Cdc24 Regulates Nuclear Shuttling and Recruitment of the Ste5 Scaffold to a Heterotrimeric G Protein in Saccharomyces cerevisiae*

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The Saccharomyces cerevisiae guanine nucleotide exchange factor Cdc24 regulates polarized growth by binding to Cdc42, a Rho-type GTPase that has many effectors, including Ste20 kinase, which activates multiple MAPK cascades. Here, we show that Cdc24 promotes MAPK signaling during mating through interactions with Ste5, a scaffold that must shuttle through the nucleus and bind to the β subunit (Ste4) of a G protein for Ste20 to activate the tethered MAPK cascade. Ste5 was basally recruited to growth sites of G1 phase cells independently of Ste4. Loss of Cdc24 inhibited nuclear import and blocked basal and pheromone-induced recruitment of Ste5. Ste5 was not basally recruited and the MAPK Fus3 was not basally activated in the presence of a Cdc24 mutant (G168D) that still activates Cdc42, suggesting that Cdc24 regulates Ste5 and the associated MAPK cascade through a function that is not dependent on its guanine nucleotide exchange factor activity. Consistent with this, Cdc24 bound Ste5 and coprecipitated with Ste4 independently of Far1 and Ste5. Loss of Cdc24 decreased Ste5-Ste4 complex formation, and loss of Ste4 stimulated Cdc24-Ste5 complex formation. Collectively, these findings suggest that Cdc24 mediates site-specific localization of Ste5 to a heterotrimeric G protein and may therefore ensure localized activation of the associated MAPK cascade.

Cell polarity underlies differentiation, proliferation, and morphogenesis in single cell and multicellular organisms and occurs through localized activation of Rho-type GTPases that are linked to many different effectors (1–3). A major question is how Rho effectors are selectively activated and coupled to appropriate signal transduction pathways in response to specific stimuli. One level of specificity is provided by physical linkages between the guanine nucleotide exchange factors (GEFs)1 and the Rho-type GTPases to upstream receptors that respond to specific stimuli. These linkages can be provided by direct binding interactions between a receptor and a GEF (e.g. Refs. 4–7) or by indirect linkages through scaffold or adapter proteins (e.g. Refs. 8–10). Currently, it is not understood how these interactions are coupled to downstream signal transduction cascades that may localize in the cytoplasm rather than at discrete sites on the plasma membrane.

Analysis in Saccharomyces cerevisiae has shed much light on how cell polarity is regulated. S. cerevisiae undergoes polarized growth when it forms a bud in the late G1 phase of the cell cycle and when it forms a projection (termed shmoo) in response to mating pheromone as a precursor to contact and fusion with a cell of the opposite mating type (2, 11). Both polarization events are mediated by the Cdc42 GTPase (12). Cdc42 activity is controlled by a single GEF, Cdc24 (13–15), and by three GTPase-activating proteins, Bem3, Rga1, and Rga3 (16, 17). Site-specific localization of Cdc24 and Cdc42 at the cell cortex underlies the initiation of cell polarity during budding and mating. During budding, this is mediated by parallel internal inputs by the Rsr1/Bud1 GTPase and the Bem1 scaffold, which associate with the plasma membrane and bind Cdc24 and Cdc42 (18–24). The initial asymmetric localization of Cdc42 may be mediated by Bem1 (25) through its Phox homology domain (26) and is independent of the actin cytoskeleton (27). Cdc24 and Bem1 also play essential roles in mediating polarized growth during mating (28, 29). The localized activation of Cdc42 by Cdc24 during mating is mediated in part by Far1, which orients the axis of polarized growth in a pheromone gradient, but is not essential for polarized growth (30, 31). Far1 associates with Cdc24-Bem1 complexes and Ste4, the β subunit of the activated G protein βγ dimer that is activated by a pheromone receptor, and is able to activate Cdc42 at the plasma membrane (8, 9, 32). Cdc24 shuttles continuously through the nucleus and is anchored in late M and G1 phase nuclei by Far1, which is exported to the cytoplasm together with Cdc24 upon activation of the mating MAPK cascade (33–35).

Cdc24 activation of Cdc42 also regulates multiple MAPK cascades through Ste20, a p21-activated kinase that activates Ste11, a MAPK kinase kinase that functions in MAPK cascades that regulate mating, invasive growth/sterile vegetative growth, and high osmolarity growth (36–40). The activation of the mating MAPK cascade is coordinated by the Ste5 scaffold, which binds the Ste4 subunit of the activated G protein βγ dimer together with Ste11, the MAPK kinase Ste7, and the MAPK Fus3 (41, 42). The binding of Ste5 to Ste4 is thought to facilitate phosphorylation of Ste11 by Cdc42-GTP-bound Ste20, which also interacts with Ste4. Although Ste5 is in the cytoplasm, it must shuttle through the nucleus to localize asymmetrically at the cell cortex and to activate Fus3 (43, 44). Interestingly, Ste4 and the Ste2 receptor are broadly distributed at the plasma membrane prior to pheromone stimulation (45, 46), whereas basal cortical recruitment of Ste5 occurs at
selective sites in G1 phase cells (44), suggesting that additional factors may regulate its stable recruitment. In addition, Ste5 rapidly accumulates at asymmetric cortical sites within an isotropic environment of mating pheromone (43), suggesting that it is an early marker of polarization that is sensitive to internal cues. Although Cdc24 was proposed to be an effector of Ste4 during mating (37), later work led to the conclusion that Cdc24 interacts with Ste4 indirectly through Far1 (8, 9). Here, we show that Cdc24 is a key regulator of Ste5 nucleocytoplasmic shuttling and recruitment and plays a direct role in the interaction between Ste5 and Ste4 that is independent of Far1.

MATERIALS AND METHODS

Yeast Strains and Media—See Tables I and II for lists of the strains and plasmids used in this study. Yeast strains were grown in standard selective (synthetic complete) medium. Cells with GAL1-driven genes were pre-grown in medium containing 2% raffinose before induction in medium containing 2% galactose. Yeast transformations were done as described (44). Quantitation of Ste5-GST and Cdc24 Regulates Ste5 Nuclear Shuttling/Recruitment to Ste4/G8

| Strains | Genotype/description | Source/Ref. |
|---------|----------------------|-------------|
| EY699   | MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 | R. Rothstein |
| EY957   | sst1Δ | 69 |
| EY1775  | sst1Δ ste5Δ::TRP1 | 52 |
| EYL1808 | sst1Δ ste5Δ::TRP1 ste43::LEU2 | 70 |
| EY2458  | sst1Δ bem1Δ::LEU2 | 44 |
| EY1262  | sst1Δ far1Δ | Elion laboratory |
| EY2019  | sst1Δ ste5Δ::TRP1 far1Δ | Elion laboratory |
| EY357   | msn5Δ::HIS3 | 43 |
| EY357   | far1Δ msn5Δ::HIS3 | This study |
| EY942   | msn5Δ::HIS3 cdc24-4 | This study |
| EY300   | leu2 ura3 his3 | F. Winston |
| EYL481  | cdc24-4 leu2 ura3 his3 | A. Bender |
| EY302   | cdc42-1 leu2 ura3 his3 | R. Li |
| EY1740  | leu2 ura3 his3 met15 | Research Genetics |
| EYL1764 | ste4Δ::KANm leu2 ura3 his3 Δ200 trp1-Δ901 lys2-801 gal2 suc2-Δ9 (pRS414 CDC24) | 9 |
| RAY1914 (EYL457) | cdc24Δ-1::LoxP HIS5 SpLoxP | 3 |
| RAY1916 (EYL458) | cdc24-m1 (pRS414 cdc24-m1) cdc24Δ-1::LoxP HIS5 SpLoxP | 9 |
| RAY1105 (EYL459) | cdc24-m2 (pRS414 cdc24-m2) cdc24Δ-1::LoxP HIS5 SpLoxP | 9 |

plasmids used in this study

| Plasmids/strain | Description | Source/Ref. |
|-----------------|-------------|-------------|
| pYM27           | ADH-TAgNLS-GFP-GFP-G2 µm URA3 | This study |
| pYMW50          | STE5-GST CEN URA3 | 44 |
| pYMW77          | STE5-GST 2 µm URA3 | 44 |
| pYMW96          | GAL1-HA-STE4 CEN LEU2 | 44 |
| pYMW146         | MBP-CDC24 | This study |
| pSKM19          | STE5-MYC6 CEN URA3 | 43 |
| pSKM21          | CUP1-STE5-GFP CEN URA3 | 43 |
| pYBS138         | STE5-STE5 CEN URA3 | 71 |
| pYEE102         | FUS3-HA, CEN HIS3 | 69 |
| pJB207          | FUS1-LacZ 2 µm LEU2 | 72 |
| EBL664          | 415MET(GFP)55::A6-CDC24 CEN LEU2 | 33 |

Expression of MBP-Cdc24 in Escherichia coli—BL21 cells harboring MBP-CDC24 (pYMW146) were induced with isopropyl β-D-thiogalactopyranoside for 3 h before harvesting. MBP-Cdc24 and MBP were detected by Coomassie Blue staining of polyacrylamide gels and confirmed by immunoblot analysis with anti-MBP antibody.

Ste5-Myc6 and MBP-Cdc24 Binding Experiments—Ste5-Myc6 and ste5a whole cell extracts were incubated with antibody 9E10, and the immunoprecipitates were then incubated in total lysates from BL21 cells harboring MBP-Cdc24 or MBP for 90 min on ice and washed five times with modified H buffer containing 0.25 M NaCl before being subjected to SDS-PAGE and immunoblot analysis. The membrane was probed with anti-MBP antibody first and then stripped and reprobed with antibody 9E10. Active Fus3 antibody was detected exactly as described (40). Immunoactivity in immunoblots was detected with horseradish peroxidase-conjugated secondary antibody using ECL (Amerham Biosciences).
Ste5 is not dependent on binding to the mating G protein and is regulated by other factors. In contrast, basal activation of the mating MAPK cascade is dependent on Ste4 and was blocked in the ste14 strain, even when Ste5-GST was overexpressed from a multicopy 2μ plasmid, as shown by negligible expression of a FUS1-lacZ reporter gene (Fig. 1C).

Temperature-sensitive Mutations in Cdc24 and Cdc42 Block Basal and a Factor-induced Recruitment of Ste5—We determined whether Bem1, Cdc24, and Cdc42 regulate basal recruitment of Ste5 by quantitating the level of Ste5-GST recruitment in a bem1Δ null strain and in temperature-sensitive cdc24-4 and cdc42-1 strains. Cortical localization of Ste5-GST still occurred in the bem1Δ mutant, but it was weaker and more diffusely spread out than in the control cells (Fig. 2, A and C). In contrast, temperature shift inactivation of either the Cdc42-1 or Cdc42-4 protein to 37 °C completely blocked Ste5-GST recruitment (Fig. 2, B and D). Cdc24 and Cdc42 were also required for mating pheromone-induced recruitment of Ste5-GST (Fig. 2D, +αF) and Ste5-Myc9 (Fig. 3, A and B) after a 15-min induction with a factor after the temperature shift. In contrast, Bem1 was not essential for a factor-induced recruitment, but was required for efficient recruitment (Figs. 2C and 3, A and B) and increased the level of recruitment of Ste5-Myc9 when overexpressed from a multicopy 2μ plasmid (Fig. 3B). Thus, Bem1 stimulates a factor-induced recruitment of Ste5, whereas Cdc24 and Cdc42 are essential for Ste5 recruitment to occur.

Basal Ste5 Recruitment and Fus3 Activation Are Blocked in the Presence of Cdc24(G168D)—The requirement for Cdc24 and Cdc42 in Ste5 recruitment was apparent at permissive temperature (room temperature) in addition to non-permissive temperature (37 °C). In particular, basal recruitment of Ste5-GST was completely blocked in both cdc24-4 and cdc42-1 strains, although some pheromone-induced recruitment of Ste5-GST and Ste5-Myc9 occurred (Figs. 2D and 3B). The level of Cdc24-1 protein is reduced compared with WT Cdc42 at permissive temperature (47), consistent with the decrease in Ste5 recruitment. In contrast, the Cdc24-4 protein, which harbors a G168D mutation in the calponin homology domain, is expressed at normal levels and retains the ability to activate Cdc42 at permissive temperature (48). This result suggests that Cdc24 regulates Ste5 recruitment through a function distinct from regulation of Cdc42.

To substantiate the findings with Ste5-GST, we analyzed the localization of a functional Ste5-GFP fusion protein in live cells expressed at WT levels of expression (43). In WT cells, Ste5-GFP was detected at cortical sites in some G2/M and G1 phase cells (Fig. 2E, left panel, arrows), consistent with the findings with Ste5-GST. Shifting the temperature to 37 °C for 3 h greatly reduced the strength of the GFP signal, preventing accurate assessment of an effect of the cdc24-4 and cdc42-1 mutations. However, the cdc24-4 and cdc42-1 mutations blocked basal recruitment of Ste5-GFP at room temperature (Fig. 2E), consistent with the findings for Ste5-GST.

Basal recruitment of Ste5 is required for basal activation of the mating MAPK cascade. To determine whether the defect in basal Ste5 recruitment correlates with a defect in MAPK activation, the level of basal activation of Fus3 was assessed. Whole cell extracts from WT, cdc24-4, and cdc42-1 strains grown at room temperature and shifted to 37 °C for 3 h were subjected to immunoblot analysis using anti-phospho-p42/p44 antibody, which recognizes the active form of Fus3 (40). Basal activation of Fus3 was greatly reduced at non-permissive temperature in both cdc24-4 and cdc42-1 strains (Fig. 2F), consistent with the observed block in Ste5 recruitment and the expected block in Ste20 recruitment. A block in Fus3 activation...
was also observed at room temperature in the cdc24-4 strain compared with a partial block in the cdc42-1 strain (Fig. 2F).

Consistent with this, overexpression of Ste5-GST did not induce morphological changes resembling shmoo formation in the cdc24-4 strain at room temperature, although it did induce them in the WT strain (Fig. 2G), as found previously (44). Collectively, these findings suggest that Cdc24 regulates Ste5 recruitment and MAPK activation through a function that is not dependent on activation of Cdc42.

Bem1, Cdc24, and Cdc42 Are Required for Nuclear Accumulation of Ste5—Because Ste5 must shuttle through the nucleus for efficient recruitment to the cell cortex, we determined whether Bem1, Cdc24, and Cdc42 also regulate nuclear shuttling of Ste5. Previous work has established that the nuclear pool of Ste5 is greatest in G1 phase cells (43). Nuclear accumulation of Ste5 was measured by determining the percentage of
FIG. 3. Nucleocytoplasmic shuttling and plasma membrane localization of Ste5 requires Bem1, Cdc24, and Cdc42. A, Ste5-Myc9 in WT, cdc24-4, cdc42-1, and bem1Δ cells. WT and bem1Δ cells were grown at 30 °C. The cdc24-4 and cdc42-1 strains and their respective WT controls were then shifted from room temperature (RT) to 37 °C for 3 h. B, quantitation of Ste5-Myc9 localization in cell polarity mutants. Cells were grown at the indicated temperatures and then treated with (+αF) or without (−αF) α factor for 15 min. C, immunoblot of Ste5-Myc9 in cell polarity mutants. D, localization of TAgNLSK128T-Ste5-Myc9 in cell polarity mutants. E, quantitation of TAgNLSK128T-Ste5-Myc9 localization in cell polarity mutants. Cells were grown as described for B. F, Cdc24 is required for nuclear accumulation of Ste5-Myc9 in an msn5Δ mutant. Shown is the indirect immunofluorescence of Ste5-Myc9 in cells shifted from room temperature to 37 °C for 3 h. The percentage of total cells with clearly greater intensity of staining of Ste5-Myc9 in the nucleus compared with the cytoplasm at 37 °C was 90% for msn5Δ, <1% for cdc24-4, and 20% for msn5Δ cdc24-4 (n = 100–150).
cells that accumulated an obvious nuclear pool of Ste5-Myc9 compared with the level of Ste5 in the cytoplasm (Fig. 3A, αF panels). Strikingly, nuclear accumulation of Ste5-Myc9 was greatly reduced in the bem1Δ strain and completely blocked in the cdc24-4 and cdc42-1 mutants at non-permissive temperature, in addition to being greatly reduced at permissive temperature (Fig. 3, A (shown is the non-permissive temperature of 37 °C) and B, % Nuclear accumulation (−α factor)). The cdc24-4 and cdc42-1 mutations did not reduce the steady-state level of Ste5-Myc9, whereas the bem1Δ mutation caused a minor decline (Fig. 3C), indicating that the defects in nuclear accumulation were not due to lower levels of Ste5-Myc9. A control protein harboring an SV40 large T antigen nuclear localization sequence (TAGNLS-GFP-GFP) was efficiently imported into all three mutants (data not shown), indicating that the decrease in nuclear accumulation was not a general one. Furthermore, a bni1Δ mutation in the formin that regulates the actin cytoskeleton did not reduce nuclear accumulation of Ste5-Myc9. Therefore, Bem1, Cdc24, and Cdc42 are specifically required for nuclear accumulation of Ste5.

The block in recruitment of Ste5-Myc9 in the bem1Δ, cdc24-4, and cdc42-1 mutants could have been a consequence of a primary defect in nuclear import. To test this possibility, we determined whether the addition of a partially functional SV40 TAGNLSK128T tag that has been shown to enhance both nuclear import and recruitment of Ste5 would (43) bypass the recruitment defects in the bem1Δ, cdc24-4, and cdc42-1 mutants. The TAGNLSK128T tag targeted Ste5-Myc9 to bem1Δ, cdc24-4, and cdc42-1 nuclei to 70–95% of WT levels at room temperature and 37 °C (Fig. 3, D and E, −αF). The shift to 37 °C somewhat increased nuclear accumulation of TAGNLSK128T-Ste5-Myc9 in the WT control, possibly from enhanced nuclear import of hsp70 (49). The recruitment of Ste5 was restored at room temperature and partially restored at non-permissive temperature in the cdc24-4 and cdc42-1 mutants (Fig. 3E). However, a clear defect in recruitment was still apparent at non-permissive temperature, with the residual recruitment in the cdc24-4 strain much weaker and not detectable in the image capturing system used (Fig. 3, D and E). These findings are consistent with reduced recruitment of Ste5 partially being the result of decreased nuclear import. The remaining defect in recruitment of TAGNLSK128T, Ste5-Myc9 in the cdc24-4 and cdc42-1 mutants at non-permissive temperature indicates that Cdc24 and Cdc42 regulate recruitment of Ste5 through a function distinct from nuclear import of Ste5.

The nuclear accumulation of a protein can decrease either as a result of reduced nuclear import or, less frequently, as a result of enhanced nuclear export (44). To determine whether the decreased nuclear accumulation of Ste5-Myc9 is the result of decreased nuclear import, we determined whether the cdc24-4 mutation would decrease the ability of a null mutation in the MSN5 exportin gene to restrict Ste5 to the nucleus. At the non-permissive temperature, ~90% of the msn5Δ cells exhibited nuclear accumulation of Ste5-Myc9, with the intensity of the nuclear pool clearly greater than that of the cytoplasmic pool (Fig. 3F, middle panel). In the msn5Δ cdc24-4 double mutant, all of the cells exhibited stronger staining of Ste5-Myc9 in the cytoplasm compared with the msn5Δ single mutant, with ~20% of the cells exhibiting significantly greater staining of Ste5-Myc9 in the nucleus compared with the cytoplasm (Fig. 3F, right panel). The decrease in nuclear accumulation in the msn5Δ cdc24-4 double mutant was dependent upon the temperature shift to 37 °C and was not apparent at room temperature (data not shown). By comparison, <1% of the cdc24-4 cells displayed any nuclear accumulation of Ste5-Myc9 whatsoever (Fig. 3F, left panel). These findings suggest that loss of Cdc24 function inhibits nuclear import of Ste5, but does not block it completely.

Ste5 Nuclear Accumulation Still Occurs in the Presence of Latrunculin A—It was possible that the inhibitory effects of the bem1Δ, cdc24-4, and cdc42-1 mutations on Ste5 nuclear accumulation are indirectly due to defects in the actin cytoskeleton. To test this possibility, Ste5-GFP nuclear accumulation and recruitment were monitored after cells were treated with latrunculin A, which disassembles the actin cytoskeleton within 2 min and has no other targets besides actin monomers in S. cerevisiae (27, 50, 51). This was done in the S288C strain background, which allows visualization of Ste5 in a greater number of nuclei compared with the W303-1a background (44). GFP-Cdc24 was used as a control because it is still recruited to the cortex of dividing cells after disruption of the actin cytoskeleton (27).

Disruption of the actin cytoskeleton with latrunculin A did not disrupt nuclear accumulation of Ste5-GFP in live cells after incubation with latrunculin A (Fig. 4, A and C), although it did disrupt the actin cytoskeleton as judged by phalloidin A staining (data not shown). The level of nuclear accumulation was somewhat reduced when Ste5-GFP was expressed for a longer period of time (Fig. 4, A and C). It was possible to detect cortical recruitment of Ste5-GFP in the presence of latrunculin A (Fig. 4A, right panel, arrow), suggesting that basal recruitment of Ste5 is not dependent on the actin cytoskeleton. GFP-Cdc24 was also still recruited to the nucleus and cell cortex in the presence of latrunculin A (Fig. 4, B and C). In contrast, a factor-induced recruitment of Ste5-GFP was inhibited by latrunculin A, consistent with the block in shmoo formation (Fig. 4D). Similar results were found with Ste5-Myc9 by indirect immunofluorescence (data not shown). These findings suggest that nuclear import of Ste5 is regulated by a mechanism that is largely, but not completely, independent of the actin cytoskeleton, whereas a factor-induced recruitment of Ste5 is dependent on the actin cytoskeleton.

Ste5 Associates with Cdc24 in Vivo and in Vitro—The strong effect of the cdc24-4 mutation on Ste5 localization and the interaction between Bem1 and Ste5 (52) raised the possibility that Cdc24 directly regulates Ste5. Cdc24 and Ste5 were tested for their ability to co-immunoprecipitate from S. cerevisiae whole cell extracts. Strikingly, Ste5-Myc9 could be detected in GFP-Cdc24 immunoprecipitates and GFP-Cdc24 could be detected in Ste5-Myc9 immunoprecipitates under conditions in which GFP-Cdc24 was only modestly overexpressed from the MET25 promoter (Fig. 5A). Furthermore, the ability to detect an interaction was dependent on the level of Ste5-Myc9 (Fig. 5A, compare lanes 1 and 2) and was not the result of nonspecific binding (compare lanes 1 and 3). In contrast, no association was detected between GFP-Cdc24 and Fus3-3xHA (Fig. 5B), which also localizes to the cell cortex in a factor-treated cells (53). Therefore, GFP-Cdc24 associates specifically with Ste5 in vivo. To determine whether the interaction between Cdc24 and Ste5 could be direct, we expressed MBP-Cdc24 in E. coli and assessed whether it could interact with Ste5-Myc9 purified from S. cerevisiae whole cell extracts. Ste5-Myc9 bound to MBP-Cdc24, but not to MBP (Fig. 5C), indicating that Ste5 and Cdc24 are likely to interact directly.

We determined whether the interaction between Ste5 and Cdc24 is restricted to a particular region of Ste5 by performing co-immunoprecipitation experiments on a variety of Ste5 mutants that span the protein (Fig. 5, D–F). GFP-Cdc24 did not associate with HA-Ste5-(24–239) (Fig. 5E), but could associate

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2 P. Maslo and E. A. Elion, unpublished data.
3 P. Maslo, Y. Wang, and E. A. Elion, unpublished data.
FIG. 4. Actin is not essential for Ste5 nuclear accumulation, but is essential for recruitment in the presence of α factor. A and B, localization of Ste5-GFP and GFP-Cdc24, respectively, in live cells treated with latrunculin A. Cells were incubated with 200 μM latrunculin A (Lat-A) for 30 min prior to a 2-h induction of Ste5-GFP from the CUP1 promoter. GFP-Cdc24 was expressed from the MET25 promoter prior to the addition of latrunculin A. C, quantitation of nuclear accumulation of Ste5-GFP and GFP-Cdc24 in the absence and presence of latrunculin A. D and E, latrunculin A blocks α factor-induced recruitment of Ste5-GFP. Cells were treated with or without 200 μM latrunculin A for 30 min and then incubated with or without α factor for 30 min.
with Ste5(C180A)-Myc9 (Fig. 5F), indicating that it does not bind the RING H2 domain. GFP-Cdc24 weakly associated with Ste5-(24–334)-Myc9 and readily associated with Ste5 constructs that span the C-terminal half of the protein (summarized in Fig. 5D). The Ste5Δ284–360 mutation (which removes a region of Ste5 with some homology to Far1) decreased association with GFP-Cdc24, suggesting that this region directly or indirectly influences association with Cdc24. In addition, the Ste5Δ474–638 mutation, which stimulates oligomerization of Ste5 through the RING H2 domain (44), increased association between Ste5 and Cdc24 (Fig. 5F). Thus, Cdc24 binds to a region overlapping the last 332 residues of Ste5 and may associate more readily with the Ste5 dimer than the monomer.

Ste5 Association with Cdc24 Is Not Dependent upon Bem1, Far1, or Msn5—It was possible that Cdc24 regulates Ste5 as part of the set of interactions that involve Far1: Cdc24 associates with Bem1 and Far1 (8, 9); Bem1 associates with Ste5 (52); and Far1 is exported by the Msn5 exportin (54), which also regulates Ste5 (43) and associates with Ste5 (Fig. 6A). This possibility was tested in co-immunoprecipitation experiments and by determining whether Cdc24 regulates Ste5 localization indirectly through Far1. Cdc24 associated with Ste5 with equal efficiency in the WT, far1Δ, msn5Δ, and bem1Δ strains (Fig. 6, B and C). In contrast, overexpression of Cdc24 decreased the level of Bem1-Ste5 complexes (Fig. 6D), whereas overexpression of Bem1 had no effect on the level of Cdc24-Ste5 complexes (data not shown), suggesting that Bem1 associates with Ste5 indirectly through Cdc24. No reproducible evidence of association could be detected between Far1 and Ste5 in multiple co-immunoprecipitation experiments (data not shown). Ste5
Ste5 and Cdc24 complexes do not involve Far1. A, Ste5 associates with Man5. WCE, whole cell extract. B, Ste5 and Cdc24 associate independently of Bem1. C, Ste5 and Cdc24 associate independently of Far1 and Man5. D, overexpression of Cdc24 inhibits association between Bem1-HA and Ste5-Myc9. WB, Western blot. E, nuclear accumulation of Ste5 is not dependent on Far1 binding to Cdc24. Note that Ste5 accumulated in nuclei to varying degrees in the different WT strain backgrounds. F, GFP-Cdc24 accumulates in ste5Δ nuclei in live cells. Collages are shown of WT, ste5Δ, far1Δ, and far1Δ ste5Δ cells expressing GFP-Cdc24. G, overexpression of Ste5 derivatives that shuttle through the nucleus or that are targeted to the nucleus increase the nuclear pool of GFP-Cdc24. N.A., nuclear accumulation; C, cytoplasmic; N, nuclear. H, quantitation of the ability of Ste5 to drive GFP-Cdc24 to the nucleus.
associated efficiently with Cdc24-m1 (Fig. 6C), which harbors a mutation that blocks binding to Far1 (9, 29), and was properly localized in the cdc24-m1 and cdc24-m2 strains (Fig. 6E). A far1Δ mutation had no effect on nuclear accumulation of Ste5, although it caused a decline in Ste5 recruitment (Fig. 6E). However, this decrease may be a secondary consequence of the far1Δ mutation caused a pronounced decrease in nuclear accumulation of GFP-Cdc24, with no decrease in the amount of cortical recruitment at growth sites during vegetative growth (Fig. 6F). In contrast, a ste5Δ mutation did not block nuclear accumulation of GFP-Cdc24 in an obvious way (Fig. 6F), indicating that Ste5 does not anchor Cdc24 in the nucleus. However, overexpression of Ste5 derivatives that localize in the nucleus (TAG-NLS-Ste5) or shuttle through the nucleus (TAG-NLS623WT-Ste5, Ste5-GST) (43, 44) could relocalize GFP-Cdc24 from the cytoplasm to the nucleus (Fig. 6, G and H). Additionally, GFP-Cdc24 accumulated in the nuclei of far1Δ msn5Δ cells (data not shown), consistent with redistribution of Ste5 to the nucleus. Collectively, these data support the possibility that Cdc24-Ste5 complexes could shuttle through the nucleus.

Cdc24 Stimulates Ste5-Ste4 Complex Formation and Associates with Ste4 Independently of Ste5 and Far1—We determined whether Cdc24 promotes the ability of Ste5 to bind to Ste4 by comparing how well Ste5 and Ste4 associate in the WT and cdc24-4 strains. Strikingly, temperature shift inactivation of the cdc24-4 protein decreased the amount of Ste5-Ste4 complexes captured in co-immunoprecipitates of HA-Ste4 (Fig. 7A and B). Inactivation of Cdc24-4 also altered the mobility of both Ste4 and Ste5 to faster migrating species (Fig. 7C), which correlate with hyperphosphorylated forms of both proteins (55, 56). Thus, Cdc24 stimulates association of Ste5 and Ste4 and may influence their phosphorylation states.

The ability of Cdc24 to stimulate Ste5-Ste4 complex formation suggested that Cdc24 binds to Ste4 independently of a bridging interaction from Far1. GFP-Cdc24 co-immunoprecipitated with HA-Ste4 in the WT and far1Δ ste5Δ strains (Fig. 7D) in addition to the ste5Δ and far1Δ strains (data not shown). Thus, Cdc24 is able to associate with Ste4 independently of Far1 and Ste5. Furthermore, Cdc24-Ste5 complex formation was increased in the ste4Δ mutant (Fig. 7E), suggesting that Ste4 inhibits the ability of Ste5 to associate with Cdc24. These findings support the possibility that Cdc24 and Ste5 both bind to a common domain on Ste4.

Loss of Bem1 and Partial Loss of Cdc42 Deregulate a Factor-induced Recruitment of Ste5—In dividing cells, Cdc24 binds Bem1 and Cdc42 at the site of polarized growth. If Ste5 is restricted to the growth site through interactions with Cdc24, then loss of Bem1 or Cdc42 should liberate the cortical pool of Cdc24 to promote recruitment of Ste5 to Ste4 in an unrestricted manner during isotropic factor stimulation (which should activate Gβγ throughout the plasma membrane). In contrast, loss of Cdc24 would not be expected to do so. This possibility was tested by examining the pattern of residual recruitment of Ste5-GST at permissive temperature in the bem1Δ and cdc42Δ strains compared with the cdc24Δ strain after a 30-min exposure to a factor. Under these conditions, the cdc42Δ-1 and cdc24Δ-4 strains exhibited a partial phenotype at the level of decreased recruitment of Ste5-GST and Ste5-Myc, whereas the bem1Δ strain exhibited only a minor defect in recruitment, although Ste5 was more broadly distributed at the cortex (Figs. 2 and 7). Remarkably, a significant number of the bem1Δ and cdc42Δ-1 cells exhibited more extensive recruitment of Ste5-GST at the plasma membrane compared with the WT control, with evidence of multiple foci of recruitment in the bem1Δ cells and recruitment around the entire plasma membrane of some cdc42Δ-1 cells (Fig. 7F). In sharp contrast, Ste5-GST remained at a single polarized site in all of the cdc24Δ-4 cells, but the residual recruitment was reduced and more diffuse compared with the WT control (Fig. 7F). These data suggest that the Cdc42-Bem1 cortical complex restricts Ste5 recruitment to Ste4 to a localized site.

DISCUSSION

Cdc24 has been thought to regulate the mating MAPK cascade solely by activating Cdc42 to allow binding of Cdc42-GTP to Ste20. Although initial genetic analysis suggested that Cdc24 might be a direct effector of Ste4, subsequent work argued against this possibility. It has also been thought that recruitment of Ste5 to the cell cortex occurs solely through an interaction between Ste5 and Ste4. The following new findings force a reconsideration of these views. First, Cdc24 is able to associate with Ste4 in the absence of Far1 and Ste5 (Fig. 7), arguing that it is a direct effector of Ste4. Second, Ste5 is basally recruited to the cell cortex independently of Ste4 through a process that is dependent on Cdc24, Cdc42, and Bem1 (Figs. 1 and 2). Third, Cdc24 binds to Ste5, and this association occurs independently of potential bridging interactions from Bem1, Far1, Msn5, and Ste4 (Figs. 5 and 6). Fourth, Cdc24 positively regulates the ability of Ste5 to associate with Ste4 (Fig. 7). Fifth, Cdc24(G168D) is unable to promote basal recruitment of Ste5 or basal activation of Fus3 even though it is able to activate Cdc42 (Fig. 2), suggesting a role for Cdc24 in Ste5 recruitment that is not dependent simply on its ability to activate Cdc42.

These data are consistent with a model in which Cdc24 directly binds Ste5 and stabilizes it at the plasma membrane at sites that also contain Bem1 and Cdc42. The ability of overexpressed Bem1 to promote cortical recruitment of Ste5 (Fig. 3B) and to stimulate basal activation of Fus3 (52) is consistent with its ability to nucleate asymmetric enrichment of Cdc42 at the plasma membrane (25), to stabilize Cdc42 recruitment at cortical sites during mating pheromone stimulation (23), and to associate with Ste5 (52). Cdc24 may facilitate Ste5 association with Ste4 by increasing the local concentration of Ste5 that is available to bind to Ste4 through its localization to Cdc42. Alternatively, Cdc24 may act as an adapter and bind to Ste4 in addition to Ste5. Remaining questions include the function of Ste4-independent basal recruitment of Ste5 and whether Cdc24 regulates Ste5 recruitment independently of its ability to serve as a GEF for Cdc42. Because basal transmission of the signal from Ste5 still requires Ste4 (Fig. 1), it is possible that the juxtaposition of Ste5 with Cdc42 facilitates recognition of Ste5-bound Ste11 by Cdc42-bound Ste20 in such a way as to take better advantage of the low level of free Ste4 that is available in the absence of pheromone stimulation to bind to Cdc42, Ste5, or Ste20. For example, binding of Ste4 to Cdc42-bound Ste5 may still be required to induce a conformational change that allows Ste5 to function properly. The fact that Cdc24(G168D) is unable to mediate basal recruitment of Ste5 and basal activation of Fus3 strongly suggests that Cdc24 regulates Ste5 independently of its ability to serve as a GEF for Cdc42. The dependence of Ste5 recruitment on Cdc24 may

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therefore be due to its role as a localization anchor for Cdc24. Bem1 and Cdc42 appear to restrict Ste5 recruitment to Ste4 to a specific site at the cell cortex during pheromone stimulation (Fig. 7); this function might be important for maintenance of an axis of polarity that is needed for continuous polarized growth (57). A basal recruitment apparatus for Ste5 that is linked to the Bud proteins might be important in the wild among progeny of ascospores and haploid cells that have switched their mating types and that are juxtaposed to favor axial interactions during mating (58).

**Fig. 7.** *Cdc24 is required for efficient association between Ste5 and Ste4.* A and B. *Cdc24* is required for efficient association of Ste5 with Ste4. Ste5-Myc9 and Ste4-HA were co-immunoprecipitated (IP) from WT and *cdc24-4* mutant cells that were grown at room temperature (RT) and shifted to 37 °C for 3 h. The amount of co-immunoprecipitation was normalized to the input in the whole cell extracts (WCE) by densitometry. WB, Western blot. C, the *cdc24-4* mutation changes the mobility of HA-Ste4 upon SDS-PAGE. Strains were incubated for 3 h at 37 °C prior to preparation of whole cell extracts. ts, temperature-sensitive. D. GFP-Cdc24 and HA-Ste4 associate in the absence of Far1 and Ste5. E, loss of Ste4 increases association between GFP-Cdc24 and Ste5-Myc9. F, Bem1 and Cdc42 restrict Ste5-GST localization during response to α factor. Shown are the results from indirect immunofluorescence of Ste5-GST in strains grown at room temperature and treated with α factor for 30 min.
Further work is needed to understand the relationship between Cdc24, Ste5, and Ste4. Cdc24 appears to bind within residues 639–917 of Ste5, a region that has only one binding partner assigned (Ste7), although additional residues within the second third of the protein may influence association. Cdc24 associated more efficiently with a derivative of Ste5 (Ste5Δ474–638) that oligomerizes more than WT Ste5, suggesting that it may bind more readily to a Ste5 dimer. This would be consistent with the fact that it dimerizes (32). The significant increase in the level of Cdc24-Ste5 complexes in a ste4Δ strain suggests that Ste5 and Cdc24 may bind to a common domain on Ste4. Ste5 is thought to bind directly to Ste4 through its RING H2 domain, but it is not currently known whether this interaction involves a specific domain of Ste4, which is mainly composed of WD40 repeats. PH domains have been shown to bind to WD40 repeats within the β subunit of G protein βγ subunits (59–61), raising the possibility that Cdc24 binds to Ste4 via its PH domain. However, initial two-hybrid tests suggest that the Cdc24 PH domain does not interact with Ste4, whereas a Cdc24 fragment spanning residues 1–289 does (37). This fragment overlaps the S189P mutation that interferes with interaction with Far1 (48) and could conceivably overlap with the region of Cdc24 that interacts with Ste4. The G168D mutation within the calponin homology domain yields a form of Cdc24 that, at room temperature, is selectively defective in bud site selection as a result of a decrease in binding to Rsr1/Bud1-GTP and that is still able to activate Cdc42 and to bind to Far1 (Rsr1/Bud1-GTP) (48). A bud1 null mutant is able to respond to mating pheromone and to form shmoos (9, 34), suggesting that the defect in Ste5 recruitment is not linked to a defect in Cdc24 binding to Rsr1/Bud1. The fact that the Cdc24(G168D) mutation in the calponin homology domain abrogates basal recruitment of Ste5 suggests that the mutation lies in a region of Cdc24 that is also recognized by Ste5. Alternatively, the binding of Rsr1/Bud1-GTP to Cdc24 may induce a conformational change that makes Cdc24 able to bind to Ste5. Furthermore, the relationship between Cdc24 and Ste5 may be different in the presence of mating pheromone when the pool of Gβγ dimers is much greater and recruitment of Cdc24 becomes dependent upon the actin cytoskeleton (9, 50).

The results also suggest that Cdc24 promotes nuclear import of Ste5 during mitotic growth. Given that loss of Cdc24 function does not cause nuclear exclusion of Ste5, it appears that Cdc24 enhances nuclear import of Ste5. Ste5 must shuttle through the nucleus to be competent for recruitment and Fus3 activation (43, 44); however, it has not been clear why this shuttling event is necessary. The enrichment of Cdc24 in the nucleus through its association with Far1 may increase the likelihood of forming a Cdc24-Ste5 complex that can facilitate cortical recruitment of Ste5. This interpretation is supported by the observation that the cortical recruitment defect of a cdc42-1 mutant is suppressed by stimulating nuclear import of Ste5. Nuclear export of Cdc24 could be controlled by exportins that bind Cdc24 or Ste5, in addition to Far1. Currently, it is unclear how Bem1 and Cdc42 promote nuclear accumulation of Ste5, although it is conceivable that they are complexed with Cdc24.

The analysis argues strongly that Cdc24-Ste5 complexes are distinct from Cdc24-Far1 complexes. Additionally, a plasma membrane-localized derivative of Ste5 that is able to recruit Ste7 and Fus3 (52) failed to recruit GFP-Cdc24 to the plasma membrane, even when expressed in the far1Δ and ste4Δ strains to remove potential competing proteins. Thus, Ste5 does not appear to have the capability to target Cdc24 to the plasma membrane, as has been found for Far1 and which is consistent with the fact that Ste5 does not substitute for Far1 to promote partner selection. Given the role for Fus3 in polarized growth during shmoo formation (62, 63), it seems more likely that stable cortical recruitment of Ste5 is important for localizing Fus3 to cell polarity proteins.

Although DbI family GEFs are well characterized in their ability to bind Rho-type GTPases, a growing body of evidence indicates that they also bind to scaffolds that complex with components of specific GTPase effector pathways (8, 9, 21, 64–66). In the case of Bem1 and Far1, it is the scaffolds that regulate the localization and activity of the GEF and associated GTPase. By binding to Ste4 and Ste5, Cdc24 appears to function both upstream and downstream of the Cdc42-Ste20 step of the mating MAPK cascade and in so doing may provide a specificity function that further ensures a tightly regulated interaction between Ste20 and Ste11 and passage of the signal through Ste5 to the bound MAPK, Fus3. This additional layer of regulation may help insulate Cdc42-Ste20 activation of Ste11 from the high osmolarity growth and filamentous pathways. Interestingly, the Pbs2 scaffold undergoes polarized localization to the cell cortex at growth sites during osmotic stress, even though Sho1 is distributed throughout the plasma membrane (67). This localization event is independent of the act cytoskeleton and therefore could also be regulated by cell polarity proteins. Additionally, the JIP1 and JIP2 scaffolds that regulate the stress-activated protein kinases also shuttle through the nucleus (68) and associate with the Rho and Rac GEFs p190RhoGEF (64) and Tiam1 and Ras-GRF1 (65). It is interesting to speculate that these interactions may facilitate localization events important for activation of the associated MAPK cascade, such as by an associated GTPase-linked protein kinase.

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