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.

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 invertebrates, 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 (Brizio 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 pseudogynes (Trueheart et al., 1987; Trueheart and Fink, 1989; Brizio 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 prn1 mutant zygotes has not been reported.

Fus2p and Rvs161p form a complex that is transported to the tips of fusing cells (Brizio et al., 1998; Paterson et al., 2008; Sheltzer et al., 2008).
Cdc42 and Fus2p in yeast cell fusion

We then counted the fully unfused zygotes (in which the septum stained with FM4-64 to visualize the plasma membrane (Figure 1D). To assess the importance of the Dbl-homology domain in vivo, Fus2p-ΔDbl-GFP was expressed at similar levels as the wild-type Fus2p-GFP (Figure 1C). To examine whether Fus2p interacts with Cdc42p, we performed 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; Kozmik 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. The Fus2p Dbl-homology domain is critically important but not absolutely required for Fus2p's function. The interaction between activated Cdc42p and Fus2p suggested that Fus2p might be an effector for Cdc42p signaling, and that Cdc42p may play a direct, specific role in cell fusion. 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 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 fus2ΔDbl 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 fus2ΔDbl 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

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).

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.

FIGURE 1: The Dbl-homology domain is required for cell fusion. (A) Fus2p-ΔDbl-GFP localizes to the shmoo tip. Cells (MY19181) 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 (DDY1300×MY10797), partial Fus2p zygote (MY10933×MY10797), and full Fus2p zygote (MY10933×MY10797). (E) Percentage of zygotes of each morphology produced in matings with the indicated genotype. Gray bars, partial Fus2p zygotes; black bars, full Fus2p zygotes. n ≥ 133 zygotes imaged in two independent experiments.
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-ACR18 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 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.

C. A. Ydenberg et al.

Molecular Biology of the Cell
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 ≥ 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 α-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 α-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 × α cdc42-138 produced 32% fusion-defective zygotes, compared with 41% for a cdc42-138 × 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).

**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

---

**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 ≥ 176 zygotes imaged 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 cytokinetic 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 polarizing mutants such as bni1 and spa2 (Paterson et al., 2008). Wild-type and cdc42-138 shmoo 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 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 zy- 
gotes (Figure 5C; 31% had plaques and 5% had invaginations, n = 48). These pheno-
types were previously shown to be unique to fus2 and rvs161 mutants and were not ob-
erved 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 ob-
erved 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 to as the zygote waistlines). In wild-type matings, 
unfused prezygotes have an average waistline of 1.0 ± 0.3 μm, which expands to 1.6 ± 0.2 μm soon after nuclear 
fusion (Gammie et al., 1998). When only unfused prezygotes were consid-
ered, cdc42-138 bilateral zygotes had waistlines measuring 1.4 ± 0.3 μm (n = 48). It is surprising that this was significantly nar-
rower than for fus2 prezygotes (1.7 ± 0.5 μm, 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 ± 0.4 μm, n = 91, vs. 1.7 ± 0.5 μm, n = 20, respectively; 
p = 0.22). Most important, the data provide additional evidence that 
the cdc42-138 mutant is morphologically dissimilar from polariza-
tion 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 more 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 interaction**

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 fu-
sion (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 muta-
tions are significantly more severe (Brizzio et al., 1996). For this ex-
periment, we used a cytoplasmic transfer assay (Figure 6A). In this 
assay, only full Fus2p 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 sig-
nificantly 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 path-
ways, then we predict that the defect of the double mutant should be 
multiplicative, normalized for the frequency observed for wild type 
(i.e., 0.22 x 0.46)/0.92. Transfer to the cdc42-138 fus2 double 
mutant was somewhat more defective than to fus2 alone (42%, n = 554, 
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 pro-
teins 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).

Next we 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 ex-
pressed 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 a effector or to play a role in localizing activated 
Cdc42p to the fusion site.

To identify the region required for binding to Cdc42p, various de-
letions of Fus2p were constructed and used in the coimmunoprecipita-
tion assay, using Cdc42p-Q61L (Figure 7, B and C). The most effi-
cient 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
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 Fus2p105-415. Top, bound Cdc42p proteins; relative levels of binding are indicated. Bottom, input Fus2p105-415. (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.

**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 Cdc42p proteins. Bottom, input Cdc42p 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 DBI-homology domain is indicated (DBI 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 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 Cdc42p proteins. Bottom, input 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 Fus2p105-415. Top, bound Cdc42p proteins; relative levels of binding are indicated. Bottom, input Fus2p105-415. (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} 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 mutants (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), formsins (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 Fusion. 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, Lqg1p, 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 lqg1p 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 polarization in response to pheromone and showed unstable localization of Fus2p-GFP (Figure 3), closely resembling spa2 and bin1. 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...
TABLE 1: Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JY428</td>
<td>α fus2Δ3 his4-34 trp1Δ1 ura3-52</td>
<td>G. Fink (Whitehead Institute, Cambridge, MA)</td>
</tr>
<tr>
<td>JY429</td>
<td>α fus1Δ1 fus2Δ3 trp1Δ1 ura3-52</td>
<td>G. Fink</td>
</tr>
<tr>
<td>JY431</td>
<td>α his4-34 trp1Δ1 ura3-52</td>
<td>G. Fink</td>
</tr>
<tr>
<td>DDY1300</td>
<td>a ura3-52 leu2-3112 hisΔ200 lys2-801 CDC42::LEU2</td>
<td>Kozminski et al. (2000)</td>
</tr>
<tr>
<td>DDY1301</td>
<td>α ura3-52 leu2-3112 hisΔ200 lys2-801 CDC42::LEU2</td>
<td>Kozminski et al. (2000)</td>
</tr>
<tr>
<td>DDY1354</td>
<td>a ura3-52 leu2-3112 hisΔ200 lys2-801 cdc42-138::LEU2</td>
<td>Kozminski et al. (2000)</td>
</tr>
<tr>
<td>DDY1355</td>
<td>α ura3-52 leu2-3112 hisΔ200 lys2-801 cdc42-138::LEU2</td>
<td>Kozminski et al. (2000)</td>
</tr>
<tr>
<td>MY9181</td>
<td>a ura3Δ0 leu2Δ0 hisΔ1 met15Δ0 fus2Δ::HIS3</td>
<td>Paterson et al. (2008)</td>
</tr>
<tr>
<td>MY10016</td>
<td>a ura3-52 leu2-3112 hisΔ200 lys2-801 CDC42::LEU2 fus2Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>MY10303</td>
<td>α ura3-52 leu2-3112 hisΔ200 ade2-1 fus2Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>MY10310</td>
<td>a ura3-52 leu2-3112 hisΔ200 lys2-801 cdc42-138::LEU2 fus2Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>MY10544</td>
<td>α ura3-52 leu2-3112 hisΔ200 lys2-801 cdc42-V36M::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>MY10554</td>
<td>α ura3-52 leu2-3112 hisΔ200 ade2-1 cdc42-V36M::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>MY10797</td>
<td>α fus2Δ3 his4-34 trp1Δ1 ura3-52 SSO1::P&lt;sub&gt;GPD1&lt;/sub&gt;-GFP::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>MY10798</td>
<td>α fus1Δ1 fus2Δ3 trp1Δ1 ura3-52 SSO1::P&lt;sub&gt;GPD1&lt;/sub&gt;-GFP::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>MY10925</td>
<td>a ura3-52 leu2-3112 hisΔ200 lys2-801 CDC42::LEU2 STE20ΔCIB</td>
<td>This study</td>
</tr>
<tr>
<td>MY10926</td>
<td>a ura3-52 leu2-3112 hisΔ200 lys2-801 cdc42-138::LEU2 STE20ΔCIB</td>
<td>This study</td>
</tr>
<tr>
<td>MY10933</td>
<td>a ura3-52 leu2-3112 hisΔ200 lys2-801 CDC42::LEU2 fus2&lt;sup&gt;Δpol&lt;/sup&gt;::GFP</td>
<td>This study</td>
</tr>
<tr>
<td>MY12402</td>
<td>a ura3-52 leu2-3112 hisΔ200 pep4::HIS3 bar1::kanMX fus2::natMX</td>
<td>This study</td>
</tr>
</tbody>
</table>

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

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 fus2Δ::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, pPPl0143 (Lamson et al., 2002) was cut with BamHI and integrated in DY1300 and DY1354, 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 BglII 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 HindIII and fus<sup>Δpol</sup>::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; Thermofisher 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 ~1.2 × 10<sup>7</sup> cells. Actin staining was performed as described with minor modifications (Adams et al., 1997), using cells treated with 6 μM α-factor for 90 min.

**Binding assays**

GST-Cdc42p and derivatives were expressed and purified from *E. coli* as described (Glafelter 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 (OD<sub>600</sub> = 0.2) and treated with 10 μg/ml synthetic mating pheromone (Syn/Seq Facility, Department of Molecular Biology, Princeton University, Princeton, NJ) for 2 h. Cell extracts were prepared as in Brizio et al. (1998). The cleared lysate was divided into three fractions, and each fraction was incubated with 5 μg of bacterially expressed GST-Cdc42 or derivative as indicated. Binding proceeded for 1 h at 4°C, with gentle rotation. After incubation, 30 μl 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
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 (YPED) 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 

\[ \frac{1}{6} \text{YPD} \times \text{CAA}\). For presentation purposes, pixel density was increased using Adobe Photoshop and Adobe Illustrator (Adobe, San Jose, CA). For presentation purposes, pixel density was increased using Adobe Photoshop and Adobe Illustrator (Adobe, San Jose, CA).

### Cell imaging

Images were acquired using the DeltaVision Microscopy System (Apllied Precision, Issaquah, WA) using a TE200 inverted microscope (Nikon, Melville, NY) and a Coolscope HQ CCD camera (Photometrics, Tucson, AZ) and a 100× 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 yeast 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.

### Acknowledgments

We thank Danny Lew, Keith Kozminski, Peter Pryciak, Eric Grote, and Robert Arkowitz for providing strains and reagents and Satoshi Yoshida and Bruce Goode for critical comments on the manuscript. This work was supported by National Institute of General Medical Sciences Grant GM37739 to M.D.R.

### References


