalanced osmotic pressure causes the fus1Δ cell to bulge into its partner. To quantify the curvature of the ZCF, we measured the radius of a circle whose chord matches the interface. A flat ZCF matches a very large circle and so has a large radius, whereas a highly curved ZCF matches a small circle with a small radius. When this measurement was performed on the GFP-containing cells, the mean radius of ZCF curvature was more than 4 times smaller for the fus1Δ parent mated to FPS1, relative to the FPS1 × FPS1 or fus1Δ × fus1Δ prezygotes (Fig. 4 C).

To determine if ZCF curvature affects Cdc42p localization, we imaged GFP–Cdc42p in the same crosses. Cdc42p was partially mislocalized when the partner containing GFP–Cdc42p had positive curvature, as in the GFP–CDC42 fus1Δ × FPS1 mating (Fig. 4, D and E). This phenotype was significantly worse than WT (P = 0.001) However, mislocalization in the fus1Δ strain was completely suppressed when the same strain was mated to an fus1Δ fus2Δ (JY429). We also found that Cdc42p localization was not affected by positive curvature: the GFP–CDC42 fus1Δ × fus1Δ mating, in which the GFP-positive cell has a negatively curved ZCF, exhibited WT Cdc42p localization. When the mating orientation was switched such that the GFP-positive cell was MATα, the same results were obtained, showing that the fusion defect and suppression are not mating type specific (Fig. 4 F). Given
that Cdc42p mislocalization in the fps1Δ strain is suppressed by differences in the osmotic pressure of its partner, we conclude that Cdc42p localization is not directly regulated by the osmolarity of the cell.

Could Cdc42p localization in the fps1Δ strain be indirectly regulated by altered pheromone signaling? As for osmotic regulation, if deletion of FPS1 affected cell fusion via pheromone signaling, then fps1Δ × fps1Δ matings should exacerbate the cell fusion defect rather than suppress it. The finding that Cdc42p mislocalization in fps1Δ × FPS1 matings but does form a focus in fps1Δ × fps1Δ matings argues that signaling defects cannot account for the difference. We conclude that the most likely difference between the shmoo tip and the ZCF is the curvature of the plasma membrane.

Because Cdc42p localization is dependent on Fus2p localization, we examined Fus2p in the same mating partners. We found that Fus2p–mCherry was localized to the ZCF in all four matings (≥94% of all prezygotes; Fig. 4 G). Given that
Fus2p was localized in all cases, even when Cdc42p was not, we conclude that curvature specifically affects the interaction between Fus2p and Cdc42p.

A role for the Fus2p/Rvs161p membrane-binding domain in Cdc42p ZCF localization

How might curvature affect the interaction between Fus2p/Rvs161p and Cdc42p? BAR domain–containing proteins have been identified as membrane curvature sensing and generating proteins for many cellular processes (Zimmerberg and Kozlov, 2006; Suetsugu et al., 2014; McMahon et al., 2015). BAR domains comprise dimers of helix bundles (Qualmann et al., 2011). Electrostatic interactions with positively charged surface residues mediate initial binding (McMahon et al., 2015). Modeling of Fus2p/Rvs161p revealed two regions that would be strongly positively charged: the concave inner surface and the lateral outer surface (Stein et al., 2015). Previous work showed that mutations of conserved lysine residues in Fus2p and Rvs161p caused mating defects but did not disrupt binding to each other, suggesting that they affect other functions (Stein et al., 2015). The “con” mutations reside on the concave surface of the predicted banana-shaped heterodimer, and the “loop” mutations reside at the tips of the heterodimer (Stein et al., 2015). Although most of these mutations are expected to disrupt localization to the membrane, others might affect the conformation of Fus2p/Rvs161p at the membrane. An initial screen of localization in shmoo (Table S3) identified one allele, fus2<sup>con</sup>, that caused a strong defect in mating (Stein et al., 2015) but retained close to WT localization (76%). We hypothesized that this mutation might alter the conformation of Fus2p/Rvs161p at the ZCF, compromising its ability to recruit Cdc42p. Fus2p<sup>con</sup> was localized to the ZCF in 81% of fus2<sup>con</sup> prezygotes (Fig. 5 A). Another mutant with a comparable mating defect, rvs161<sup>loop</sup>, exhibited only 14% Fus2p localization (Fig. 5 A). In both the fus2<sup>con</sup> and rvs161<sup>loop</sup> mutants, Cdc42p was broadly distributed along the ZCF, similar to fus2Δ (Fig. 5, A and B). These data suggest that the mating defect in the fus2<sup>con</sup> mutant is due to the failure of Fus2p to recruit Cdc42p to the ZCF.

These data combine to produce a model in which Fus2p/Rvs161p localize to the curved shmoo tip in polarizing cells, with the complex in an orientation in which it does not interact with Cdc42p. As prezygote formation occurs, the change from positive to zero curvature would cause Fus2p/Rvs161p to undergo a conformational change, allowing interaction with Cdc42p.

The BAR protein superfamily comprises multiple sub-families that adopt conformations with a variety of intrinsic curvatures. BAR domains are 16–23 nm in length (Qualmann et al., 2011), implying that they should be sensitive to curvature at the nanometer scale (Zimmerberg and Kozlov, 2006). However, the Cdc42p focus is much larger, 1 μm in diameter. Recently, it was found that fungal septins can distinguish micrometer-scale curvature; single septin complexes bind preferentially to micrometer-scale curved membranes and are stabilized by polymerization (Bridges et al., 2016). BAR domains also oligomerize, forming filamentous polymers in vitro (McMahon et al., 2015; Suetsugu, 2016). Possibly, like septins, polymerization of BAR-domain proteins would allow membrane curvature sensing over many tens or hundreds of nanometers.

Flattening of the ZCF membrane may facilitate Fus2p/Rvs161p polymerization, perhaps relying on electrostatic interactions between the concave face of one dimer and the convex face of the other (Stein et al., 2015). Polymerization may induce a conformation change in Fus2p/Rvs161p, promoting Cdc42p binding and focus formation. As cell fusion continues, the Fus2p/Rvs161p/Cdc42p complex remains associated with the edge of the remnant cell walls, even as free Fus2p returns to the nucleus (Paterson et al., 2008; Ydenberg and Rose, 2009). The edge of the remnant cell wall has strong negative curvature, a natural fit for the concave face of the dimer. After the remnant cell walls are removed, the cortex of the zygote is once again smooth with overall positive curvature.

Because yeast live in hypoosmotic conditions, cell wall removal during mating must be tightly regulated. During growth, cell wall damage is quickly sensed and repaired, as shown by the rapid localization of Pkc1p to sites of laser damage (Kono et al., 2012). Therefore, yeast cells must distinguish between removal at the shmoo tip, where degradation would be fatal, and at the ZCF. Recently, it was suggested that cell wall degradation at the ZCF is driven solely by the contact-dependent increase in the concentration of cell wall remodeling enzymes due to reduced diffusion at the ZCF (Huberman and Murray, 2014). Multiple lines of evidence suggest this is not sufficient for cell
fusion. First, several mutations block cell fusion without affecting prezygote formation or general cell wall remodeling processes (e.g., fus1Δ and fus2Δ). Second, the behavior of fps1Δ suggests that cell fusion is regulated. Finally, the localization of Cdc42p suggests that cells use curvature to distinguish whether they have formed a prezygote. Sensing sites of zero or negative curvature may be a general mechanism of regulation. BAR-domain proteins have roles in hemagglutinin-promoted cell–cell fusion (Richard et al., 2011) and mouse myoblast fusion (Ho et al., 2004; Simionescu-Bankston et al., 2013; George et al., 2014; Kim et al., 2015). Therefore, curvature sensing may be a conserved mechanism in regulating and/or promoting cell fusion.

Materials and methods

General yeast techniques

Yeast media, general methods, and transformations were performed as described previously (Amberg et al., 2005), with minor modifications. Cells were grown in yeast extract peptone dextrose or selective media to maintain plasmids. Transformations were performed by growing cells to log phase (OD600 0.4–0.6), washing once with water, and resuspending in transformation mixture (33% w/v polyethylene glycol 4000, 0.1 M Li-acetate, and 0.2 mg/ml single-stranded carrier DNA), together with 1 µg plasmid DNA or 10 µl of a PCR in a final volume of 360 µl. Strains and plasmids are listed in Tables S1 and S2. All transformations were performed as described in the previous section on yeast mating assays

Yeast mating assays

Quantitative filter-matings were performed as described previously, with minor alterations (Gammie and Rose, 2002), by mixing early exponential MATα cells with MATα cells in a 1:4 ratio to reach a total of 107 cells/ml. This ratio was determined to be optimal for mating efficiency of the MATα cells, while showing the lowest variance. The cells were mixed together, concentrated on 25-mm, 0.45-µm nitrocellulose filter disks (Millipore Corporation), and incubated on rich media plates for 2.5–3 h at 30°C.

Microscopy

For imaging of pheromone-induced cells with fluorescent proteins, cells were induced by addition of 10 µg/ml α-factor for 90 min, fixed for 10 min with 2% formaldehyde at 30°C, washed in 1× PBS, and then imaged in PBS. All images except those in Fig. 3 (G and H) and Fig. 5 were acquired at 23°C using a deconvolution microscopy system (DeltaVision; Applied Precision, LLC) equipped with an inverted microscope (TE200; Nikon), a 100× objective with numerical aperture of 1.4, and a CoolSnap HQ2 Camera (Photometrics). Deconvolution and image analysis were performed using softWoRx and ImageJ (National Institutes of Health). Data for Fig. 3 (G and H) and Fig. 5 were acquired at 23°C using a TI-E inverted microscope (Nikon) equipped with a 100× objective with numerical aperture of 1.45 and an Orca Flash4 camera. NIS Elements (Nikon) software was used for image acquisition along with NIS Elements automatic 3D deconvolution and ImageJ for analysis.

For imaging of prezygotes containing fluorescent proteins, mating mixtures were prepared as described in the previous section on yeast mating assays, resuspended in synthetic media, fixed for 10 min with 2% formaldehyde at 30°C, and imaged as above. Quantification of fluorescence was performed using ImageJ software (Schindelin et al., 2012, 2015). Values along the ZCF were interpolated using cubic interpolation in MATLAB (The MathWorks). The Mann-Whitney test for two independent samples was used to obtain p-values for localization curves unless otherwise stated. Quantification of colocalization of Cdc42p and Fus2p was performed by quantifying fluorescence across a line perpendicular to the shmoo tip or ZCF using ImageJ software. Fus2p and Cdc42p intensities were determined by measuring fluorescence intensity at the ZCF using an elliptical region of interest in ImageJ and normalized to fluorescence intensity at the opposite end of the cell. Error bars represent the SEM for all cells imaged. A two-tailed, two-sample unequal t test was used to determine significance.

Microscopic assays of FM4-64-stained mating mixtures were performed as described previously (Grote, 2008). Mating mixtures were prepared as described in the previous section on yeast mating assays, and then resuspended in 1 ml TAF buffer (20 mM Tris-HCl, 20 mM NaNO3, and 20 mM NaF in water) and kept on ice. FM4-64 (Molecular Probes/Invitrogen) was added to mating mixtures to a final concentration of 4 µM, and stained zygotes were imaged as described in the first paragraph of this section.

Live imaging was performed by mixing 0.02 OD600 of each mating type and allowing cells to mate on a 2% agarose pad at 23°C. Images were taken at 3-min intervals using the DeltaVision system described in the first paragraph under Microscopy.

Online supplemental material

Video 1 shows live imaging of GFP–Cdc42p during cell fusion of WT mating pairs. Cdc42p forms a focus just before cell fusion. Fig. S1 shows that deletion of Kel1p, a fusion protein known to interact with Fus2p, disrupts Cdc42p localization to the ZCF. Fig. S2 shows that removal of the C terminus of Fus2p does not disrupt its localization to the ZCF. Yeast strains and plasmids used are listed in Tables S1 and S2, respectively. Table S3 reports the mating efficiency and localization of Fus2p–GFP in mutants affecting the Fus2p/Rvs161p BAR domain.

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