

Cdc42p and Fus2p act together late in yeast cell fusion

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ABSTRACT Cell fusion is the key event of fertilization that gives rise to the diploid zygote and is a nearly universal aspect of eukaryotic biology. In the yeast *Saccharomyces cerevisiae*, several mutants have been identified that are defective for cell fusion, and yet the molecular mechanism of this process remains obscure. One obstacle has been that genetic screens have mainly focused on mating-specific factors, whereas the process likely involves housekeeping proteins as well. Here we implicate Cdc42p, an essential protein with roles in multiple aspects of morphogenesis, as a core component of the yeast cell fusion pathway. We identify a point mutant in the Rho-insert domain of *CDC42*, called *cdc42-138*, which is specifically defective in cell fusion. The cell fusion defect is not a secondary consequence of ineffective signaling or polarization. Genetic and morphological data show that Cdc42p acts at a late stage in cell fusion in concert with a key cell fusion regulator, Fus2p, which contains a Dbl-homology domain. We find that Fus2p binds specifically with activated Cdc42p, and binding is blocked by the *cdc42-138* mutation. Thus, in addition to signaling and morphogenetic roles in mating, Cdc42p plays a role late in cell fusion via activation of Fus2p.

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INTRODUCTION

Cell fusion is widespread in the eukaryotic kingdom. In addition to the familiar fertilization events necessary to restore the diploid state after meiosis (Primakoff and Myles, 2002), fusion occurs throughout the development of many multicellular organisms, including the formation of muscle, bone, and the mammalian placenta (Vignery, 2000; Potgens *et al.*, 2002; Horsley and Pavlath, 2004; Chen and Olson, 2005). In contrast to virus–cell fusion and fusion between intracellular membranes, the molecular mechanism of cell fusion is not well understood.

Cell fusion in budding yeast occurs during mating events between haploid cells (Marsh and Rose, 1997; Ydenberg and Rose, 2008). Yeast haploid cells of the *a* and α mating types each secrete pheromones sensed by a receptor expressed by the opposite cell type.

This induces characteristic gene expression and developmental changes resulting in polarized growth or “shmooing” in the direction of the mating partner, followed by cell fusion and nuclear fusion. Although numerous mutants have been identified that block fusion, many of them are now known to have prior defects in pheromone signaling and polarization, suggesting that the cell fusion defect is a secondary consequence of inefficient completion of earlier mating events (Brizzio *et al.*, 1996; Dorer *et al.*, 1997; Paterson *et al.*, 2008).

In contrast, four proteins have been identified that appear to play a direct role in yeast cell fusion without having detectable defects in earlier steps of mating. Mutations in *FUS1*, *FUS2*, and *RVS161* block the removal of cell wall material between mating pre-zygotes (Trueheart *et al.*, 1987; Trueheart and Fink, 1989; Brizzio *et al.*, 1998), whereas *PRM1* mutants block fusion after cell wall removal but before plasma membrane fusion (Heiman and Walter, 2000). The molecular function of these proteins is not known, but it may involve the trafficking and/or fusion of mating-specific vesicles that are visualized at the cell fusion zone (Gammie *et al.*, 1998). These vesicles are delocalized in *fus1* mutants, as well as in polarization mutants (e.g., *bni1* and *spa2*). The vesicles are properly localized in *fus2* and *rvs161* mutants, suggesting that these mutations affect later stages of cell fusion than *fus1*. The behavior of the vesicles in *prm1* mutant zygotes has not been reported.

Fus2p and Rvs161p form a complex that is transported to the tips of fusing cells (Brizzio *et al.*, 1998; Paterson *et al.*, 2008; Sheltzer

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Abbreviations used: BAR, Bin1, amphiphysin, Rvs; GAP, GTPase activating protein; GEF, GTP exchange factor.

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and Rose, 2009). Rvs161p is a BAR domain-containing protein that plays a separate role in endocytosis (Crouzet *et al.*, 1991) by stabilizing membrane curvature (Peter *et al.*, 2004). Fus2p contains a Dbl-homology domain, suggesting that it interacts with a Rho-type G protein. In support of this, a large-scale two-hybrid screen identified an interaction between Fus2p and Cdc42p in the GTP-bound form (Nelson *et al.*, 2004).

Yeast Cdc42p plays multiple essential roles during growth and morphogenesis (Johnson, 1999; Richman *et al.*, 1999; Kozminski *et al.*, 2000; Adamo *et al.*, 2001), including during mating (Simon *et al.*, 1995; Zhao *et al.*, 1995). In late G1, Cdc42p establishes polarized growth toward a specific area of the cell surface to grow a new bud. During mating, the budding axis is overridden by cues that direct Cdc42p activity along the axis of the pheromone gradient (Nern and Arkowitz, 1998, 1999). Cdc42p has been implicated in cell fusion (Barale *et al.*, 2006), but the stage at which it acts and its molecular functions are not clear. Here we present evidence that Cdc42 has multiple roles in cell fusion, that Fus2p is a direct effector of Cdc42p signaling, and that Cdc42p and Fus2p act together late in the cell fusion pathway. Our results have implications for the molecular mechanism of yeast cell fusion, the function of an uncharacterized domain in Cdc42p, and conservation between fungal and metazoan cell fusion mechanisms.

RESULTS

The Fus2p Dbl-homology domain promotes cell fusion

Fus2p contains a Dbl-homology domain (Paterson *et al.*, 2008), suggesting that it may act as a guanine-nucleotide exchange factor for one of the six yeast Rho proteins. Conversely, a two-hybrid study suggested that Fus2p interacted with Cdc42p-Q61L, a GTP-locked allele (Nelson *et al.*, 2004). These results suggested that Fus2p may be an effector for Cdc42p, since it interacted exclusively with the active form of the GTPase.

To assess the importance of the Dbl-homology domain *in vivo*, we examined the phenotype caused by a deletion of this domain. Fus2p-ΔDbl-green fluorescent protein (GFP) localized to the nucleus in mitotic cells and at the shmoo tip in pheromone-arrested cells, similar to wild-type Fus2p-GFP (Patterson *et al.*, 2008). Therefore the Dbl-homology domain is required for neither localization in mitotic and pheromone-arrested cells nor the regulated change in localization (Figure 1A). Both proteins were detected at similar levels (Figure 1B). When this deletion was integrated into the genome to replace the endogenous *FUS2* locus, the resulting strain was significantly defective for the formation of diploids, although not quite as defective as a complete deletion mutant (*fus2Δ*; Figure 1C). To examine the morphology of the zygotes formed by this strain, wild-type and *fus2-ΔDbl* strains were mated to a *fus2Δ* tester strain and stained with FM4-64 to visualize the plasma membrane (Figure 1D). We then counted the fully unfused zygotes (in which the septum

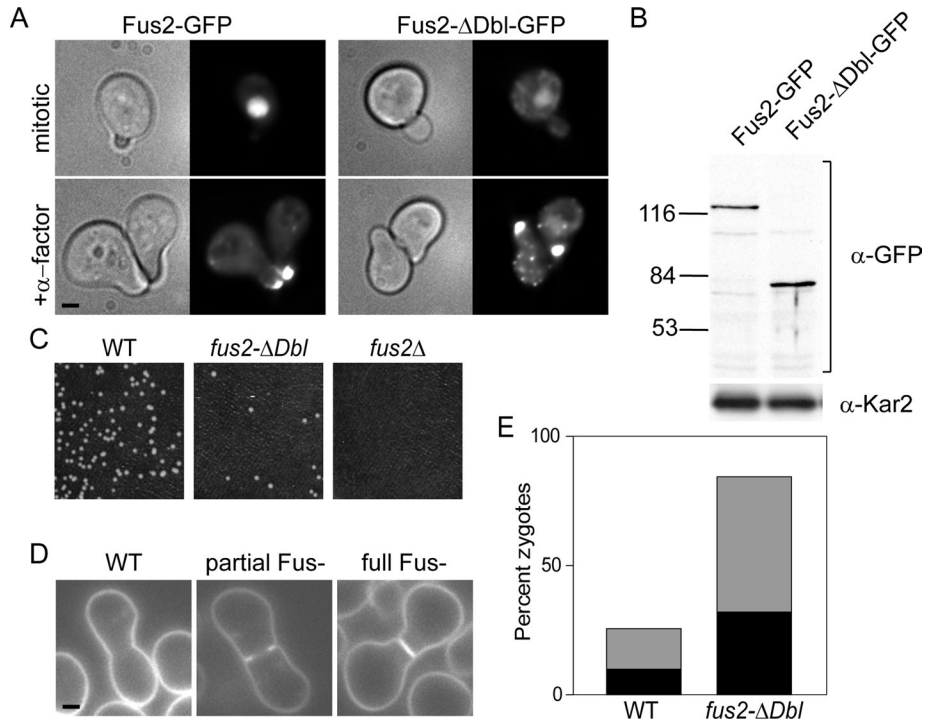


FIGURE 1: The Dbl-homology domain is required for cell fusion. (A) Fus2p-ΔDbl-GFP localizes to the shmoo tip. Cells (MY9181) transformed with a plasmid expressing either Fus2p-GFP (pMR5469) or Fus2p-ΔDbl-GFP (pMR5883) were treated with pheromone as indicated for 2 h and examined. (B) Fus2p-ΔDbl-GFP is expressed at similar levels as the wild-type Fus2p-GFP protein. The same strains as in A were analyzed by Western blot using anti-GFP. (C) Diploid formation is defective in *fus2-ΔDbl*. Wild type (DDY1300), *fus2Δ* (MY10016), and *fus2-ΔDbl* (MY10933) were mated to *fus1 fus2* (MY10798) for 4 h at 30°C. (D, E) Cell fusion is defective in *fus2-ΔDbl*. The same strains as in C were mated to *fus2* (MY10797) for 2 h, washed into azide, and stained with FM4-64 to label the plasma membrane. (D) Examples of fusion-defective zygotes. Wild-type zygote (DDY1300xMY10797), partial Fus⁻ zygote (MY10933xMY10797), and full Fus⁻ zygote (MY10933xMY10797). (E) Percentage of zygotes of each morphology produced in matings with the indicated genotype. Gray bars, partial Fus⁻ zygotes; black bars, full Fus⁻ zygotes. n ≥ 133 zygotes imaged in two independent experiments.

between the mating partners remains intact) and partially unfused zygotes (in which there is a gap in the septum that fails to open completely). Both types of unfused zygotes are indicative of the defect in Fus2p function. The *fus2^{ΔDbl}* mutant produced a large number of unfused and partially unfused zygotes (Figure 1E). Again, the phenotype is not as strong as a null *fus2Δ* strain, in which the majority of zygotes are fully unfused (compare to Figure 2B). These results indicate that the *fus2^{ΔDbl}* allele is hypomorphic and that this domain is critically important but not absolutely required for Fus2p's cell fusion activity.

Mutations in the Rho-insert domain are defective in cell fusion

The interaction between activated Cdc42p and Fus2p suggested that Cdc42p may play a direct, specific role in cell fusion. Cdc42p has at least two defined roles in the mating pathway already: it is required for response to pheromone by activation of the PAK Ste20p (Moskow *et al.*, 2000) and for defining the site of polarization of the shmoo (Nern and Arkowitz, 2000). One mutant allele of *cdc42* that exhibits a defect in cell fusion has been previously characterized, *cdc42-V36M* (Barale *et al.*, 2006). However, given the many potential roles for Cdc42p in mating, the specific defect of *cdc42-V36M* remained unclear. To look for additional alleles of *CDC42* that might be specifically defective for Fus2p-mediated cell fusion, we used an

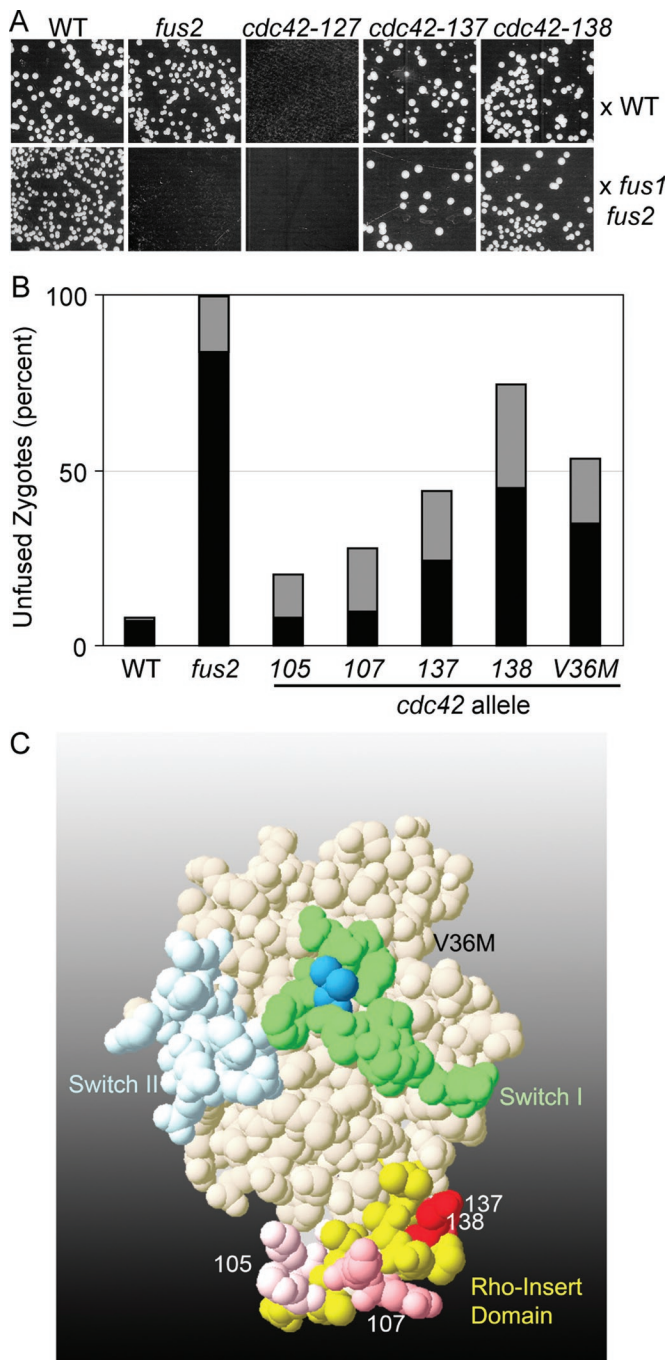


FIGURE 2: Mutants in the Cdc42p Rho-insert domain are defective for cell fusion. (A, B) A screen for cell fusion-specific alleles of Cdc42p. Wild type (WT; DDY1300), *fus2* (MY10016), and 20 alleles from a Cdc42p alanine-scanning set were mated to an anti-WT strain (JY431; A) or an anti-*fus1 fus2* strain (JY429; B). Shown are the alleles discussed in the text. Note that the matings against JY431 are more dilute than against JY429, so that colony formation against the two testers cannot be compared with one another. Images are representative of two independent experiments. (B) Microscopic examination of zygotes. Bilateral matings of the indicated genotype were mated for 2 h at 30°C, washed into azide, and stained with FM4-64. Shown is the percentage of zygotes of each morphology in the indicated genotypes (WT, DDY1300 × DDY1301; *fus2*, MY10016 × MY10303; *cdc42-138*, DDY1354 × DDY1355; *cdc42-V36M*, MY10547 × MY10554; see Kozminski *et al.*, 2000, for other strain numbers). Gray bars, partial *Fus*⁻ zygotes; black bars, full *Fus*⁻ zygotes. $n \geq 122$ zygotes imaged in at least two independent experiments.

alanine-scanning collection created previously (Kozminski *et al.*, 2000). Twenty mutant alleles, annotated as wild type for growth, were mated to a wild-type strain or a fusion-defective tester strain and examined for the ability to form diploids by a plate mating assay (Figure 2A and Supplemental Figure S1). Because the *fus2Δ* single mutant exhibits an obvious defect when mated against a compromised partner (Figure 2A) but not when mated to the wild-type strain, we reasoned that this approach would allow us to identify alleles that were specifically defective in cell fusion. One allele, *cdc42-127*, did not mate with either the *fus1 fus2* or the wild-type partner, indicating that it has a sterile (*Ste*⁻) phenotype (Figure 2A). This mutation, V33A, lies within the Switch I region and was not analyzed further. Two alleles, *cdc42-137* and *-138*, exhibited significant mating defects with the *fus1 fus2* tester strain but not with the wild-type strain, suggesting that they represent cell fusion-specific (*Fus*⁻) alleles (Figure 2B). The two mutations, D121A and D122A, respectively, lie within the Rho-insert domain (Figure 2C) and alter consecutive negatively charged residues.

To confirm that the mating defect against the compromised strain was due to reduced cell fusion efficiency, we quantified zygote morphology in these mutants. For these experiments, we used a bilateral mating configuration in which both partners contain the same mutation of interest. Under these conditions, wild-type zygotes typically have no visible septum between the mating partners, whereas *cdc42-137* and *138* zygotes frequently had all or part of the septum intact, indicating that they had not fully fused (Figure 2B; 44% of *cdc42-137* zygotes and 75% of *cdc42-138* zygotes were fusion defective compared with 8% of wild-type zygotes).

Given the location of these alleles within the Rho-insert domain, we examined two other mutations constructed in the alanine-scanning collection that altered residues in this region but that had not exhibited a defect in the plate mating screen. Both mutants showed a significant cell fusion defect; *cdc42-105* (E127A, K128A) produced 20% fusion defective zygotes, and *cdc42-107* (R131A, R133A, R135A) produced 28% fusion defective zygotes (Figure 1C). These defects were not as strong as those for *cdc42-137* and *cdc42-138*, explaining why they were missed in the less sensitive plate mating assay. Together the data indicate that the Rho-insert domain of Cdc42p, which had not previously been shown to have a specific function, plays a significant and unexpected role in cell fusion. To further characterize the defect in cell fusion, we focused on the more potent *cdc42-138* allele.

The fusion defect in *cdc42-138* is not due to defective pheromone signaling

Cdc42p plays a known role in the pheromone-signaling pathway (Simon *et al.*, 1995; Zhao *et al.*, 1995). One possibility was that the defect in cell fusion is simply due to partly compromised signaling (Brizzio *et al.*, 1996). To isolate Cdc42p from any effects on the pheromone pathway, we used a *STE20-ΔCRIB* mutation (Lamson *et al.*, 2002), which removes the Cdc42p-binding domain in Ste20p, thereby rendering the protein and the pheromone response

(C) Structure of Cdc42p indicating mutations affecting cell fusion. Mutations are mapped on a space-filling model of human Cdc42p (Brookhaven Protein Database, accession number 1AN0). The Rho-insert domain is shown in yellow, with mutations indicated in shades of red, correlated with the severity of the defect. The Switch I domain is shown in green, with the V36M mutation in dark blue. The Switch II domain is in light blue. Pseudo-wild-type mutations with no effect on cell fusion are not shown.

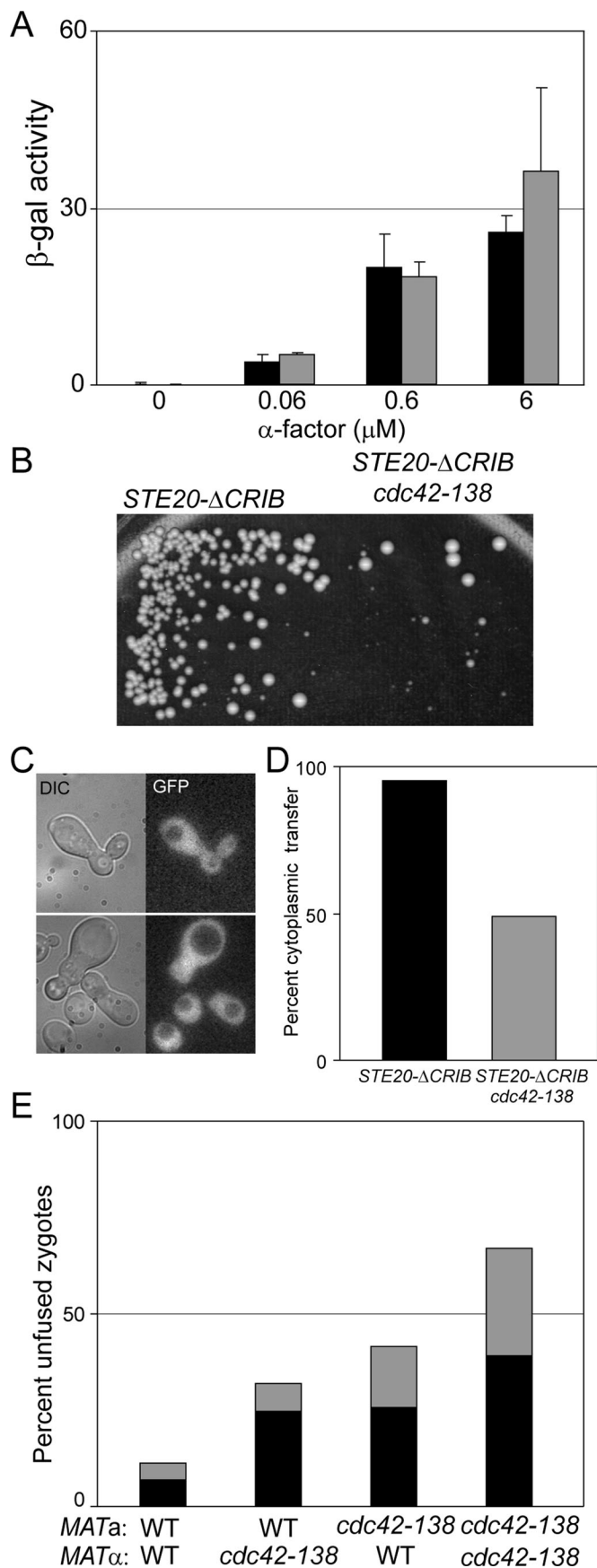


FIGURE 3: The cell fusion defect in *cdc42-138* is not due to defective pheromone signaling. (A) *cdc42-138* has no defect in signaling in the context of *STE20-ΔCRIB*. *STE20-ΔCRIB* (MY10925; black bars) and

pathway insensitive to signaling from Cdc42p. In the *STE20-ΔCRIB* background, *cdc42-138* had no effect on pheromone signaling (Figure 3A). In contrast, mating between *STE20-ΔCRIB cdc42-138* double mutants was significantly more defective than matings between the *STE20-ΔCRIB* controls (Figure 3B). Therefore *cdc42-138* is defective in an aspect of mating that is independent of signaling to Ste20p. We used cytoplasmic transfer assays to confirm that the mating defect was due to a cell fusion defect (Figure 3, C and D). When a *fus2Δ* tester strain expressing cytoplasmic GFP was mated to a *STE20-ΔCRIB* strain, the GFP was transferred to the partner in 95% of the zygotes (corresponding to Fus⁺ and partial Fus⁻ zygotes). When the *STE20-ΔCRIB* strain also contained the *cdc42-138* mutation, cytoplasmic transfer was observed in only 49% of zygotes, indicating that *cdc42-138* had a full cell fusion defect in more than half of the zygotes in this strain ($n \geq 469$). We conclude that *CDC42* plays a role in cell fusion that is independent of its role in signal transduction.

A second possible explanation for the cell fusion defect in *cdc42-138* is that the mutants produce reduced levels of *a*-factor or α -factor, leading to a fusion defect due to reduced signaling in the partner (Brizzio *et al.*, 1996). Because the genes required for production and secretion of *a*-factor and α -factor are different, cell fusion mutations that affect pheromone levels are mating-type specific (Brizzio *et al.*, 1996). In crosses between *cdc42-138* strains and wild-type (WT) strains, the efficiency of cell fusion was independent of whether the *a*-cell or the α -cell contained the mutation (Figure 3E; *a* WT \times α *cdc42-138* produced 32% fusion-defective zygotes, compared with 41% for *a* *cdc42-138* \times α WT). Moreover, the defect was significantly stronger when both strains contained the *cdc42-138* mutation, showing that Cdc42p function plays a role in cell fusion in both parents. These data indicate that the fusion defect in *cdc42-138* is not due to defective pheromone signaling.

CDC42 plays independent roles in cell polarization and fusion

Previously a point mutation within the Switch I domain of Cdc42p (*cdc42-V36M*) was reported to cause a cell fusion-defective phenotype (Barale *et al.*, 2006). To ensure that any differences observed between *cdc42-V36M* and *cdc42-138* would not be due to strain background, we re-created this allele in our strain background. We confirmed that *cdc42-V36M* caused a cell fusion defect, producing a significant number of unfused zygotes (Figure 2B; 53% had fusion defects).

STE20-ΔCRIB cdc42-138 (MY10926; gray bars), transformed with *fus1-LacZ* (pSB231), were treated with the indicated concentrations of α -factor for 90 min and their response measured via β -galactosidase assay. Error bars represent SD of three independent experiments. (B–D) *cdc42-138* still exhibits a cell fusion defect in the *STE20-ΔCRIB* background. (B) *STE20-ΔCRIB* (MY10925) and *STE20-ΔCRIB cdc42-138* (MY10926) were mated to *fus2* (MY10797) for 4 h at 30°C, and diploids were selected by replica plating to selective media (C, D). Cytoplasmic transfer assays were performed using the same strains as in B, in which one partner expresses cytoplasmic GFP. (C) Examples of zygotes showing transfer (top) or in which cell fusion was blocked (bottom). The latter phenotype corresponds to the “Full” Fus⁻ cell fusion defect. (D) Quantification of the data in C. More than 460 zygotes were imaged in two independent experiments. (E) The defect in *cdc42-138* is not mating-type specific. WT and *cdc42-138* cells were mated in the orientations indicated. Gray bars, partial Fus⁻ zygotes; black bars, full Fus⁻ zygotes. $n \geq 176$ zygotes imaged in two independent experiments.

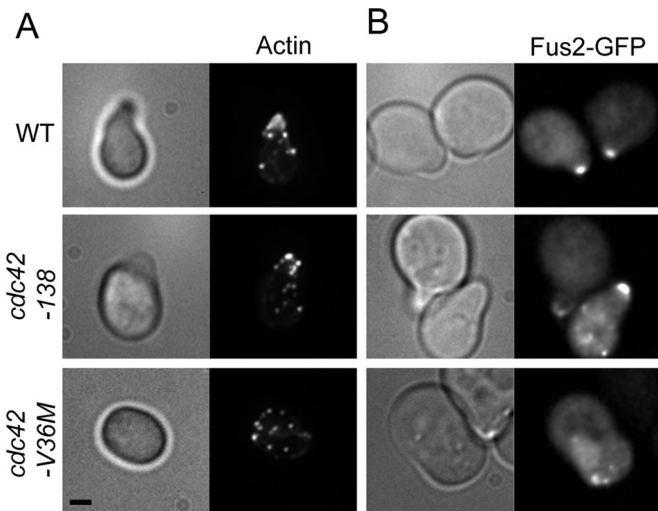


FIGURE 4: *cdc42-138* is not defective in cell polarization in response to pheromone. (A) Actin localization in *cdc42-138* and *cdc42-V36M*. WT (DDY1300), *cdc42-138* (DDY1354), and *cdc42-V36M* (MY10547) were treated with α -factor for 90 min, fixed, and stained with Texas red-phalloidin. Representative images are shown. $n \geq 241$ cells in three independent experiments. (B) Fus2p-GFP localization in *cdc42-138* and *V36M*. The same strains were transformed with *FUS2::GFP₁₀₄* (pMR5482) and imaged after treatment with α -factor for 90 min. Representative images are shown. $n \geq 55$ cells in two independent experiments.

Mutations that affect the polarization of cells in response to pheromone (e.g., *spa2*, *bni1*, *pea2*) exhibit corresponding defects in cell fusion (Dorer *et al.*, 1997; Gammie *et al.*, 1998). Because Cdc42p is critically important for polarity during both mating and mitotic growth (Chant, 1999; Pruyne and Bretscher, 2000; Casamayor and Snyder, 2002), we next examined the morphological responses of *cdc42-138* and *cdc42-V36M* to pheromone.

When wild-type or *cdc42-138* cells were treated with pheromone, they formed characteristic pointed mating projections (Figure 4A). The actin cytoskeleton became polarized, with actin patches concentrated in the projection and cables oriented toward the shmoo tip (Figure 4A; 85% of wild-type cells and 81% of *cdc42-138* cells had polarized actin). In contrast, *cdc42-V36M* cells were round or oblong, without an obvious projection of any kind (Figure 4A). Actin patches were not tightly localized at any particular point but instead were dispersed throughout the cell (Figure 4A; only 18% had polarized actin).

We also examined the localization of Fus2p. Fus2p-GFP localizes tightly to the shmoo tip in wild-type cells but is usually dispersed or nuclear in polarization mutants such as *bni1* and *spa2* (Paterson *et al.*, 2008). Wild-type and *cdc42-138* shmooes contained predominantly tip-localized Fus2p-GFP (Figure 4B; Fus2p-GFP was predominantly polarized in 98 and 80% of cells, respectively). The small but significant ($p = 0.004$, Fisher's exact test) decrease in Fus2p-GFP localization is consistent with an interaction with Cdc42p. In contrast, in the majority of *cdc42-V36M* cells (56%), Fus2p-GFP was dispersed or partially nuclear. In only 44% of *cdc42-V36M* cells was Fus2p-GFP localized to a point on the cortex (usually at one of the ends of the oval-shaped cells). However, even in these cells, localization was not wild type; time-lapse imaging of Fus2p-GFP in *cdc42-V36M* showed that the cortical spots were unstable, moving rapidly away from the cortex and showing significant nuclear localization (Supplemental Movie S2). Similar unstable localization was observed in *spa2* and *bni1* mutants (Paterson *et al.*, 2008).

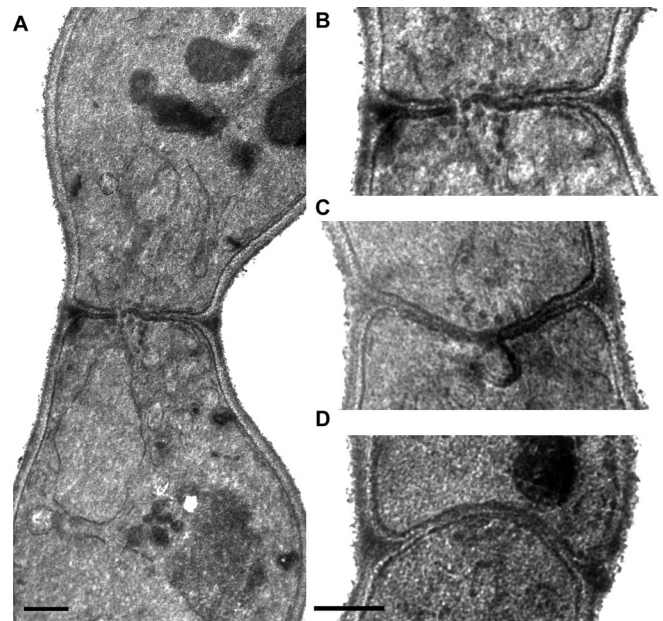


FIGURE 5: EM of *cdc42-138* and *fus2* zygotes. *cdc42-138* bilateral zygotes (DDY1354 \times DDY1355) were examined by electron microscopy. (A) A zygote, showing the unfused nuclei and septum, containing cell wall material. Image is representative of 91 zygotes. (B) The waist regions of the same zygotes, showing vesicles clustered at the zone of cell fusion. Images are representative of 32 zygotes in which vesicles were observed. The vesicles were clustered in 91% of this class. (C) Dark plaques and a membrane invagination at the cell fusion zone. These phenotypes were observed in 21 and 10% of all zygotes examined, respectively. (D) A *fus2* zygote, for comparison. Scale bar, 0.5 μ m.

Together, these data indicate that the *cdc42-138* mutant responds to pheromone and polarizes normally. Therefore, the block in cell fusion is not likely to be a secondary consequence of defects in earlier events in mating. In contrast, the *cdc42-V36M* mutant polarizes poorly in response to pheromone, suggesting that the fusion defect may be largely due to indirect effects on the cytoskeleton during cell polarization.

Cdc42-138 acts late in cell fusion

Several classes of cell fusion mutants are now known that can be distinguished by their morphological characteristics. Mutations in *SPA2*, *FUS1*, *FUS2*, and *RVS161* all block prezygotes at the stage of cell wall removal (Gammie *et al.*, 1998). As discussed earlier, *SPA2* and *BNI1* primarily affect polarization, and mutants form broad adhesion zones between the fusing cells. The respective phenotypes can be distinguished at the level of electron microscopy; *fus2* and *rvs161* mutants have vesicles tightly clustered at the zone of cell fusion, similar to wild-type zygotes prior to fusion. In contrast, *fus1* and *spa2* mutants contain broadly dispersed vesicles that do not localize to a particular point. Double-mutant and suppression analysis showed that *fus1* and *fus2* work in overlapping but distinct pathways, whereas *fus2* and *rvs161* work together (Trueheart *et al.*, 1987; Brizzio *et al.*, 1998; Gammie *et al.*, 1998).

Electron microscopy of *cdc42-138* bilateral zygotes revealed that they contain intact cell walls between the mating partners (Figure 5). Vesicles were observed to be tightly clustered in a single region at the zone of cell fusion (Figure 5, B and C; 35% of sections through *cdc42-138* prezygotes had vesicles, $n = 48$; of these, 89% had clustered vesicles, $n = 19$). This phenotype is very similar to that of *fus2*

(Figure 5D) and *rvs161* but distinct from that of *spa2* and *fus1* (Gammie et al., 1998). We also observed dark-staining plaques and membrane invaginations in *cdc42-138* zygotes (Figure 5C; 31% had plaques and 5% had invaginations, $n = 48$). These phenotypes were previously shown to be unique to *fus2* and *rvs161* mutants and were not observed in *fus1* and *spa2* mutants (Gammie et al., 1998). Furthermore, the formation of these structures was dependent on *FUS1* and *SPA2* function because they were not observed in *fus1 fus2* or *spa2 fus2* double mutants. The fact that *cdc42-138* formed these structures provides evidence that the *SPA2* and *FUS1* pathways remain functional.

To further characterize the morphology of *cdc42-138*, we measured the breadth of the zygote cell fusion zones (referred as the zygote waistlines). In wild-type matings, unfused prezygotes have an average waistline of $1.0 \pm 0.3 \mu\text{m}$, which expands to $1.6 \pm 0.2 \mu\text{m}$ soon after nuclear fusion (Gammie et al., 1998). When only unfused prezygotes were considered, *cdc42-138* bilateral zygotes had waistlines measuring $1.4 \pm 0.3 \mu\text{m}$ ($n = 48$). It is surprising that this was significantly narrower than for *fus2* prezygotes ($1.7 \pm 0.5 \mu\text{m}$, $n = 19$, $p = 0.008$, Student's *t* test). We reasoned that because the *cdc42-138* mutant has a weaker phenotype than the *fus2* mutant, a significant portion of the zygotes with broad zones may have fused, leading to a shorter average waistline. In support of this, when the entire population of fused and unfused zygotes was considered, there was no difference between the average waistline of the *cdc42-138* and *fus2* mutant zygotes ($1.6 \pm 0.4 \mu\text{m}$, $n = 91$, vs. $1.7 \pm 0.5 \mu\text{m}$, $n = 20$, respectively; $p = 0.22$). Most important, the data provide additional evidence that the *cdc42-138* mutant is morphologically dissimilar from polarization mutants, which have significantly wider waistlines than the *fus2* mutant (Gammie et al., 1998). Together the data show that the *cdc42-138* mutant is morphologically most similar to *fus2* and *rvs161* mutants. Furthermore, they show that *CDC42* plays a role in the very latest stages of cell fusion.

FUS2 and CDC42 work together

To test whether *FUS2* and *CDC42* act in the same pathway for cell fusion, we constructed a *fus2Δ cdc42-138* double mutant and compared its phenotype to that of the two single mutants. We reasoned that if *cdc42-138* specifically compromised Fus2p function in cell fusion (or vice versa), then the double mutants should show no more severe a defect than the most defective single mutant. Such behavior is observed between *rvs161* and *fus2* mutations, whereas double mutants between either of these mutations and *fus1* or *spa2* mutations are significantly more severe (Brizzio et al., 1996). For this experiment, we used a cytoplasmic transfer assay (Figure 6A). In this assay, only full Fus⁻ zygotes with no plasma membrane fusion are scored as being defective. The *fus2* donor strain was able to transfer cytoplasmic GFP to 92% of wild-type cells ($n = 764$) but was significantly defective for transfer to *fus2* (46% transfer, $n = 778$, $p = 0.001$, chi-square) and *cdc42-138* (72% transfer, $n = 710$, $p = 0.001$, chi-square; Figure 6B). If the two proteins act in two independent pathways, then we predict that the defect of the double mutant should be multiplicative, normalized for the frequency observed for wild type (i.e., $[0.72 \times 0.46]/0.92$). Transfer to the *cdc42-138 fus2* double mutant was somewhat more defective than to *fus2* alone (42%, $n = 554$,

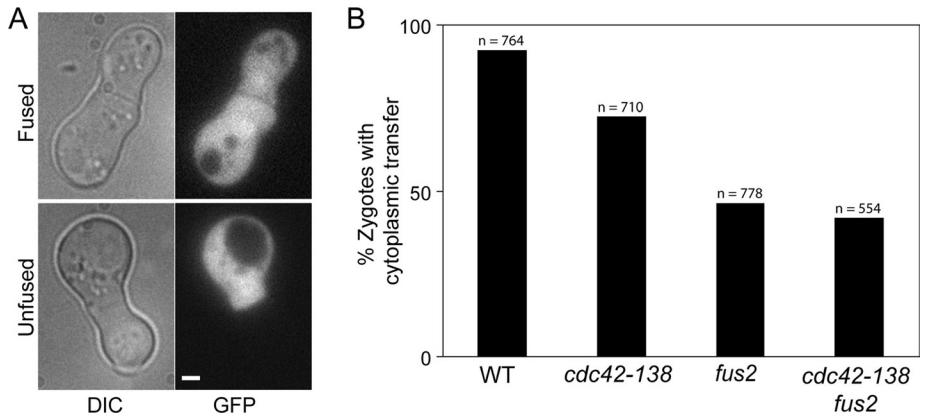


FIGURE 6: Fus2p and Cdc42p work in the same pathway. (A) Cytoplasmic transfer assay. Strains of different genotypes were mated to MY10797 (*fus2* P^{GPD1}-GFP) for 2 h at 30°C. An example of a zygote displaying cytoplasmic transfer (top) and one not displaying transfer (bottom) are shown. (B) WT (DDY1300), *cdc42-138* (DDY1354), *fus2* (MY10016), and *fus2 cdc42-138* (MY10310) were mated to MY10797, and the percentage of zygotes showing transfer were counted. The total number of zygotes counted in three independent experiments are shown.

$p = 0.04$, chi-square) but significantly different from the multiplicative prediction for independent pathways (36%, $p = 0.004$, chi-square). Because of the significant difference from the multiplicative model, we conclude that the *cdc42-138* and *fus2* mutations largely affect the same pathway for cell fusion. However, the smaller difference from the phenotype of the single mutant suggests that one or both proteins may also contribute an independent function to cell fusion.

Fus2p Interacts with GTP-bound Cdc42p

Our genetic data indicate that Fus2p and Cdc42p work in the same pathway; they do not distinguish which protein acts upstream. Because Fus2p has a Dbl-homology domain required for cell fusion, one possibility is that Fus2p is a guanine nucleotide exchange factor (GEF) for Cdc42p. However, numerous attempts to demonstrate GEF activity have been unsuccessful, even under conditions where Cdc24p (the GEF for Cdc42p) showed strong activity (Ydenberg, Stein, Andrianantoandro, and Rose, unpublished data). We next considered the possibility that Fus2p is a Cdc42p effector, interacting specifically with activated, GTP-bound Cdc42p.

We began by confirming the Fus2p-Cdc42p interaction in an *in vitro* system. Wild-type Cdc42p, Cdc42p-Q61L, the GTP-locked mutant, and Cdc42p-T17N, the GDP-locked mutant, were expressed as GST fusions in *Escherichia coli*. Equal amounts of purified GST-Cdc42p were added to yeast extracts containing full-length Fus2p, which was internally FLAG-epitope tagged at residue 104. This protein was shown previously to support wild-type levels of cell fusion, *in vivo* (Paterson et al., 2008). All three forms of Cdc42p coimmunoprecipitated with Fus2p-FLAG; however roughly 20-times-higher levels of Cdc42p-Q61L (GTP bound) were bound compared with either Cdc42p-T17N (GDP bound) or wild type (Figure 7A). These results confirm that Cdc42p can bind to Fus2p and suggest that Fus2p is not a GEF for Cdc42p. Indeed, because the interaction was strongest with activated Cdc42p, we conclude that that Fus2p is more likely to be an effector or to play a role in localizing activated Cdc42p to the fusion site.

To identify the region required for binding to Cdc42p, various deletions of Fus2p were constructed and used in the coimmunoprecipitation assay, using Cdc42p-Q61L (Figure 7, B and C). The most efficient binding was observed with full-length protein and the deletion lacking residues 580–677. Of interest, although the Dbl-homology domain was sufficient to see some binding to Fus2p, it was not

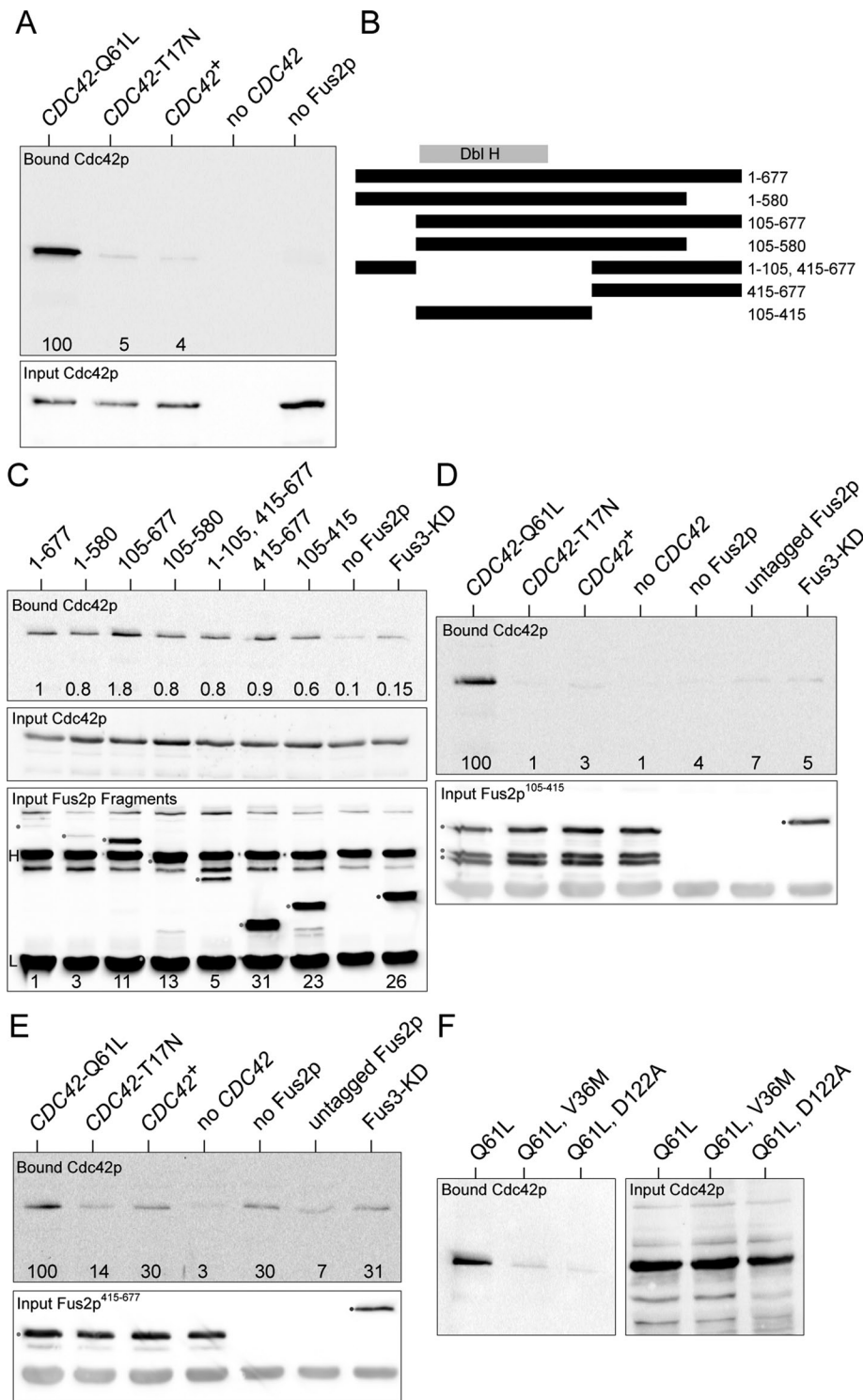


FIGURE 7: Fus2p binds to Cdc42p in vitro. (A) Fus2p binds most strongly to the activated GTP-bound form of Cdc42p. Wild-type, Cdc42p-Q61L (GTP-locked), and Cdc42p-T17N (GDP-bound) proteins were expressed as GST fusions in *E. coli* and purified on glutathione Sepharose. The Cdc42p-GST fusions were incubated with yeast cell extracts expressing FLAG epitope-tagged Fus2p for 1 h at 4°C, and interacting proteins were precipitated with anti-FLAG Sepharose. The Cdc42p-GST fusions were detected by Western blot using anti-GST and chemiluminescent imaging. Top, bound Cdc42 proteins. Bottom, input Cdc42 proteins. Numbers indicate the relative level of binding of each Cdc42p species normalized to Cdc42p-Q61L. In the rightmost “no Fus2p” lane, Cdc42p-Q61L was used. (B) Map of Fus2p deletion mutants. The Dbl-homology domain is indicated (Dbl H). The extents of the deletions, in amino acid residues, are indicated. Note that the FLAG epitope is inserted at residue 104. (C) Cdc42p binds to more than one domain of Fus2p. Yeast strains expressing the deletion constructs shown in B were

required for binding; binding was also observed for protein fragments containing residues 415–677. These results suggest that more than one region of Fus2p interacts with Cdc42p. To analyze this further, we examined the nucleotide specificity of binding to the two regions of Fus2p. Binding to the DBH domain showed strong specificity for binding to the GTP-bound Cdc42p-Q61L but not to the other forms of Cdc42p (Figure 7D). The 415–677 fragment showed lower specific binding, although it still bound preferentially to Cdc42p-Q61L (Figure 7E). We conclude that the region of Fus2p that binds Cdc42 extends beyond the DBH domain.

As a final test, we examined whether the *cdc42* mutations affected the interaction with Fus2p. The *cdc42*-V36M and *cdc42*-138 alleles were constructed in the plasmid expressing the GTP-locked Q61L mutation and the purified proteins tested for binding to Fus2p-FLAG (Figure 7F). Both proteins showed a significant decrease in binding relative to wild-type Cdc42p (*cdc42*-V36M and *cdc42*-138 exhibited 7 and 4% binding, respectively). These results corroborate the

used in binding experiments with GST-Cdc42p-Q61L. The “no Fus2p” control used yeast extracts in which Fus2p was not epitope tagged. FLAG-tagged Fus3-KD (kinase dead) was used as an unrelated protein control. Top, relative levels of bound GST-Cdc42p-Q61L proteins are indicated. Middle, input GST-Cdc42p-Q61L. Bottom, the levels of the input FLAG-tagged Fus2p deletion proteins. Proteins bound to the anti-FLAG Sepharose were eluted and detected by Western blotting with anti-FLAG antibody. The deletion proteins are indicated by a red dot and the Fus3-KD protein with a blue dot, and the heavy and light immunoglobulin chains are indicated (H and L, respectively). The levels of the proteins relative to full-length Fus2p are indicated at the bottom. (D) Nucleotide specificity of binding to the DBH domain. The indicated Cdc42p proteins were incubated with Fus2p¹⁰⁵⁻⁴¹⁵. Top, bound Cdc42p proteins; relative levels of binding are indicated. Bottom, input Fus2p¹⁰⁵⁻⁴¹⁵. In this experiment some degradation of the Fus2p deletion fragment was observed. In the rightmost three lanes, Cdc42p-Q61L was used. (E) Nucleotide specificity of binding to 415–677. As in D, except that the Cdc42p proteins were incubated with Fus2p⁴¹⁵⁻⁶⁷⁷. Top, bound Cdc42p proteins; relative levels of binding are indicated. Bottom, input Fus2p⁴¹⁵⁻⁶⁷⁷. (F) Cdc42p mutations diminished Fus2p binding. The two mutations affecting cell fusion were introduced into the Cdc42p-Q61L construct and used for binding to wild-type Fus2p-FLAG.

genetic data and suggest that *cdc42-138*, as well as *cdc42-V36M*, may interfere with cell fusion by disrupting the interaction between Cdc42^{GTP}p and Fus2p.

DISCUSSION

Several lines of evidence previously suggested an interaction between Cdc42p and Fus2p. First, they interact by two-hybrid assay (Nelson *et al.*, 2004). Second, *BEM1*, encoding a scaffold for Cdc42p and its effectors, is a high-copy suppressor of *fus2* (Fitch *et al.*, 2004). Third, high-copy *FUS2* suppresses the mating defects of *bem1* mutations (Leberer *et al.*, 1996). Finally, Fus2p contains a Dbl-homology domain (Paterson *et al.*, 2008), which is shared with activators of Rho-GTPases. However, Cdc42p has numerous functions and interaction partners and so could potentially have multiple direct and indirect roles during mating.

To implicate *CDC42* specifically in cell fusion, we sought mutant alleles defective in cell fusion but not other steps of mating. One mutation, *cdc42-138*, caused a strong defect in cell fusion but not in mitotic growth, which was not due to impaired pheromone signaling or cell polarization. The mutant phenotype closely resembled *fus2* mutations and analysis of double mutants showed that *cdc42-138* and *fus2* mutations conferred defects in the same step in cell fusion.

The Dbl-homology domain in Fus2p is required for as much as 90% of Fus2p's function in cell fusion. The presence of residual function suggests that Fus2p may have other roles in cell fusion or more than one interaction with Cdc42p. Indeed, we found that Cdc42p interacts with the Dbl-homology domain and additional sequences in Fus2p.

Although Fus2p contains a Dbl-homology domain found in other Cdc42p GEFs, we did not detect GEF activity (unpublished data). Instead, we found that Fus2p interacted more strongly with Cdc42-Q61L, the GTP-bound form of Cdc42p, *in vitro*, consistent with the two-hybrid data (Nelson *et al.*, 2004). Because GEFs are expected to bind more strongly to the GDP-bound G-protein, these findings suggest that Fus2p is not a GEF but may instead serve either as a mating-specific effector for Cdc42p or to localize the activated Cdc42p to the cell fusion zone. It is notable that another putative GEF, Lte1p, requires its GEF domain for cortical localization and not for activation of Tem1p (Geymonat *et al.*, 2009).

If Fus2p is an effector, what might be the role of the Fus2p–Cdc42p interaction in promoting cell fusion? Fus2p binds the BAR domain-containing protein Rvs161p, and the interaction is essential for cell fusion (Brizzio *et al.*, 1998). BAR-domain proteins preferentially bind to curved membranes (Peter *et al.*, 2004), and Rvs161p, together with Rvs167p, binds to membranes and promotes curvature *in vitro* (Friesen *et al.*, 2006; Youn *et al.*, 2009). Mutations in Rvs161p that inhibit membrane binding also inhibit mating (Youn *et al.*, 2009), suggesting that Fus2p/Rvs161p's role in mating requires membrane interaction.

Fus2p localizes near clustered vesicles, and both Fus2p and the vesicles are partly delocalized in *fus1* mutants (Gammie *et al.*, 1998; Paterson *et al.*, 2008). Deletion of neither Fus2p's Dbl-homology domain nor *cdc42-138* affected Fus2p localization. Moreover, vesicles clustered normally in *cdc42-138*. These data suggest that the Cdc42p interaction might regulate an activity associated with Fus2p/Rvs161p rather than its localization. Recent work showed that membrane bending by the vesicle protein synaptotagmin is required for vesicle fusion (Hui *et al.*, 2009). Perhaps Cdc42p regulates the degree of curvature induced by Fus2p/Rvs161p, deforming vesicle and/or target membranes in aid of membrane fusion. Alternatively, Fus2p/Rvs161p might interact with the curved outer surface of the vesicle fusion pore, stabilizing it to promote fusion. In either case,

Fus2p/Rvs161p may play a role in facilitating the exocytosis of secreted proteins required for cell wall dissolution.

A role for the Rho-insert domain

The Rho-insert domain is a ~20-residue loop containing a 13-residue α -helix unique to the Rho subfamily of small G proteins (Feltham *et al.*, 1997). The residues of Cdc42p required for cell fusion (D121 and D122) are within four charged amino acids at one end of the Rho-insert domain. Previous work in yeast was not informative as to possible function; individual mutations to alanine did not affect growth; mutation of all four was lethal (Kozminski *et al.*, 2000). A role in cell fusion represents the first specific function identified for this region of the protein in yeast.

Although little is known about Cdc42p's Rho-insert domain in yeast, hints may be gleaned from human Cdc42 and Rac1, where it is involved in the interactions with a variety of proteins, including Rho GDP dissociation inhibitors (Wu *et al.*, 1997; Richman *et al.*, 2004), formins (Richman *et al.*, 2004; Lammers *et al.*, 2008), and IQGAPs (McCallum *et al.*, 1996; Li *et al.*, 1999; Owen *et al.*, 2008). It is possible that in yeast other protein interactions may be affected in addition to Fus2p. Although the yeast formin Bni1p is required for cell fusion, it also is required for cell polarization (Evangelista *et al.*, 1997; Matheos *et al.*, 2004; Paterson *et al.*, 2008), likely acting prior to the function identified by *cdc42-138*. Of interest, the yeast IQGAP, Iqg1p, regulates exocytosis by localizing Sec3p to polarized growth sites (Osman *et al.*, 2002), and Sec3p interacts with activated Cdc42p (Zhang *et al.*, 2001, 2008). Continuous secretion has been shown to be required for the late stages of cell fusion (Grote, 2010). However, a specific requirement for either Iqg1p or Sec3p in cell fusion has not been tested.

Cdc42 plays roles at multiple stages of mating and cell fusion

Analyzing the many roles of GTPases has required extensive use of point mutations to specifically interfere with individual functions (Mosch *et al.*, 1999; Kozminski *et al.*, 2000; Adamo *et al.*, 2001; Saka *et al.*, 2001; Barale *et al.*, 2006; Heinrich *et al.*, 2007). For example, this approach demonstrated that *cdc42-V36M* affects cell fusion but not mitotic growth (Barale *et al.*, 2006). We found that *cdc42-V36M* did not polarize in response to pheromone and showed unstable localization of Fus2p-GFP (Figure 3), closely resembling *spa2* and *bni1*. Fus1p-GFP also failed to localize in *cdc42-V36M* (Barale *et al.*, 2006). We conclude that *cdc42-V36M* is primarily defective for polarization and may affect cell fusion indirectly, possibly through Bni1p. The *cdc42-V36M* mutant may also have a specific defect in cell fusion, which is masked by the earlier polarization defect.

In contrast, *cdc42-138* mutant cells polarized normally and were specifically defective in cell fusion. Epistasis analysis showed that Spa2p acts upstream of Fus1p, which in turn acts upstream of Fus2p (Gammie *et al.*, 1998). The morphology of *cdc42-138* most closely resembled *fus2*, arguing that this allele disrupts a function downstream of Fus1p and Spa2p. In confirmation, double-mutant analysis showed that *fus2* and *cdc42-138* act at the same step.

We conclude that Cdc42p plays multiple roles during cell fusion. Roles in pheromone signaling, via Ste20p (Simon *et al.*, 1995; Zhao *et al.*, 1995), and polarization, via Bni1p (Evangelista *et al.*, 1997), are well established. We propose a separate function in cell fusion mediated by the Rho-insert domain in association with Fus2p/Rvs161p.

A conserved role of Cdc42 homologues in eukaryotic cell fusion?

Among metazoan cell fusion events, *Drosophila* myoblast fusion may be the best understood (Chen and Olson, 2004). Ultrastructural

Strain	Genotype	Reference
JY428	α <i>fus2</i> Δ 3 <i>his4-34</i> <i>trp1</i> Δ 1 <i>ura3-52</i>	G. Fink (Whitehead Institute, Cambridge, MA)
JY429	α <i>fus1</i> Δ 1 <i>fus2</i> Δ 3 <i>trp1</i> Δ 1 <i>ura3-52</i>	G. Fink
JY431	α <i>his4-34</i> <i>trp1</i> Δ 1 <i>ura3-52</i>	G. Fink
DDY1300 ^a	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>CDC42::LEU2</i>	Kozminski et al. (2000)
DDY1301	α <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>CDC42::LEU2</i>	Kozminski et al. (2000)
DDY1354	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>cdc42-138::LEU2</i>	Kozminski et al. (2000)
DDY1355	α <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>cdc42-138::LEU2</i>	Kozminski et al. (2000)
MY9181	<i>a</i> <i>ura3</i> Δ 0 <i>leu2</i> Δ 0 <i>his3</i> Δ 1 <i>met15</i> Δ 0 <i>fus2</i> Δ :: <i>HIS3</i>	Paterson et al. (2008)
MY10016	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>CDC42::LEU2</i> <i>fus2</i> Δ :: <i>HIS3</i>	This study
MY10303	α <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>ade2-1</i> <i>fus2</i> Δ :: <i>HIS3</i>	This study
MY10310	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>cdc42-138::LEU2</i> <i>fus2</i> Δ :: <i>HIS3</i>	This study
MY10547	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>cdc42-V36M::LEU2</i>	This study
MY10554	α <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>ade2-1</i> <i>cdc42-V36M::LEU2</i>	This study
MY10797	α <i>fus2</i> Δ 3 <i>his4-34</i> <i>trp1</i> Δ 1 <i>ura3-52</i> <i>SSO1::P^{GPD1}-GFP::URA3</i>	This study
MY10798	α <i>fus1</i> Δ 1 <i>fus2</i> Δ 3 <i>trp1</i> Δ 1 <i>ura3-52</i> <i>SSO1::P^{GPD1}-GFP::URA3</i>	This study
MY10925	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>CDC42::LEU2</i> <i>STE20</i> Δ <i>CRIB</i>	This study
MY10926	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>cdc42-138::LEU2</i> <i>STE20</i> Δ <i>CRIB</i>	This study
MY10933	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>lys2-801</i> <i>CDC42::LEU2</i> <i>fus2</i> ^{<i>Δ</i>dbl} :: <i>GFP</i>	This study
MY12402	<i>a</i> <i>ura3-52</i> <i>leu2-3112</i> <i>his3</i> Δ 200 <i>pep4::HIS3</i> <i>bar1::kanMX</i> <i>fus2::natMX</i>	This study

^aStrains containing *cdc42-105*, *cdc42-107*, and *cdc42-137* are isogenic to DDY1300 (or DDY1301). See Kozminski et al. (2000).

TABLE 1: Strains used this study.

studies revealed clustered, electron-dense vesicles across the cell fusion zone (Doberstein et al., 1997), similar to yeast cell fusion and vertebrate myoblast fusion (Lipton and Konigsberg, 1972). Recent work showed that, as in yeast, fusion occurs at a single site, coincident with accumulation of actin (Sens et al., 2010). Cdc42p homologues Drac1 and Drac2 are also required for myoblast fusion (Hakeda-Suzuki et al., 2002) and most likely activated by an unconventional GEF (Erickson et al., 1997; Brugnera et al., 2002). Moreover, Cdc42p is required for mouse myoblast fusion (Vasyutina et al., 2009). The conserved requirement for Cdc42p-related proteins in cell fusion suggests that yeast cell fusion will yield important insights into the mechanism in higher cells.

MATERIALS AND METHODS

General yeast methods, strains, and plasmid construction

Yeast media, general methods, and transformations were performed as described previously (Adams et al., 1997) with minor modifications. Strains and plasmids are listed in Tables 1 and 2, respectively.

To create MY10016, the *fus2* Δ ::*HIS3* deletion was amplified from MY9181 and transformed into DDY1300. Correct integration was confirmed by PCR across the *FUS2* locus. MY10547 and MY10554 were created as described (Kozminski et al., 2000). To create MY10925 and MY10926, pPP1043 (Lamson et al., 2002) was cut with *Bam*HI and integrated in DDY1300 and DDY1354, respectively, by the loop-in/loop-out procedure. Correct loop-outs were confirmed by PCR across the *STE20* locus. To create MY10797 and MY10798, pEG746 (Jin et al., 2004) was cut with *Bgl*II and transformed into JY428 and JY429, respectively. The integration was confirmed by examining the resulting strains for GFP fluorescence.

pMR5883 was created by a multistep cloning procedure in the vector pRS416 (Sikorski and Hieter, 1989). A similar procedure was

used to create pMR5917, which is equivalent but in the integration vector pRS406. pMR5917 was cut with *Hind*III and *fus2*^{*Δ*105-414}::*GFP* integrated in DDY1300 by the loop-in/loop-out procedure. A loop-out containing GFP fluorescence was denoted MY10933.

To create pMR6115 and pMR6116, mutations were introduced into pB2082 by site-directed PCR mutagenesis (Phusion; Thermo Fisher Scientific, Waltham, MA). Deletion mutants in *FUS2* were generated by site-directed PCR mutagenesis using pMR5480 (Paterson et al., 2008) as a template. Deletions were confirmed by sequencing, and pheromone-induced protein expression was confirmed by trichloroacetic acid (TCA) precipitation–Western blot.

β -Galactosidase assays were performed essentially as described (Adams et al., 1997), using cell extracts prepared from $\sim 1.2 \times 10^8$ cells. Actin staining was performed as described with minor modifications (Adams et al., 1997), using cells treated with 6 μ M α -factor for 90 min.

Binding assays

GST-Cdc42p and derivatives were expressed and purified from *E. coli* as described (Gladfelter et al., 2002) with minor modifications. For expression of Fus2p-FLAG in yeast, MY9181, transformed with pMR5480 (or derivative), was grown to early exponential phase (OD₆₀₀ = 0.2) and treated with 10 μ g/ml synthetic mating pheromone (Syn/Seq Facility, Department of Molecular Biology, Princeton University, Princeton, NJ) for 2 h. Cell extracts were prepared as in Brizzio et al. (1998). The cleared lysate was divided into three fractions, and each fraction was incubated with 5 μ g of bacterially expressed GST-Cdc42 or derivative as indicated. Binding proceeded for 1 h at 4°C, with gentle rotation. After incubation, 30 μ l of equilibrated anti-FLAG Sepharose (Sigma-Aldrich, St. Louis, MO) was added to each reaction and the incubation continued at 4°C for 1 h. The anti-FLAG Sepharose was washed five times and finally

Plasmid	Markers	Reference
pSB231	CEN URA3 <i>fus1-LacZ</i>	Trueheart et al. (1987)
pMR5469	CEN URA3 <i>P^{GAL1}-FUS2::GFP₁₀₄</i>	Paterson et al. (2008)
pMR5482	CEN URA3 <i>FUS2::GFP₁₀₄</i>	Paterson et al. (2008)
pMR5883	CEN URA3 <i>P^{GAL1}-fus2^{Δ105-414}::GFP</i>	This study
pMR5480	<i>FUS2::FLAG₁₀₄ URA3 CEN</i>	Paterson et al. 2008
pMR6404	<i>FUS2¹⁻⁵⁸⁰::FLAG₁₀₄ URA3 CEN</i>	This study
pMR6405	<i>FUS2¹⁰⁵⁻⁶⁷⁷::FLAG₁₀₄ URA3 CEN</i>	This study
pMR6406	<i>FUS2¹⁰⁵⁻⁵⁸⁰::FLAG₁₀₄ URA3 CEN</i>	This study
pMR6407	<i>FUS2¹⁻¹⁰⁴-FLAG₁₀₄-FUS2⁴¹⁵⁻⁶⁷⁷ URA3 CEN</i>	This study
pMR6408	<i>FUS2⁴¹⁵⁻⁶⁷⁷::FLAG₁₀₄ URA3 CEN</i>	This study
pMR6409	<i>FUS2¹⁰⁵⁻⁴¹⁵::FLAG₁₀₄ URA3 CEN</i>	This study
pMR6115	<i>GST-CDC42^{V36M, Q61L}</i>	This study
pMR6116	<i>GST-CDC42^{Q61L D122A}</i>	This study
pB2082	<i>GST-CDC42^{Q61L}</i>	Gladfelter et al. (2002)
pB2083	<i>GST-CDC42^{T17N}</i>	Gladfelter et al. (2002)
pB2084	<i>GST-CDC42</i>	Gladfelter et al. (2002)

TABLE 2: Plasmids used in this study.

resuspended in SDS loading buffer. Experiments using the Fus2p deletion mutants were performed similarly, except that constructs were transformed into MY12402, which harbored deletions of the *fus2*, *pep4*, and *bar1* genes.

Mating and cell fusion assays

Plate mating assays were performed as described (Gammie and Rose, 2002) by replica plating strains together on yeast extract/peptone/dextrose (YEPD) and selecting diploids by replica plating to minimal media or drop-in media. Microscopic examination of zygotes and cytoplasmic transfer assays were performed as described (Grote, 2008), using $\sim 1.0 \times 10^6$ cells of each mating type. Zygotes were processed for electron microscopy as described (Gammie et al., 1998), using $\sim 1.2 \times 10^8$ cells of each mating type, separated over five filters and mated on YEPD for 2 h.

Cell imaging

Images were acquired using the DeltaVision Microscopy System (Applied Precision, Issaquah, WA) using a TE200 inverted microscope (Nikon, Melville, NY) and a Coolsnap HQ CCD camera (Photometrics, Tucson, AZ) and a 100 \times objective with a numerical aperture of 1.4. Fus2p-GFP images were acquired as previously described (Ydenberg and Rose, 2009). Figures were prepared for publication using Adobe Photoshop and Adobe Illustrator (Adobe, San Jose, CA). For presentation purposes, pixel density was increased using bicubic resampling, where necessary.

Western blotting

For Western analysis of Fus2p-GFP, protein was prepared from $\sim 6.0 \times 10^6$ exponential phase cells by TCA precipitation, separated

by SDS-PAGE, and transferred to a nitrocellulose filter. GFP-tagged Fus2p was detected using mouse anti-GFP (Roche, Indianapolis, IN) at 1:1000. FLAG-tagged Fus2p was detected with anti-FLAG (Sigma-Aldrich) at 1:5000. Rabbit anti-Kar2 (our laboratory) was used at 1:50,000. Glutathione S-transferase (GST)-tagged Cdc42p was detected with anti-GST (Cell Signaling Technologies, Beverly, MA) at 1:1000.

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