Essential Role of the NH$_2$-Terminal Region of Cdc24 Guanine Nucleotide Exchange Factor in Its Initial Polarized Localization in Saccharomyces cerevisiae

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The cortical recruitment and accumulation of the small GTPase Cdc42 are crucial steps in the establishment of polarity, but this process remains obscure. Cdc42 is an upstream regulator of budding yeast Cdc42 that accelerates the exchange of GDP for GTP in Cdc42 via its Dbl homology (DH) domain. Here, we isolated five novel temperature-sensitive (ts) cdc24 mutants, the green fluorescent protein (GFP)-fused proteins of which lose their polarized localization at the nonpermissive temperature. All amino acid substitutions in the mutants were mapped to the NH$_2$-terminal region of Cdc24, including the calponin homology (CH) domain. These Cdc24-ts mutant proteins did not interact with Bem1 at the COOH-terminal PB1 domain, suggesting a lack of exposure of the PB1 domain in the mutant proteins. The cdc24-ts mutants were also defective in polarization in the absence of Bem1. It was previously reported that a fusion protein containing Cdc24 and the p21-activated kinase (PAK)-like kinase Cla4 could bypass the requirement for Bem1 in polarity cue-independent budding (i.e., symmetry breaking). Cdc24-ts–Cla4 fusion proteins also showed ts localization at the polarity site. We propose that the NH$_2$-terminal region unmasks the DH and PB1 domains, leading to the activation of Cdc42 and interaction with Bem1, respectively, to initiate cell polarization.

The establishment of cell polarity is crucial for many biological processes in both unicellular and multicellular organisms (11, 38). One protein that plays a key role in the establishment of cell polarity is Cdc42, a Rho family small GTPase. CDC42 was first identified as a gene required for the establishment of cell polarity in budding yeast (Saccharomyces cerevisiae) (1), and subsequent studies demonstrated that Cdc42 is also a key regulator of cell polarity in various other eukaryotes (13, 18, 19, 25, 33).

Budding yeast exhibits polarized growth at several stages of its life cycle (42, 45). During vegetative growth, yeast cells undergo polarized cell growth by budding. In this process, polarization of the actin cytoskeleton toward a single cortical position in the cell (the incipient bud site) leads to bud formation. A key regulator of the polarization of the actin cytoskeleton during budding is Cdc42, which is activated by its sole guanine nucleotide exchange factor (GEF), Cdc42. The GTP-bound form of Cdc42 interacts with its effectors, including the formin Bni1p and p21-activated kinase (PAK)-like kinases Cla4 and Ste20, to organize the dynamic assembly of the actin cytoskeleton, which in turn targets the fusion of secretory vesicles to the site of polarized growth (25, 42, 45).

Cdc24 contains two functional domains that are found in all GEFs for Rho family GTPases: a Dbl homology (DH) domain (residues 283 to 452), which shows a high degree of similarity to the Dbl family of exchange factors, and a nearby pleckstrin homology (PH) domain (residues 472 to 681) (52, 62). The DH domain is an $\alpha$-helical catalytically active domain (30), while the PH domain is thought to serve as a membrane-targeting signal (28). Additionally, Cdc24 contains a calponin homology (CH) domain at its NH$_2$ terminus (residues 137 to 241), which in some proteins has been implicated in actin binding (15), and a Phox Bem1 (PB1) domain at its COOH terminus (residues 780 to 854) (23, 59). The Cdc24 PB1 domain interacts with scaffold protein Bem1, and this interaction enhances the polarized localization of Cdc24 (3, 4, 50).

How polarity regulators are localized during the initial stage of cell polarization is an important question. Because Cdc24 is an upstream activator of Cdc42, Cdc24 should be among the polarity proteins that are localized first. Cdc42 could be localized to a polarity site in a polarity cue-dependent or -independent manner. In polarity cue-dependent budding (e.g., haploid-specific axial or diploid-specific bipolar budding), the Ras-like GTPase Rsr1/Bud1 recruits Cdc24 to the incipient bud site (43, 44, 50). In polarity cue-independent polarization, even when RSR1 is deleted, cells can still polarize and form buds efficiently, albeit at random positions (7). In this spontaneous cell polarization (56), which is also called symmetry breaking (22), it is unknown how Cdc24 is localized to the polarity site.

The polarized localization of Cdc24 has been examined in various deletion mutants or using truncated versions of CDC42, and Cdc24 domains important for polarization have been identified (52, 53). However, these mutants had to be expressed in the presence of endogenous Cdc24 because CDC42 is an essential gene, and this situation complicated the interpretation of the results. For example, one mutant could not localize to a polarity site due to competition with wild-type Cdc24, even though it could localize by itself, albeit with low efficiency. On the other hand, another mutant that was unable to localize to a polarity site by itself could polarize by binding to another polarity regulator. For example, the PB1 domain of Cdc24 could be localized to a polarity site by binding to Bem1, which was recruited by endogenous Cdc24 and GTP-Cdc42. Thus, to establish which Cdc24 domain is essential for the initial polarized localization of the molecule, the full-length mutant proteins had to be analyzed in the absence of wild-type Cdc24.
A promising approach to overcome these problems is the isolation of a temperature-sensitive (ts) cdc24 mutant, the product of which exhibits ts localization to a polarity site in cdc24 cells.

In this study, we screened randomly mutagenized GFP-CDC24 for new alleles, the products of which could not localize to the budding site in a temperature-dependent manner. We isolated five alleles, all of which encoded proteins with one to three amino acid alterations within the NH2-terminal region. All five mutant Cdc24 proteins lost the ability to interact with Bem1, but, interestingly, they were also defective in polarization in the absence of Bem1. Thus, the NH2-terminal region of Cdc24 has an essential regulatory function in its initial localization to a site of polarization.

**MATERIALS AND METHODS**

Strains, growth conditions, and plasmids. The yeast strains used are listed in Table 1. Deletion of the open reading frames (ORFs) in CDC24 and BEM1 was performed using a PCR-based method, as described previously (31). RSR1 was disrupted as described previously (26). Unless otherwise specified, the strains were grown in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose, and 0.01% adenine). Strains carrying the plasmids were selected in synthetic medium (SD) containing the required nutritional supplements (46). 5-Fluoroorotic acid (5-FOA) medium was prepared as described previously (46). Standard yeast genetic manipulations were performed as described previously (48). Yeast transformation was done using the lithium acetate method (12, 14).

The plasmids used are listed in Table 2. The details of the constructs and sequences of the oligonucleotide DNAs are available on request. Site-directed mutagenesis for construction of the cdc24-D824K/D831R, CDC42-D57V/C188S, and RSR1-G12V/C2695 alleles were performed by PCR and confirmed by sequencing. Escherichia coli strains DH5α and XL1-Blue were used for the construction and amplification of the plasmids.

Random mutagenesis of GFP-CDC24. Full-length GFP-CDC24 was randomly mutagenized by a PCR-based method as described previously (54). The ~4.1-kb mutagenized products were mixed with an equal amount of pKT1154ΔBamHI, which had been linearized by restriction enzyme digestion. This mixture of DNA was introduced into strain YKT1012 (cdc24::kanMX6 [pRS316-CDC24]) to allow homologous recombination between the PCR products and linearized plasmid. Transformants were selected on SD plates lacking leucine (SD-Leu plates) at 25°C and then replica plated onto 5-FOA plates to select for loss of the plasmid pRS316-CDC24. The uracil-requiring colonies were recovered and tested for growth on YPD plates at 25 and 37°C to allow the identification of ts mutants. Of 4,157 uracil-requiring clones, 132 clones were isolated as ts mutants. The plasmids were recovered from the mutants and reintroduced into YKT1012 to confirm that the ts growth defect depended on the plasmids. Of 132 mutants, 59 reproducibly exhibited a ts growth defect. The 59 ts mutants isolated were examined for the localization of green fluorescent protein (GFP)-Cdc24 after growth at 37°C for 1 h. Of these, 22 mutants exhibited polarized GFP-Cdc24 while 15 had no GFP signal. Of the remaining 22 mutants, 17 exhibited a polarization defect in GFP-Cdc24 even at the permissive temperature (25°C). In the remaining five mutants, GFP-Cdc24 did not localize to the site of polarization at 37°C but did so at 25°C.

To determine the mutation sites responsible for the ts growth defect, we constructed chimeric genes consisting of the wild-type CDC24-derived region and cdc24-ts mutant-derived region using Af11 (see Fig. 1F) and analyzed their growth phenotype and the localization of GFP-Cdc24. In all five mutants, a region upstream of the Af11 site was responsible for the phenotype. Sequence analysis of this region revealed that cdc24-ts158, -ts210, and -ts233 encoded a single amino acid substitution while cdc24-ts37 and -ts136 encoded three amino acid substitutions. We next constructed chimeric genes to determine the mutation site responsible for the ts phenotype in the last two mutants. The chimeric gene containing a region upstream of the EcoT22I site (Fig. 1F) from cdc24-ts158 conferred the ts growth defect, indicating that M242L and/or L297S encoded by this region, but not V508I, were responsible. Neither the chimeric gene containing the cdc24-ts136-derived region upstream of the EcoT22I site, which included the mutation responsible for A67T, nor that containing the cdc24-ts136-derived region downstream of the BstXI site (Fig. 1F), which included the mutations responsible for E423G and A440V, conferred a ts growth defect, indicating that both A67T and E423G and/or A440V were responsible for the phenotype. Sequencing confirmed that there was no alteration in the GFP region in each cdc24 mutant. The region downstream of the Af11 site was also sequenced to examine whether there were other mutations within this region. The cdc24-ts37, -ts210, and -ts233 mutations did not cause any amino acid alterations, but cdc24-ts136 and -ts158 mutants encoded I76M and I640V substitutions, respectively. We constructed new cdc24-ts136 and -ts158 versions, in which the region downstream of the Af11 site was replaced with that of the wild type, and these new versions were used in all experiments except for Fig. 1.

Some mutations in the DH domain were found in the collection of ts mutants as follows. From the 22 mutants that exhibited polarized GFP-Cdc24 at 37°C, 10 were randomly selected, and the nucleotide sequences encoding their DH domains were determined. Five mutants contained amino acid substitution(s) within the DH domain; their entire CDC24 ORFs were sequenced. Two clones that contained multiple amino acid substitutions outside the DH domain were eliminated. The cdc24-ts127 and -ts160 clones contained single and double amino acid substitutions only within the DH domain, respectively. The cdc24-ts94 clone contained a single amino acid substitution at position 75 (S75G) in addition to Y417C within the DH domain. To remove the S75G mutation, the region upstream of the EcoT22I site in cdc24-ts94 was replaced with that of wild-type CDC24. The GFP-cdc24-ts94 (Y417C) mutant exhibited ts growth, and GFP-Cdc24-ts94 (Y417C) was polarized at 37°C. We renamed the original cdc24-ts94 mutant cdc24-ts94(original), while cdc24-ts94 (Y417C) (referred to as cdc24-ts94) was used for further study.

Two-hybrid assays. Two-hybrid assays were performed using L40 cells containing the LexA DNA-binding domain fusion plasmid and Gal4 activation domain fusion plasmid (20). Quantitation of β-galactosidase activity was performed using o-nitrophenyl-β-D-galactopyranoside as a substrate (16). β-Galactosidase activity is expressed in Miller units (34).

**Microscopic observation.** Cells were observed using an Eclipse E800 microscope (Nikon Instec, Tokyo, Japan) equipped with an HB-10103AF super-high-pressure mercury lamp and 1.4-numerical aperture (NA), 100X Plan Apo oil immersion objective (Nikon Instec) with appropriate fluorescence filter sets (Nikon Instec) or differential interference contrast (DIC) optics. Images were acquired using a digital cooled charge-coupled device (CCD) camera (C4742-95-12NR; Hamamatsu Photonics K. K., Hamamatsu, Japan) and AQUACOSMOS software (Hamamatsu Photonics K. K.).

To visualize GFP-tagged proteins in living cells, cells were grown to the early logarithmic phase, harvested, and resuspended in SD medium. The cells were then mounted on microslide glass and observed immediately using a GFP band-pass filter set (excitation, 460 to 500 nm; dichroic mirror, 505 nm; emission, 510 to 560 nm). When cells incubated at 37°C were observed, the samples were kept at 37°C using a Thermolo Plate (Tokai HIT Co. Ltd., Fujiinomiya, Japan) during observation.

**Immunoblotting.** Yeast whole-cell extracts were prepared as follows. Four OD600 U of cells grown to the early logarithmic phase were harvested and washed once with water. The cells were then lysed in 150 μl of 0.2 M NaOH—5% β-mercaptoethanol on ice for 15 min. Chilled acetone (1 ml) was added to the cell extracts, and the extracts were incubated at −20°C for 30 min. Denatured proteins were precipitated by centrifugation, washed once with chilled acetone, and resuspended in SDS sample buffer. Immunoblotting was performed with mouse anti-therm Haglutinin (anti-HA; HA.11) (BabCO, Richmond, CA) or mouse anti-HA (12CA5) mono-
### TABLE 1. *S. cerevisiae* strains used in this study

| Straina | Genotypeb | Reference or source |
|---------|-----------|---------------------|
| YEF473  | MATa/MATa his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 lys2/lys2 | This study |
| YKT38   | MATa his3/leu2 trp1 ura3 lys2 | This study |
| YKT275  | MATa cdc24::KanMX6 [p415-GFP-CDC24] | This study |
| YKT795  | MATa bem1Δ::His3MX6 | This study |
| YKT1012 | MATa cdc24::KanMX6 [pRS316-CDC24] | This study |
| YKT1013 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts37] | This study |
| YKT1014 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts136] | This study |
| YKT1015 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts158] | This study |
| YKT1016 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts210] | This study |
| YKT1017 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts235] | This study |
| YKT1018 | MATa cdc24::KanMX6 [p415-GFP-HA-CDC24] | This study |
| YKT1019 | MATa cdc24::KanMX6 [p415-GFP-HA-cdc24-ts37] | This study |
| YKT1020 | MATa cdc24::KanMX6 [p415-GFP-HA-cdc24-ts136] | This study |
| YKT1021 | MATa cdc24::KanMX6 [p415-GFP-HA-cdc24-ts158] | This study |
| YKT1022 | MATa cdc24::KanMX6 [p415-GFP-HA-cdc24-ts210] | This study |
| YKT1023 | MATa cdc24::KanMX6 [p415-GFP-HA-cdc24-ts233] | This study |
| YKT1024 | MATa cdc24::KanMX6 [pRS316-CDC24] [p415-GFP-CDC24] | This study |
| YKT1025 | MATa cdc24::KanMX6 [pRS316-CDC24] [p415-GFP-cdc24Δ(683-854)] | This study |
| YKT1026 | MATa cdc24::KanMX6 [pRS316-CDC24] [p415-GFP-cdc24Δ(231-854)] | This study |
| YKT1027 | MATa cdc24::KanMX6 [pRS316-CDC24] [p415-GFP-cdc24Δ(1-152)] | This study |
| YKT1028 | MATa cdc24::KanMX6 [pRS316-CDC24] [p415-GFP-cdc24Δ(1-267)] | This study |
| YKT1029 | MATa cdc24::KanMX6 [pRS316-CDC24] [p415-GFP-cdc24Δ(1-372)] | This study |
| YKT1030 | MATa cdc24::KanMX6 [pRS316-cdc24-ts37] | This study |
| YKT1031 | MATa bem1Δ::His3MX6 cdc24::KanMX6 [pRS316-cdc24-ts158] | This study |
| YKT1032 | MATa cdc24::KanMX6 [pRS316-cdc24-ts158] | This study |
| YKT1033 | MATa bem1Δ::His3MX6 cdc24::KanMX6 [pRS316-cdc24-ts158] | This study |
| YKT1034 | MATa cdc24::KanMX6 [pRS316-cdc24-ts210] | This study |
| YKT1035 | MATa bem1Δ::His3MX6 cdc24::KanMX6 [pRS316-cdc24-ts210] | This study |
| YKT1039 | MATa cdc24::KanMX6 URA3::GFP-CDC24 | This study |
| YKT1040 | MATa bem1Δ::His3MX6 cdc24::KanMX6 URA3::GFP-CDC24 | This study |
| YKT1041 | MATa cdc24::KanMX6 URA3::GFP-cdc24-ts37 | This study |
| YKT1042 | MATa cdc24::KanMX6 URA3::GFP-cdc24-ts136 | This study |
| YKT1043 | MATa cdc24::KanMX6 URA3::GFP-cdc24-ts158 | This study |
| YKT1044 | MATa cdc24::KanMX6 URA3::GFP-cdc24-ts210 | This study |
| YKT1045 | MATa cdc24::KanMX6 URA3::GFP-cdc24-ts233 | This study |
| YKT1046 | MATa bem1Δ::His3MX6 URA3::GFP-CDC24 | This study |
| YKT1047 | MATa bem1Δ::His3MX6 URA3::GFP-cdc24-ts37 | This study |
| YKT1048 | MATa bem1Δ::His3MX6 URA3::GFP-cdc24-ts136 | This study |
| YKT1049 | MATa bem1Δ::His3MX6 URA3::GFP-cdc24-ts233 | This study |
| YKT1050 | MATa cdc24::KanMX6 [p415-GFP-cdc24-DKDR] | This study |
| YKT1051 | MATa cdc24::KanMX6 [pRS314-GFP-CDC24] | This study |
| YKT1052 | MATa cdc24::KanMX6 [pRS314-GFP-cdc24-DKDR] | This study |
| YKT1053 | MATa cdc24::KanMX6 [pRS314-GFP-cdc24-ts158] | This study |
| YKT1054 | MATa cdc24::KanMX6 [pRS314-GFP-cdc24-ts158-DKDR] | This study |
| YKT1057 | MATa cdc24::KanMX6 [p415-GFP-HA-cdc24-ts94] | This study |
| YKT1058 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts127] | This study |
| YKT1059 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts160] | This study |
| YKT1060 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts160] | This study |
| YKT1061 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts160] | This study |
| YKT1062 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts160] | This study |
| YKT1063 | MATa cdc24::KanMX6 [p415-GFP-cdc24-ts160] | This study |

a YKT strains are isogenic derivatives of YEF473. 
b For YKT strains, only relevant genotypes are described.
TABLE 2 Plasmids used in this study

| Plasmid | Characteristics (reference or source) |
|---------|----------------------------------------|
| pRS306  | URA3 (51) |
| pRS314  | TRP1 CEN6 (51) |
| pRS315  | LEU2 CEN6 (51) |
| pRS316  | URA3 CEN6 (51) |
| pRS316-CD24 (pKT1439) | CDC24 URA3 CEN6 |
| p415-GFP-CD24 (pKT1154) | pCdc24-GFP-A8-cdc24 LEU2 CEN6 |
| pKT1534BanMII (pKT1902) | P_cdc24-GFP-A8-cdc24 ts17 LEU2 CEN6 |
| p415-GFP-cdc24-ts17 (pKT1504) | P_cdc24-GFP-A8-cdc24 ts17 LEU2 CEN6 |
| p415-GFP-cdc24-ts136 (pKT1505) | P_cdc24-GFP-A8-cdc24 ts16 LEU2 CEN6 |
| p415-GFP-cdc24-ts158 (pKT1507) | P_cdc24-GFP-A8-cdc24 ts18 LEU2 CEN6 |
| p415-GFP-cdc24-ts210 (pKT1508) | P_cdc24-GFP-A8-cdc24 ts20 LEU2 CEN6 |
| p415-GFP-cdc24-ts333 (pKT1509) | P_cdc24-GFP-A8-cdc24 ts33 LEU2 CEN6 |
| p415-GFP-HA-CD24 (pKT1510) | P_cdc24-GFP-A8-cdc24 ts16 LEU2 CEN6 |
| p415-GFP-HA-cdc24-ts37 (pKT1511) | P_cdc24-GFP-A8-cdc24 ts17 LEU2 CEN6 |
| p415-GFP-HA-cdc24-ts136 (pKT1512) | P_cdc24-GFP-A8-cdc24 ts16 LEU2 CEN6 |
| p415-GFP-HA-cdc24-ts158 (pKT1514) | P_cdc24-GFP-A8-cdc24 ts18 LEU2 CEN6 |
| p415-GFP-HA-cdc24-ts210 (pKT1515) | P_cdc24-GFP-A8-cdc24 ts20 LEU2 CEN6 |
| p415-GFP-HA-cdc24-ts233 (pKT1516) | P_cdc24-GFP-A8-cdc24 ts23 LEU2 CEN6 |
| p415-GFP-HA-cdc24-t94 (original) (pKT1888) | P_cdc24-GFP-A8-cdc24 ts94 LEU2 CEN6 |
| p415-GFP-HA-cdc24-t94 (pKT1889) | P_cdc24-GFP-A8-cdc24 ts94 LEU2 CEN6 |
| p415-GFP-cdc24-ts127 (pKT1890) | P_cdc24-GFP-A8-cdc24 ts127 LEU2 CEN6 |
| p415-GFP-cdc24-ts160 (pKT1891) | P_cdc24-GFP-A8-cdc24 ts160 LEU2 CEN6 |
| p415-GFP-cdc24 (683-854) (pKT1517) | P_cdc24-GFP-A8-cdc24 ts85 LEU2 CEN6 |
| p415-GFP-cdc24 (231-854) (pKT1518) | P_cdc24-GFP-A8-cdc24 ts62 LEU2 CEN6 |
| p415-GFP-cdc24 (1-152) (pKT1519) | P_cdc24-GFP-A8-cdc24 ts31 LEU2 CEN6 |
| p415-GFP-cdc24 (1-267) (pKT1520) | P_cdc24-GFP-A8-cdc24 ts27 LEU2 CEN6 |
| p415-GFP-cdc24 (1-372) (pKT1521) | P_cdc24-GFP-A8-cdc24 ts21 LEU2 CEN6 |
| pRS316-cdc24-ts37 (pKT1527) | P_cdc24-GFP-A8-cdc24 ts37 LEU2 CEN6 |
| pRS316-cdc24-ts37 (pKT1529) | P_cdc24-GFP-A8-cdc24 ts37 LEU2 CEN6 |
| pRS316-cdc24-ts150 (pKT1530) | P_cdc24-GFP-A8-cdc24 ts150 LEU2 CEN6 |
| pRS306-CD24 (pKT1332) | P_cdc24-GFP-A8-cdc24 ts136 LEU2 CEN6 |
| pRS306-GFP-cdc24-ts37 (pKT1533) | P_cdc24-GFP-A8-cdc24 ts37 LEU2 CEN6 |
| pRS306-GFP-cdc24-ts136 (pKT1534) | P_cdc24-GFP-A8-cdc24 ts136 LEU2 CEN6 |
| pRS306-cdc24-ts136 (pKT1922) | P_cdc24-GFP-A8-cdc24 ts136 LEU2 CEN6 |
| pRS306-cdc24-ts210 (pKT1537) | P_cdc24-GFP-A8-cdc24 ts210 LEU2 CEN6 |
| pRS306-cdc24-ts333 (pKT1538) | P_cdc24-GFP-A8-cdc24 ts333 LEU2 CEN6 |
| p415-GFP-cdc24-5DKDR (pKT1539) | P_cdc24-GFP-A8-cdc24 ts33 LEU2 CEN6 |
| pRS314-GFP-CD24 (pKT1440) | P_cdc24-GFP-A8-cdc24 ts127 LEU2 CEN6 |
| pRS314-GFP-cdc24-5DKDR (pKT1541) | P_cdc24-GFP-A8-cdc24 ts127 LEU2 CEN6 |
| pRS314-GFP-cdc24-ts158 (pKT1542) | P_cdc24-GFP-A8-cdc24 ts158 LEU2 CEN6 |
| pRS314-GFP-cdc24-ts158-5DKDR (pKT1543) | P_cdc24-GFP-A8-cdc24 ts158 LEU2 CEN6 |
| pRS315-GFP-CD24-CLA4 (pKT1892) | P_cdc24-GFP-A8-cdc24 ts136 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts37-CLA4 (pKT1893) | P_cdc24-GFP-A8-cdc24 ts37 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts136-CLA4 (pKT1923) | P_cdc24-GFP-A8-cdc24 ts136 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts158-CLA4 (pKT1924) | P_cdc24-GFP-A8-cdc24 ts158 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts210-CLA4 (pKT1896) | P_cdc24-GFP-A8-cdc24 ts210 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts233-CLA4 (pKT1897) | P_cdc24-GFP-A8-cdc24 ts233 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts494-CLA4 (pKT1898) | P_cdc24-GFP-A8-cdc24 ts494 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts127-CLA4 (pKT1899) | P_cdc24-GFP-A8-cdc24 ts127 CLA4 LEU2 CEN6 |
| pRS315-GFP-cdc24-ts160-CLA4 (pKT1900) | P_cdc24-GFP-A8-cdc24 ts160 CLA4 LEU2 CEN6 |

**Note:**
- *P_Cdc24* is the promoter of CDC24.
- *DBDlexA* is the DNA-binding domain of LexA.
- *ADGAL4* is the transcriptional activating domain of Gal4.

Clonal antibody (Roche Applied Science, Indianapolis, IN) as described previously (36). To visualize the total loaded proteins, the membrane filter after immunoblotting was stained with 0.1% Amido black 10B (Wako Pure Chemical Industries, Osaka, Japan) in 5% acetic acid followed by several rinses with water.

**Cell cycle synchronization.** G_{1}-G_{2} release experiments were carried out as follows. Cells were inoculated in 5 ml of YPDA or SD-Leu medium and grown at 25°C for 2 to 3 days. A completely saturated culture (2 ml) was combined with 8 ml of fresh YPDA or SD-Leu medium, and the culture was incubated overnight. The cells were pelleted and resuspended in YP Sor medium (1% yeast extract, 2% Bacto peptone, and 1 M sorbitol), then spun at 500 × g for 1 min. Next, 8 ml of the supernatant fraction, in which unattached cells were added, was combined with 2 ml of YPSor medium and centrifuged again (500 × g, 1 min). These procedures were...
repeated three times until a uniform population of unbudded cells was obtained. The cells were pelleted, resuspended in SC or SD-Leu medium at 1.0 × 10^7 cells/ml, and released at the appropriate temperature.

RESULTS
Isolation of ts cdc24 mutants in which the mutant Cdc24 proteins are not localized to budding sites. One straightforward approach to understanding how Cdc24 initially localizes to a site for polarity establishment would be to isolate a mutant that fails to do so. Random mutagenesis of the entire CDC24 gene should reveal a region or amino acid that is required for the polarized localization of Cdc24. NH2-terminally GFP-tagged Cdc24 (encoded by GFP-CDC24) was functional for growth of cdc24Δ cells at 25, 30, and 37°C, and GFP-Cdc24 was localized to polarized sites, including the presumptive bud site and bud tip during budding and the bud neck during cytokinesis (our unpublished results; 52). We randomly mutagenized GFP-CDC24 on a low-copy-number vector by error-prone PCR and screened for mutants that failed to complement the growth defect of the cdc24Δ mutant at 37°C. Of these ts mutants (59 isolates), we further screened for mutants in which GFP-Cdc24 was not localized to the presumptive bud site or bud tip at 37°C. We identified five mutant alleles, designated cdc24-ts37, -ts136, -ts158, -ts210, and -ts233 (Fig. 1A). The bud tip localization of mutant GFP-Cdc24 proteins was not observed after 1 h of incubation at 37°C (Fig. 1B). To quantitatively examine the localization of the mutant GFP-Cdc24 proteins, asynchronous cultures at 25°C were shifted to 37°C for 1 h and the unbudded and small-budded cells with polarized GFP-Cdc24 were counted. Wild-type GFP-Cdc24 was polarized in ~75% of the cells at 25 and 37°C. In contrast, the GFP-Cdc24-ts proteins were polarized in only ~2 to 11% of the cells at 37°C (Fig. 1C).

To confirm that the mislocalization of mutant Cdc24 was due to defects in its initial localization to an incipient bud site, GFP-Cdc24 localization was examined in cells that had been arrested at G1 by nutrient deprivation at 25°C and were subsequently released at 37°C. These experiments were performed in strains in which the GFP-cdc24-ts alleles were integrated at the URA3 locus, because the expression level of GFP-Cdc24 varied depending on the plasmid-bearing strain. At 120 min after release, >40% of the cells with wild-type GFP-Cdc24 had budded (data not shown) and GFP-Cdc24 was localized to the presumptive bud site or bud tip in 75% of the unbudded and small-budded cells (Fig. 1D). In contrast, the GFP-Cdc24-ts proteins failed to localize to the incipient bud site; thus, these mutant cells could not form a bud (data not shown). Polarized localization of the mutant Cdc24 proteins was not observed with shorter release times (30, 60, and 90 min) either (data not shown).

We next confirmed that the defects in polarized localization were not due to degradation of the mutant proteins at 37°C. Because wild-type GFP-Cdc24 was not detected in cell lysates by immunoblotting with commercially available anti-GFP antibodies (data not shown), we constructed a new cdc24-ts allele encoding a protein in which three HA epitopes (3×HA) were inserted between GFP and the product of the CDC24 ORFs. Wild-type GFP-3×HA-Cdc24 was functional for growth at 25, 30, and 37°C of cdc24Δ cells, and it localized to polarized sites, whereas all of the GFP-3×HA-cdc24-ts mutants exhibited ts growth and the mutant proteins exhibited temperature-dependent mislocalization, similar to the HA-untagged versions (our unpublished data). Immunoblotting was performed on cell lysates prepared from cells incubated at 37°C for 1 h. Mutant and wild-type Cdc24 were produced at comparable levels at 37 and 25°C (Fig. 1E). Together, these results suggest that the mutant proteins were defective in their initial localization to a site for polarity establishment.

The mutation sites are located in the NH2-terminal region of Cdc24. The DNA sequences of the cdc24-ts alleles were determined to identify their mutation sites. We first determined the DNA segment that was responsible for the ts growth and defect in polarized localization. Swapping experiments between wild-type and mutant CDC24 using Aff1 revealed that the change in all five mutant alleles affected the NH2-terminal two-thirds of Cdc24 (Fig. 1F). Three alleles, cdc24-ts138, cdc24-ts210, and cdc24-ts233, carried a single mutation that resulted in an amino acid substitution (C47Y, D156V, or S198P, respectively) in the NH2-terminal region of Cdc24 (Fig. 1F, oval) (in this paper, “NH2-terminal region” refers to the region from the NH2 terminus of Cdc24 to the NH2-terminal end of the DH domain). D156 and S198 were located within the CH domain, whereas C47 was located near the NH2 terminus. The cdc24-ts37 and cdc24-ts136 alleles bore three mutations that resulted in amino acid substitutions (Fig. 1F, rectangles). The mutations responsible for the ts growth phenotype were further assessed by swapping experiments using BstXI and/or EcoT221. In the product of the cdc24-ts37 allele, M242L and/or L297S, located near or in the DH domain, respectively, was responsible for the ts growth, while V508I was not (data not shown). The cdc24-ts136 allele led to A67T near the NH2 terminus and E423G and A440V in the COOH-terminal region of the DH domain. Both A67T and E423G/A440V were required for the ts growth phenotype (data not shown). These results demonstrate the importance of the NH2-terminal region in the initial polarized localization of Cdc24. The DNA sequence of the region downstream of the Aff1 site was also determined for each mutant. The cdc24-ts37, -ts210, and -ts233 mutations did not cause any amino acid alterations, but cdc24-ts136 and -ts158 mutants encoded I766M and I640V substitutions, respectively. We constructed new cdc24-ts136 and -ts158 versions, in which the region downstream of the Aff1 site was replaced with that of the wild type. These new cdc24-ts mutants exhibited temperature-sensitive growth and defects in polarized localization comparable to those of the original mutants (data not shown). These new versions were used here.

The Cdc24-ts proteins exhibit reduced interactions with Bem1. The interaction of the COOH-terminal PB1 domain in Cdc24 with Bem1 plays an important role in the maintenance of Cdc24 at the polarized site (4, 17). Interestingly, the interaction of all five Cdc24-ts proteins with full-length Bem1 was significantly reduced at 30°C, and even at the permissive temperature (25°C), as estimated by two-hybrid analysis (Fig. 2A). Immunoblotting of these Cdc24-ts proteins verified that the expression levels were comparable to that of wild-type Cdc24 in the two-hybrid reporter strain (Fig. 2A). These results were surprising, because the PB1 domain of Cdc24 was sufficient for the interaction with Bem1 (4, 23).

Our results suggest that wild-type Cdc24 was present in a conformation that enabled it to bind Bem1, whereas the mutant Cdc24 proteins were not. To further assess the function of the NH2-terminal region in the interaction with Bem1 and polarized localization of Cdc24, we constructed five truncation mutants of Cdc24 with deletions in the NH2- or COOH-terminal region (Fig. 2B). Three NH2-terminal deletion mutants were examined by two-hybrid analysis for the ability to interact with Bem1. Clone
Isolation of novel cdc24-ts mutants unable to localize to budding sites. (A) ts growth defects in cdc24Δ cells expressing GFP-cdc24-ts. cdc24Δ cells bearing GFP-cdc24-ts on a plasmid were streaked onto YPDA plates, followed by incubation at 25 or 37°C for 2 days. The strains used were YKT725 (CDC24), YKT1013 (cdc24-ts37), YKT1014 (cdc24-ts136), YKT1015 (cdc24-ts158), YKT1016 (cdc24-ts210), and YKT1017 (cdc24-ts233). (B) Localization of the GFP-Cdc24-ts proteins. Cells of the strains described for panel A were grown to the early logarithmic phase at 25°C, followed by incubation at 25 or 37°C for 1 h before observation by fluorescence microscopy. Bar, 5 μm. (C) Quantification of bud site-localized GFP-Cdc24. Unbudded and small-budded cells (n=100 for each) in panel B were examined for GFP-Cdc24 localization to the presumptive bud site or bud tip. The percentages of cells with polarized GFP-Cdc24 are shown. (D) GFP-Cdc24-ts mutant protein localization in cells released from a G0 arrest. Cells were arrested at 25°C in the stationary phase (G0) and released at 37°C by resuspension in prewarmed fresh medium, as described in Materials and Methods. The localization of GFP-Cdc24 was examined at 120 min after release. The percentage of cells with polarized GFP-Cdc24 is shown below each panel. The numbers in parentheses are the total numbers of unbudded and small-budded cells. Arrows indicate polarized GFP-Cdc24. The strains used were YKT1039 (wild type [WT]), YKT1041 (ts37), YKT1042 (ts136), YKT1043 (ts158), YKT1044 (ts210), and YKT1045 (ts233). Bar, 5 μm. (E) Mutant Cdc24 protein levels. cdc24Δ cells bearing GFP-HA-cdc24-ts on a plasmid were grown to the mid-logarithmic phase at 25°C and then incubated at 25 or 37°C for 1 h. Whole-cell extracts, prepared as described in Materials and Methods, were analyzed by immunoblotting for GFP-HA-Cdc24 with the monoclonal antibody 12CA5. The bands corresponding to GFP-HA-Cdc24 are indicated with arrows. The asterisks indicate a nonspecific cross-reacting band that could be used as a loading control. This band is present in a control strain that did not express GFP-HA-Cdc24 (–). The strains used were YKT1018 (WT), YKT1019 (ts37), YKT1020 (ts136), YKT1021 (ts158), YKT1022 (ts210), YKT1023 (ts233), and YKT1038 (–). (F) Domain structure of Cdc24 and amino acid substitutions in the Cdc24-ts proteins. The restriction sites used to map the mutations in the CDC24 coding sequence are indicated beneath. CH, calponin homology domain; DH, Dbl homology domain; PH, pleckstrin homology domain; PB1, Phox Bem1 domain. Mutants with single and triple amino acid substitutions are indicated with ovals and rectangles, respectively. An amino acid substitution that was not responsible for the growth phenotype is indicated with a dashed rectangle.
FIG 2 The NH2-terminal region of Cdc24 is involved in binding of the PB1 domain to Bem1. (A) Two-hybrid interaction of the Cdc24-ts proteins with Bem1. An activation domain fusion plasmid (pGAD-C1-HA-CDC24, pGAD-C1-HA-cdc24-ts, or control vector) was cotransformed with a LexA DNA-binding domain fusion plasmid (pBTM116-HA-BEM1) into L40 cells. The interaction was tested by growth on SD plates lacking Trp, Leu, and His (SD-Trp-Leu-His plates) supplemented with 5 mM 3-aminotriazole (3-AT) at 30°C for 4 days. Quantitative β-galactosidase activity assays using cells grown at 25 or 30°C were performed as described in Materials and Methods. Each value represents the average and standard deviation for three independent transformants. Protein expression in a reporter strain was confirmed by immunoblotting with the anti-HA antibody 12CA5 (right). The bands for Gal4AD-HA-Cdc24 and LexA-HA-Bem1 are shown. The asterisk indicates a nonspecific cross-reacting band that can serve as a loading control. (B) Schematic representation of the various constructs for the Cdc24 truncated mutants. (C) Two-hybrid interaction of the NH2-terminally truncated mutants of Cdc24 with Bem1. A DNA fragment encoding full-length Cdc24 or the NH2-terminally truncated mutants was cloned into an activating domain vector (pGAD-C2). The resulting plasmids or a control vector was cotransformed with a LexA DNA-binding domain fusion plasmid (pBTM116-HA-BEM1) into L40 cells. The interaction was tested by growth on SD-Trp-Leu-His plates at 30°C. Quantitative β-galactosidase activity assays were performed as in panel A. Cdc24-Δ(1-267) gave a weak interaction in the plate assay, but it was undetectable in the β-galactosidase assay. (D) Localization of the GFP-Cdc24 truncated mutants. cdc24 cells containing pKT1439 (pRS316-CDC24) were transformed with a plasmid bearing the gene encoding the indicated derivative of Cdc24 fused to GFP at its NH2-terminal end. The resulting transformants were grown to the early logarithmic phase in SD-Leu medium at 30°C. The cells were observed by fluorescence microscopy. The strains used were YKT1024 (full), YKT1025 [Δ(683-854)], YKT1026 [Δ(231-854)], YKT1027 [Δ(1-152)], YKT1028 [Δ(1-267)], and YKT1029 [Δ(1 to 372)]. The percentages of cells showing polarized localization of the GFP-Cdc24 truncated mutants are indicated. The numbers in parentheses are the total numbers of unbudded and small-budded cells. Bar, 5 μm.
Cdc24-Δ(1-152), in which the NH2-terminal region of the CH domain was removed, did not interact with Bem1 (Fig. 2C). Cdc24-Δ(1-267), with a further 115-amino-acid deletion, interacted weakly with Bem1, while Cdc24-Δ(1-372), the deletion in which extended into the DH domain, interacted with Bem1 at a level comparable to that for wild-type Cdc24. We confirmed that the truncated mutants were produced at a level comparable to that for full-length Cdc24 by immunoblotting (data not shown).

We examined the localization of a GFP-tagged version of these truncated cdc24 mutants using wild-type cells; truncated Cdc24 mutants should localize to the bud tip as long as they can interact with Bem1. As expected, GFP-Cdc24-Δ(683-854) and GFP-Cdc24-Δ(231-854), which lacked the COOH-terminal PB domain, did not localize to the incipient bud site or bud tip (Fig. 2D). Localization to polarized sites was not observed with GFP-Cdc24-Δ(1-152), was observed partially with GFP-Cdc24-Δ(1-267), and was seen clearly with GFP-Cdc24-Δ(1-372), consistent with our two-hybrid analysis results. Production of these truncated mutant proteins was confirmed using a 3xHA-tagged version, in which the 3xHA coding sequence was inserted between GFP and CDC24; all of the truncated mutants were detected at a level comparable to that for full-length Cdc24 by immunoblotting (data not shown). These results suggest that the CH domain plays an inhibitory role in exposing the PB1 domain and that the most NH2-terminal domain relieves this inhibition. Thus, the Cdc24 NH2-terminal region may regulate the unmasking of the PB1 domain, and our cdc24-ts mutants appear to be defective in exposing the PB1 domain.

Cdc24-ts proteins are defective in localization to a polarization site independent of Bem1. The interaction between Cdc24 and Bem1 has been implicated in enhanced assembly of these proteins and Cdc42 at a bud site (3, 4). Thus, the failure of the Cdc24-ts proteins to interact with Bem1 may explain the observed defects in the cdc24-ts mutants. However, Cdc24 seems to have an essential functional independent of Bem1, because Cdc24 is essential for budding, whereas Bem1 is not.

We next examined whether the Cdc24-ts proteins were defective in their initial polarized localization independent of Bem1. When three of the cdc24-ts mutations, the cdc24-ts37, cdc24-ts158, and cdc24-ts210 mutations, were combined with the bem1Δ mutation, synthetic growth defects were observed with cdc24-ts37 and cdc24-ts158 at 35°C, but not with cdc24-ts210 (Fig. 3A). This suggests that Cdc24-ts37 and Cdc24-ts158 proteins were impaired independent of interaction with Bem1. Our results do not indicate that Cdc24-ts210 is not impaired in this function, because it is possible that this function of Cdc24-ts210 is relatively normal at 35°C.

We next tested whether GFP-Cdc24 could localize to a bud site in the absence of Bem1. Gulli et al. (17) reported that Bem1 was not necessary for Cdc24 to localize to a bud site but was required for the maintenance of Cdc24 at the bud tip. To observe the localization of Cdc24 to the bud site in the bem1Δ mutant in our strain background, we synchronized wild-type with bem1Δ cells harboring GFP-CDC24 at G0 by nutrient deprivation and followed GFP-Cdc24 by fluorescence microscopy upon release in fresh medium at 25°C. GFP-Cdc24 localized to the incipient bud site or bud tip in bem1Δ cells within 120 min after release, although its efficiency was lower than that in wild-type cells; polarized localization of GFP-Cdc24 was observed in 76.5% of 119 unbudded and small-budded cells and 26.3% of 171 wild-type and bem1Δ cells (Fig. 3B).

To examine whether the Cdc24-ts proteins were defective in their initial polarized localization independent of Bem1, we compared the initial localization of GFP-Cdc24-ts proteins with that of wild-type GFP-Cdc24 in bem1Δ cells. To rule out the effects of synthetic defects between the bem1Δ and cdc24-ts mutations, the GFP-Cdc24-ts proteins were expressed in the presence of endogenous wild-type Cdc24. Because the bem1Δ mutant exhibited a ts growth defect (Fig. 3A), we conducted the experiments at a semirestrictive temperature (33°C). bem1Δ cells expressing GFP-CDC24 or GFP-cdc24-ts were synchronized by nutrient deprivation at 25°C and released into fresh medium at 33°C. At 120 min after release, wild-type GFP-Cdc24 localized to the site of growth.
polarization to some degree (7.8%), whereas almost no polarized localization was observed for the GFP-Cdc24-ts proteins (Fig. 3C). These data support the notion that the NH2-terminal region of Cdc24 plays a significant role in its initial polarized localization independent of Bem1.

The experiments in Fig. 3 could not be performed at 37°C, the restrictive temperature for the cdc24-ts mutants, because the bem1Δ mutant does not grow at 37°C. Thus, in our next set of experiments, we used a mutant Cdc24 specifically impaired in its interaction with Bem1. Substitutions in the conserved residues of the PC motif in the PB1 domain of Cdc24, D824K/D831R, abolished the interaction with the COOH-terminal PB1 domain of Bem1 (23). We confirmed that GFP-tagged full-length Cdc24(D824K/D831R) failed to interact with Bem1 at 25°C using two-hybrid analysis (Fig. 4A). GFP-Cdc24(D824K/D831R), when expressed as the sole copy of Cdc24, localized to the incipient bud site and bud tip 120 min after release at 25°C from the G0 arrest, although the efficiency (31.5% of the cells) was lower than that of the wild type (72.5%) (Fig. 4B). The cdc24(D824K/D831R) mutant exhibited a subtle ts growth defect compared with the severe growth defects in the cdc24-ts158 mutant (Fig. 4C). Consistent with this, GFP-Cdc24(D824K/D831R) was localized to the incipient bud site or bud tip in 29.8% of the cells 120 min after release at 37°C, whereas almost no polarized localization of GFP-Cdc24-ts158 (D824K/D831R) was observed (<0.4%) (Fig. 4D). These results suggest that the Cdc24-ts158 protein was impaired for polarized localization beyond an inability to interact with Bem1.

The Cdc24-ts proteins show defective localization to a polarization site in the absence of Rsr1 and Bem1. The results described above were obtained in a strain in which the polarization of Cdc24 occurs in a polarity cue-dependent manner. In symmetry breaking, Cdc24 accumulates at a cortical site in the absence of a polarity cue (e.g., the rsl1Δ mutant). It was recently shown that the major function of Bem1 in symmetry breaking is to physically link Cdc24 to the Cdc42 effector Cla4, a PAK; a protein fusion of Cdc24 with Cla4 bypassed the requirement for Bem1 in symmetry breaking (27). Thus, the role of the Cdc24 NH2-terminal region in the polarization of Cdc24 could be examined in the absence of Rsr1 and Bem1 using this system.

To examine the effects of cdc24-NH2-terminal-ts (referred to as cdc24-NT-ts) mutations on Cdc24 polarization in symmetry breaking, we constructed GFP-HA-cdc24-NT-ts-CLA4 fusion genes and expressed them in the rsl1Δ bem1Δ mutant. Wild-type GFP-HA-Cdc24-Cla4 was localized to polarized sites in the rsl1Δ bem1Δ mutant (Fig. 5A). cdc24-NT-ts-CLA4 suppressed the lethality of the rsl1Δ bem1Δ mutant at 25°C, but not at 37°C (Fig. 5B). Initial localization to a polarity site was examined in cells released after 120 min from nutrient deprivation. When cells were released at 25°C, the GFP-HA-Cdc24-NT-ts-Cla4 proteins were polarized to the incipient bud site or bud tip, although the efficiency was somewhat lower in cdc24-ts136-CLA4 and cdc24-ts121-CLA4 cells (Fig. 5C). In contrast, when cells were released at 37°C, the mutant proteins failed to localize to a polarity site, resulting in large, unbudded cells. We confirmed that these phenotypes were not due to degradation of the mutant proteins (Fig. 5D).

These results suggest that, in addition to defective interaction with Bem1, cdc24-NT-ts mutations cause another defect that leads to polarization defects in symmetry breaking.

The DH domain is also required for the polarization of Cdc24 in symmetry breaking. Our results suggest that the NH2-terminal region of Cdc24 has a novel function in the initial polarization of Cdc24. We assumed that the NH2-terminal region itself could be localized to a polarity site, but GFP-Cdc24(1-282) did not localize to a bud site (data not shown), consistent with previous data (52). Thus, the cdc24-NT-ts mutants seem to be defective
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in some regulatory function. Besides being defective in exposing the PB1 domain to Bem1, the cdc24-NT-ts mutants may also be defective in exposing the DH domain to Cdc42. It has been proposed that, in symmetry breaking, the GEF activity of Cdc24 plays an essential role in its polarized accumulation in a positive feedback loop: Cdc24 promotes the GTP loading of Cdc42, which recruits the Cdc24-Bem1-Cla4 complex by binding to Cla4, and newly recruited Cdc24 activates more Cdc42 (27). Thus, we next examined the effects of DH domain mutations on the polarized localization of Cdc24 in symmetry breaking.

Because the DH domain is essential (6), we expected that we would find DH domain mutants that showed normal polarization. Three such mutants, the cdc24-ts94, cdc24-ts160, and cdc24-ts127 mutants, were found (see Materials and Methods). These cdc24-DH-domain-ts mutants are referred to as cdc24-DH-ts mutants. DH domains are characterized by three conserved regions (CR1 to CR3); CR3 forms an exposed surface, which constitutes the major contact site for GTPases (47, 57). The cdc24-ts94 mutant contained a Y417C substitution in CR3, whereas cdc24-ts160 and cdc24-ts127 mutants contained R351G/E378G and S359P substitutions, respectively, in the less conserved intervening region between CR2 and CR3 (Fig. 6A). These mutants were arrested at G0 at 25°C and released at 37°C for 120 min. The mutants showed polarization of GFP-Cdc24, although the level of localization was relatively low (33.8%) in the cdc24-ts127 mutant (Fig. 6B). Because the polarization of the GFP-Cdc24-DH-ts proteins could be due to the action of Rsr1, we examined the localization of these DH domain mutants in the cdc24Δ rsr1Δ mutant. However, these mutants were similarly polarized in the absence of Rsr1 (Fig. 6C).

We next examined the effects of these DH mutations on the polarization of Cdc24 in symmetry breaking by expressing the CLA4-fused mutant genes in the rsr1Δ bemi1Δ mutant. Unexpectedly, the polarization efficiency varied depending on the mutant allele: the polarization of GFP-HA-Cdc24-ts127-Cla4 was decreased to 2.3%, whereas GFP-HA-Cdc24-ts94-Cla4 and GFP-HA-Cdc24-ts160-Cla4 were efficiently polarized (Fig. 6D). We speculated that a mutant Cdc24-DH-ts-Cla4 could oligomerize with endogenous wild-type Cdc24 via its DH domain to be localized to a polarity site, as described previously (35). Thus, GFP-HA-cdc24-DH-ts-Cla4 genes were expressed in the cdc24Δ rsr1Δ bemi1Δ mutant. Interestingly, cdc24-ts94-CLA4 and cdc24-ts127-CLA4 strains exhibited extremely slow growth even at 25°C (Fig. 6E), suggesting that DH domain function was important in symmetry breaking in these strains. Consistent with this, although the cdc24-ts160-CLA4 mutant could grow at 25°C, it completely lost its polarized localization when released at 37°C (Fig. 6F). Thus, at least for symmetry breaking with CLA4-fused CDC42 genes in the cdc24Δ rsr1Δ bemi1Δ mutant, the DH domain was essential for the polarized localization of Cdc24. Defective polarization of the Cdc24-NT-ts-Cla4 proteins in symmetry breaking may be due to impaired activation of the GEF. In symmetry breaking with Cdc24-DH-ts (nonfused normal type) in the cdc24Δ rsr1Δ mutant, BEM1 may possess a suppressor function for the cdc24-DH-ts mutations.

Our results implicate that the cdc24-NT-ts mutants are also defective in exposing the DH domain to Cdc42. Interaction of Cdc24 and Cdc42 could be estimated by the two-hybrid method using Cdc42-D57Y, a mutant locked in the GDP-bound form (10). As shown in Fig. 7, Cdc24-ts proteins did not interact with Cdc42-D57Y at 30°C. Thus, polarization defects in Cdc24-NT-ts
Cdc24 DH domain mutants are defective in polarized localization in symmetry breaking. (A) Identification of DH domain mutants in Cdc24. Amino acid substitutions in Cdc24-ts94, Cdc24-ts127, and Cdc24-160 are shown, with an alignment of DH domains from the Rho-GEFs of various organisms. CR1 to CR3 are conserved regions. The amino acid sequences were initially aligned using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html) and then shaded according to BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The GenBank accession numbers are as follows: Cdc24, NP_009359; Scd1, NP_594221 (Schizosaccharomyces pombe); Dbl, NP_001093325 (Homo sapiens); Vav3, NP_006104 (H. sapiens); Tiam1, AAA98443 (H. sapiens); Sos-1, NP_005624 (H. sapiens).
proteins may be caused by simultaneous defects in the interaction with Bem1 and Cdc42. If Cdc24-NT-ts proteins mimic an inactive conformation before budding initiation, they may also interact with Rsr1, an upstream regulator of Cdc24, in polarity cue-breaking, in which GFP-Cdc24-Cla4 is polarized independent of budding. These results suggest that Cdc24-NT-ts proteins are in an inactive conformation but not in a misfolded form.

**DISCUSSION**

The NH2-terminal domain of Cdc24 plays an essential role in its polarized localization. In this study, we isolated new mutant alleles of CDC24 among a collection of ts cdc24 mutations, based on the failure of their products to localize to a polarity site. Our approach should shed light on an important region for Cdc24 polarization without bias, because these mutants were selected from randomly mutagenized full-length CDC24. The amino acid substitutions in these mutants were mapped to the NH2-terminal region, the role of which in the regulation or function of Cdc24 is poorly understood.

The NH2-terminal 282 amino acids in Cdc24 contain the CH domain (residues 137 to 241). The role of the CH domain was characterized in the landmark-directed localization of Cdc24. Cells expressing cdc24-G168D exhibited a random budding pattern due to reduced interactions with Rsr1 (50), whereas cells expressing cdc24-S189P or cdc24-S189F were defective in mating due to reduced interactions with Far1p (39, 40). However, our cdc24-210 (D156V) and cdc24-ts233 (S198P) mutants, both of which possessed mutations in the CH domain, exhibited normal bud site selection and normal mating, at least at 30°C (data not shown), but exhibited temperature-dependent growth defects. The amino acid alterations in the cdc24-ts mutants were distributed throughout the NH2-terminal region (Fig. 1F). Thus, the entire NH2-terminal domain, rather than the CH domain, seems to play an essential role in the polarized localization of Cdc24.

The NH2-terminal domain of Cdc24 is required for its interaction with Bem1. Although the Cdc24-ts proteins had intact PB1 domains, all of them failed to interact with Bem1. This did not seem to result from denaturation of the Cdc24-ts proteins, because Cdc24-ts proteins interacted with Far1 (Fig. 7). Thus, exposure of the PB1 domain in the Cdc24-ts proteins may be defective. A simple explanation for this is that the NH2-terminal domain has an inhibitory effect on the PB1 domain and that wild-type Cdc24 can be released from this inhibition by an upstream (e.g., cell cycle) signal, while mutant Cdc24 proteins cannot. A similar model was proposed for the Rsr1-mediated activation of Cdc24 (50). Consistent with this, truncation of the NH2-terminal 152 amino acids impaired the protein’s interaction with Bem1, while further truncation (to residue 372) partially restored the interaction (Fig. 2B, C). These results suggest that the defective polarization of the Cdc24-ts proteins was due to the loss of interaction with Bem1. However, this does not seem to be the ultimate reason for the defective polarization of Cdc24: wild-type Cdc24 was weakly polarized in the absence of Bem1, while mutant Cdc24 proteins were not.

The NH2-terminal domain of Cdc24 is required for its polarized localization in symmetry breaking. We also examined the polarized localization of GFP-Cdc24-ts proteins in symmetry breaking, in which GFP-Cdc24-Cla4 is polarized independent of Bem1 and Rsr1. The GFP-Cdc24-ts-Cla4 proteins still localized to the polarity site in a temperature-dependent manner. Thus, the
NH₂-terminal region of Cdc24 possesses a novel function in polarization, independent of Bem1 and Rsr1. The NH₂-terminal region was not polarized by itself, suggesting that it plays a regulatory role. We propose that the NH₂-terminal region may also regulate the function of the DH domain, because a functional DH domain was required for the polarization of GFP-Cdc24-Cla4 in symmetrical breaking in the cdc24Δ rrs1Δ bem1Δ mutant and because Cdc24-ts proteins did not interact with Cdc42-D57Y. Consistent with this, Kozubowski et al. (27) suggested that the GEF activity of Cdc24 plays an essential role in polarization of the Cdc24-Bem1-Cla4 complex in symmetry breaking. Additionally, Cdc24 was shown to form oligomers via its DH domain, and mutational analysis of the DH domain showed that oligomerization was required for the polarized localization of Cdc24, independent of its catalytic activity (35). Because the NH₂-terminal region is required for exposure of the PB1 domain in Bem1 binding, it is conceivable that the NH₂-terminal region is also required for exposure of the DH domain during the interaction with Cdc42. Interestingly, a functional DH domain was not required for the polarization of GFP-Cdc24 in the cdc24Δ rrs1Δ mutant. In symmetry breaking in this strain, BEM1 may possess a suppressor function for the cdc24-DH-ts mutations in addition to its adaptor function linking Cdc24 to Cla4.

Cdc24-ts proteins did not interact with Bem1, Cdc42, and Rsr1 but interacted with Far1 at 30°C, suggesting that Cdc24-ts proteins are defective in conversion to an active conformation. When the two-hybrid experiments were performed at 35°C, Cdc24-ts proteins did not interact with Far1 (data not shown). However, the two-hybrid results seem to underestimate the functionality of the Cdc24-ts proteins, because cdc24-ts mutants grew efficiently and exhibited an axial budding pattern at 30°C, a temperature at which the two-hybrid interactions between Cdc24 and Bem1/Cdc42/Rsr1 were impaired. Therefore, interactions between Cdc24-ts proteins and Far1 may not be completely impaired at the restrictive temperature. We propose that Cdc24-ts proteins are kept in an inactive conformation, in which the NH₂-terminal Rsr1-interacting domain masks the DH domain as well as the PB1 domain, but we cannot exclude the possibility that Cdc24-ts proteins are rather misfolded at 37°C.

Intramolecular autoinhibition is widely observed in mammalian GDP/GTP exchange factors for the Rho family (8, 32, 37, 60, 61). The GDP/GTP exchange activity of these GEFs is inhibited by a small internal region that interacts with the DH domain. Interaction with activating proteins or phosphorylation relieves these GEFs from inhibition, resulting in exposure of the DH domain to Rho GTPases. Of these mammalian GEFs, the Vav1 oncprotein has a CH domain at its NH₂-terminus like Cdc24, and this CH domain participates in autoinhibition together with the acidic domain, which lies between the CH and DH domains (60). Because the CH domain of Cdc24 showed an inhibitory effect on the interaction with Bem1 (Fig. 2), it may also play an inhibitory role in the activation of the DH domain as in Vav1. Interestingly, a BLAST search using the CH domain of Cdc24 against human protein sequence databases indicated that it is most similar to the CH domain of Vav proteins (28% identity and 42% similarity; data not shown).

How is the NH₂-terminal region involved in the conversion of Cdc24 to an active conformation? Phosphorylation is one plausible mechanism. The relief of autoinhibition by phosphorylation has been reported for several mammalian GEFs in the Rho family, including Vav1 (2, 29, 58). The GEF activity of Vav1 is autoinhibited by the binding of a helix from the acidic domain to the active site in the DH domain. This inhibitory interaction is relieved by the phosphorylation of Tyr174 in the inhibitory helix (2). Mass spectrometry indicated that Cdc24 is, in fact, phosphorylated at numerous sites in vivo, including the NH₂-terminal region. However, exhaustive mutagenesis of these phosphorylated sites did not produce a change in cell growth and morphology (55). However, we cannot exclude the possibility that substoichiometric phosphorylation is responsible for the activation of Cdc24. Interestingly, mutation of the Tyr200 residue in the CH domain to a nonphosphorylatable Phe residue resulted in aberrant localization of GFP–Cdc24-Y200F (9). Cla4 has been shown to phosphorylate Cdc24 in a Cdc42-GTP-dependent manner, both in vitro and in vivo (3, 17). Conflicting models, stimulatory (3) and inhibitory (17), have been proposed for the role of Cla4-mediated Cdc24 phosphorylation in the assembly of polarity proteins. In symmetry breaking, it has been proposed that, once Cdc42-GTP is produced by Cdc24, Cdc42-GTP activates Cla4, which in turn activates Cdc24 in the same complex by phosphorylation, and then active phospho-Cdc24 produces more Cdc42-GTP, resulting in a positive feedback loop (27). Thus, interesting questions remain as to whether Cla4 phosphorylates the NH₂-terminal domain of Cdc24 to activate the DH domain and release the PB1 domain and whether Cdc24-ts proteins are defective in this activation.

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