Cdc42 activation state affects its localization and protein levels in fission yeast

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Abstract

Rho GTPases control polarized cell growth and are well-known regulators of exocytic and endocytic processes. Cdc42 is an essential GTPase, conserved from yeast to humans, that is critical for cell polarization. Cdc42 is negatively regulated by the GTPase-activating proteins (GAPs) and the GDP dissociation inhibitors (GDIs), and positively regulated by guanine nucleotide exchange factors (GEFs). Cdc42 GTPase can be found in a GTP- or GDP-bound state, which determines the ability to bind downstream effector proteins and activate signalling pathways. Only GTP-bound Cdc42 is active. In this study we have analysed the localization of the different nucleotide-bound states of Cdc42 in Schizosaccharomyces pombe: the wild-type Cdc42 protein that cycles between an active and inactive form, the Cdc42G12V form that is permanently bound to GTP and the Cdc42T17N form that is constitutively inactive. Our results indicate that Cdc42 localizes to several membrane compartments in the cell and this localization is mediated by its C-terminal prenylation. Constitutively active Cdc42 localizes mainly to the plasma membrane and concentrates at the growing tips where it is considerably less dynamic than wild-type or GDP-bound Cdc42. Additionally we show that the activation state of Cdc42 also participates in the regulation of its protein levels mediated by endocytosis and by the exocyst complex.

INTRODUCTION

Cdc42, a member of the Rho family of small GTPases, plays a central role in the regulation of polarity in all eukaryotic cells [1]. As a Rho GTPase, Cdc42 can be found in a GDP- or GTP-bound state, which determines the ability to bind downstream effector proteins and activate signalling pathways. Only GTP-bound Cdc42 is active. As with other Rho GTPases, Cdc42 activity is positively regulated by GEFs (guanine-nucleotide-exchange factors) and negatively regulated by GAPs (GTPase-activating proteins) and GDIs (GDP-dissociation inhibitors). GDIs interact with membrane-bound GTPases to mask their prenylated C-terminal domain, resulting in the extraction from the plasma membrane [2].

To promote cell polarization, Cdc42 localization and activation need to be precisely regulated both spatially and temporally (for a review see [3]). In mammals, during wound-induced directed cell migration, Cdc42 is recruited to the wound edge to induce the polarization of the cells toward the wound [4, 5]. In the yeast Saccharomyces cerevisiae, Cdc42 accumulates at the presumptive growth site, where it promotes polarized cell growth [6] or the formation of mating projection in response to pheromone [1]. In the fission yeast Schizosaccharomyces pombe, Cdc42 function is also required for polarized growth, and loss of Cdc42 function results in defects in both cell growth and morphology [1, 7, 8]. Active Cdc42 accumulates to the growth sites, poles and septum [9]. Once activated, Cdc42 interacts with its effectors, organizing the actin cytoskeleton and membrane trafficking to promote polarized secretion and growth. In turn, polarized secretion may reinforce the delivery of Cdc42 GEFs to the growth area generating a positive feedback mechanism that plays a crucial role in maintaining the concentrated and polarized distribution of active Cdc42 [10]. In Saccharomyces cerevisiae, this positive feedback requires a complex containing the GEF Cdc24, and the scaffold Bem1 [11, 12]. Additionally, a negative feedback is activated by the Cdc42-stimulated effector kinase Cla4 that also binds to Bem1 and negatively regulates Cdc42 by phosphorylating Cdc24 [13]. Septins also contribute to negative feedback signalling by recruiting Cdc42 GAPs to the polarity cap, where they destabilize Cdc42 [14]. Vesicle trafficking in budding yeast not only...
controls Cdc42 GEF localization and function, but also controls Bem3, one of the Cdc42 GAPs. Bem3 polarization depends on the integrity of both the endocytic and secretory pathways [15]. In *Schizosaccharomyces pombe*, Cdc42 activates the formin For3, responsible for actin cable nucleation during interphase [8, 16], and participates in the localization of the exocyst, a complex involved in tethering the secretory vesicles to the plasma membrane [7, 17]. The kinase Pak1, homologue of Cla4, might regulate Cdc42 activity, likely by altering the intracellular distribution of Scd2, a Bem1 homologue, and the Cdc42-GEF Scd1 [18].

In addition to the regulation of Cdc42 activity, the concentration of this GTPase at the plasma membrane is also tightly controlled in *Saccharomyces cerevisiae*. Two mechanisms for Cdc42 recycling have been proposed, one through endocytosis and the other dependent on the GDI regulator that extracts inactive Cdc42 from the membrane [19]. A recent work also proposes that an actin-independent pathway of Cdc42 recycling acts in parallel with the GDI [20]. In *Schizosaccharomyces pombe* it has been recently described that Cdc42 is more concentrated in the growth areas, where it is mainly active and bound to GTP, because active Cdc42 is less mobile than inactive Cdc42 located to the cell sides [21]. In contrast to *Saccharomyces cerevisiae*, recycling mechanisms such as GDI or endocytosis do not play a major role in concentration of *Schizosaccharomyces pombe* Cdc42 at the growth areas of the plasma membrane [21].

In this study, different mechanisms involved in the localization and concentration of wild-type and mutant alleles of Cdc42 were analysed using *Schizosaccharomyces pombe* mutant strains affecting the endocytosis and exocytosis processes.

**METHODS**

**Strains, growth conditions and genetic methods**

Standard *Schizosaccharomyces pombe* media and genetic manipulations were used [22]. All the strains used were isogenic to wild-type strains 972 h⁻ and 975 h⁺, and are described in Table 1. GFP-cdc42, GFP-cdc42G12V and GFP-cdc42T17N under the control of the cdc42 promoter were cloned into the pJK148 plasmid and integrated at the leu1¹ locus. *Schizosaccharomyces pombe* was transformed by the lithium acetate method [23]. When needed, strains were constructed by either tetrad dissection or random spor germination. Cells were grown in rich medium (YES) with appropriate supplements.

*Escherichia coli* DH5α was used as host for propagation of plasmids. Bacteria were cultured in LB medium supplemented with 50 µg ml⁻¹ ampicillin.

**Western blot analysis**

Extracts from 1 x 10⁹ cells expressing different tagged proteins were obtained as described [24], using 200 µl lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 % NP-40) containing protease inhibitors (2 µg ml⁻¹ aprotinin, 2 µg ml⁻¹ leupeptin, 2 µg ml⁻¹ pepstatin, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride). Proteins were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and blotted with the corresponding antibodies and the ECL detection kit (GE Healthcare). Twenty micrograms of protein were loaded for Western blot analysis and hybridized with anti-GFP monoclonal antibody (JL-8; Clontech) at 1 : 2000 dilution or with high affinity anti-HA (Roche) at 1:10 000 dilution. Cdc2 and tubulin were used as loading controls and were detected with anti-Cdc2 monoclonal antibody (Y1004; Abcam) at 1 : 5000 dilution and monoclonal anti-tubulin monoclonal antibody (DM1A; Sigma) at 1 : 10 000 dilution, respectively.

**Sucrose density gradient centrifugation**

Cell lysates were prepared and analysed as described by Santos and Snyder [25]. Briefly, cells were grown in YES (0.5 % yeast extract, 3 % glucose plus adenine, leucine, lysine, uracil and histidine 225 mg l⁻¹), to mid log phase (4–5 x 10⁶ cells ml⁻¹), and 5 x 10⁶ cells were resuspended in 6 ml of 17 % sucrose (w/v) in 50 mM Tris-HCl pH 7.5/1 mM EDTA containing protease inhibitors and 6 ml of acid-washed glass beads (G8772; Sigma). Cells were bead-beaten on a FastPrep (Millipore; five pulses of 30 s at 6.5 rpm), and the crude extracts were centrifuged at 1500 g for 10 min. Supernatants were layered on top of a 33 ml linear sucrose gradient (10–65 %, w/v) in 50 mM Tris-HCl/1 mM EDTA, pH 7.5. The tubes were centrifuged in a SW28 rotor at 25 000 r.p.m. for 20 h at 4 °C (Beckman Instruments). One-millilitre fractions were collected from the bottom of the tube with the use of a peristaltic pump, assessed for sucrose concentration with an optical densitometer to compare the fractions of similar sucrose concentration (fraction 1 is the bottom of the tube) and subjected to Western blot analysis. Blots were hybridized with antibodies against Pma1 (kindly provided by Dr Roncero, IBFG, Salamanca, Spain), GFP monoclonal antibody (JL-8; Clontech) to detect Gtr1-GFP, SPAC1B2.03c-GFP (endoplasmic reticulum marker) and GFP-Syb1 fusion proteins, and DsRed polyclonal antibody (Living Colors, Clontech) to detect Anp1-mCherry.

**Microscopy techniques**

Cell samples were observed using an Olympus IX71 microscope equipped with a personal Delta Vision system and a Photometrics CoolSnap HQ2 monochrome camera. Stacks of 11 z-series sections were acquired at 0.4 µm intervals. Fluorescence images were deconvolved with softWoRx software (Applied Precision) for posterior analysis. All fluorescence images shown are median planes of the cells. Measurements were made using Image software (National Institutes of Health). Fluorescence recovery after photobleaching (FRAP) experiments were performed on an Olympus IX81 spinning disk confocal microscope. A 3.5 µm² circular region of the cell tip or the cell side was bleached with a sequence of three high-intensity laser iterations following two pre-bleach acquisitions. Post-bleaching images were taken every second over a period of 30 s. For FRAP analysis, intensities of bleached regions were compared to the control and expressed as percentage recovery (towards 100 %) relative to the fluorescence level at baseline.
associates with different cellular membranes through the
prenylation of the C-terminal CAAX box. Although Cdc42 is
primarily transported to the plasma membrane, a significant
fraction of Cdc42 localizes to the Golgi in mammalian cells [27, 28], and it has also been described in the yeast vacuole
membrane where it controls actin polymerization activity
during vacuole fusion [29]. By microscopic studies, fission yeast
Cdc42 labelled with GFP is also observed in several cell membranes [7, 21]. To further analyse with which endomembranes
Cdc42 is associated, we performed sucrose gradient assays
with lysates of cells of different strains containing functional
HA-Cdc42 and different known labelled proteins as markers
for specific cellular membrane compartments (Fig. 1a): Pma1
is an ATPase that localizes to the plasma membrane (fractions
3–6, peak in fraction 4) [30, 31]; Syb1 is a v-SNARE localized
to the plasma membrane, secretory vesicles and endosomes
(fractions 3–7, peak at fraction 5) [32]; Anp1 is part of a man-
osyl-transferase complex located at the Golgi apparatus (fractions 4–10, peak at fraction 7–8) [33]; SPAC1B2.03c is a SUR4
family protein used as a marker for the endoplasmic reticulum
(ER) (fractions 9–11, peak at fraction 10) [33]; and Gtr1 is a
vacuolar membrane protein (fractions 10–12, peak at fraction

Table 1. Strains used in this study

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11) [34]. HA-Cdc42 was detected along the gradient from fraction 2 to fraction 11 (Fig. 1a), indicating that it is present at several cellular membranes, including plasma membrane, ER, Golgi apparatus and vacuoles. It is therefore possible that the different functions that Cdc42 exerts in cell polarity and cell traffic regulation are due to its association with different protein complexes and cell compartments.

**Active Cdc42 preferentially localizes to the plasma membrane**

Cdc42 acts as a molecular switch in its active state bound to GTP or in its inactive state bound to GDP. Simultaneous analysis of mCherry-Cdc42 and CRIB-GFP, that recognizes the GTP-bound form of Cdc42, suggested a strong correlation between Cdc42 enrichment and the active Cdc42 form at the growth areas [21]. To further analyse whether the localization of the GTPase in the cell depends on its activation state, we constructed strains containing N-terminal GFP-tagged versions of Cdc42, Cdc42G12V (bound to GTP) and Cdc42T17N (bound to GDP) expressed under the cdc42 promoter. These constructs were integrated at the leu1 locus of a wild-type strain carrying the endogenous copy of cdc42 because the GTP/GDP-locked alleles are lethal when they are the only source of Cdc42. These strains do not display growth defects (Fig. S1, available with the online Supplementary Material). N-terminal tagging GTPases are partially functional, but the published localization of the fully functional Cdc42-GFP [21] is similar to the one presented here. As previously described [7], wild-type GFP-Cdc42 localized to several cell membranes but more

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**Fig. 1.** Cdc42 localizes to various membrane compartments in the cell. (a) Cell lysates were prepared from yeast strains and fractionated in 10–65 % sucrose density gradients by centrifugation for 20 h. Fractions were collected from the bottom of the gradient and analysed by immunoblot analysis with the use of corresponding antibodies (HA, Pma1, GFP or dsRed). The following strains were used: PPG47.11, HA-Cdc42; PPG115.79, SPAC1B2.03-GFP; PPG85.65, Anp1-mCherry and GFP-Syb1; and PPG106.57, Gtr1-GFP). The equivalence of the different gradients was made by measurement of the sucrose concentration of the fractions. (b) The mean of five sucrose gradient fractionation experiments is shown. (c) Graphics showing the gradient position and the protein amount of the different markers compared with HA-Cdc42 profile.
perceptibly to the plasma membrane, and the concentration was increased at the growing poles and the septum (Fig. 2a). The constitutively active form of Cdc42 (GFP-Cdc42G12V) localized mainly to the plasma membrane, and, like the wild-type Cdc42, the fluorescence signal was stronger at the growth poles (Fig. 2a). On the contrary, localization of the inactive form GFP-Cdc42T17N at the plasma membrane was not increased over internal membranes and concentration at the poles was not observed (Fig. 2a). Quantification of the amount of Cdc42 protein at the plasma membrane is presented in Fig. 2b. Active Cdc42 is enriched at the plasma membrane, whereas inactive Cdc42 shows reduced accumulation at growth sites.

Western blot analysis demonstrated that the total protein levels of wild-type Cdc42, constitutively active Cdc42G12V and inactive Cdc42T17N were similar (Fig. 2c). Therefore, the higher signal observed for Cdc42G12V at the plasma membrane was not due to an increased amount of total protein. These results further demonstrate that active GTP-bound Cdc42 concentrates at the plasma membrane.

**Active Cdc42 is more stably associated to the plasma membrane than wild-type or inactive Cdc42**

It has been described that Cdc42 is highly mobile at the plasma membrane, but displays reduced mobility at cell tips [21]. These differences in mobility correlate with a higher concentration of GTP-bound Cdc42 at the cell tips, as measured using the CRIB-GFP probe [9, 21]. To further explore these observations we tested the dynamics of the different Cdc42 forms at the plasma membrane by performing FRAP experiments where one of the cell tips was photo-bleached and signal recovery was followed. Using the same bleach spot in all experiments, we found that fluorescence recoveries of the wild-type GFP-Cdc42 and the dominant negative GFP-Cdc42T17N version were similar (Fig. 3); halftime recovery was about 5 s and 70% of the protein fluorescence was recovered after 25 s. By contrast the

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**Fig. 2.** Active Cdc42 localizes mainly at the plasma membrane. (a) Fluorescence micrographs of cells grown at 28°C carrying a copy of GFP-cdc42 (PPG98.05), GFP-cdc42G12V (PPG98.61) and GFP-cdc42T17N (PPG98.63) integrated at the leu1 locus. Bar, 5 µm. N, nucleus; V, vacuole. (b) Quantification of the amount of Cdc42 forms at the plasma membrane was performed using the maximum value in a line scan perpendicular to the cell pole minus the background mean value. Values were normalized considering wild-type levels as reference. Data correspond to more than 120 cells in three independent experiments. (c) Levels of GFP-Cdc42, GFP-Cdc42G12V and GFP-Cdc42T17N in cell extracts were analysed by Western blot. Tubulin was used as loading control. Quantification of relative protein levels is shown. Error bars correspond to standard deviation.
fluorescence recovery of the GFP-Cdc42G12V form was much slower; it took more than 30 s to get 40% recovery (Fig. 3). This observation suggests that the constitutively active form remains longer at the plasma membrane. These results corroborate that GTP-bound Cdc42 exhibits slower mobility than GDP-bound Cdc42 [21] and suggest that the active form of the GTPase is less efficiently removed from the plasma membrane than the wild-type or the inactive form.

**Cdc42 membrane localization requires C-terminal geranyl-geranylation**

Cdc42 carries a C-terminal CAAX-box that serves as a prenylation signal and inserts a geranyl-geranyl group at the cysteine residue. Cwg2 is the β subunit of the geranyl-geranyl transferase that is essential for the cell [35]. To analyse whether the membrane localization of Cdc42 is dependent on the prenyl group, we tested the localization of GFP-Cdc42, GFP-Cdc42G12V and GFP-Cdc42T17N in a cwg2-1 thermosensitive mutant. As expected, no membrane localization of the GFP constructs was observed even at permissive temperature, suggesting that localization of Cdc42 to the plasma or internal membranes requires the geranyl-geranyl group at the C-terminus (Fig. 4). However, GFP-Cdc42G12V was still clearly detected at the division area of the cwg2-1 mutant (Fig. 4) suggesting that additional factors can localize active GTP-Cdc42 at this region. One of those factors could be the BAR protein Hob3, which is involved in the early localization of Cdc42 and Gef1 to the ring during septum formation in *Schizosaccharomyces pombe* [36]. Analysis of the different GFP-Cdc42 versions in *hob3Δ* cells confirmed that Cdc42 is localized to internal and plasma membranes but does not concentrate to the septum area of the cells at the beginning of cytokinesis (Fig. S2). Notably, the expression of GFP-Cdc42G12V was able to suppress the multiseptation defect of *hob3Δ* cells, suggesting that constitutively active Cdc42 could still be partially localized and exert its function during septation in the absence of Hob3, (Fig. S2). In fact, GFP-Cdc42G12V was able to localize to the division area in a *cwg2-1 hob3Δ* double mutant (Fig. S3). This result indicates that Cdc42 localization at the division area involves prenylation and Hob3 and suggests that other proteins might also be involved in the localization of the active Cdc42.

**Endocytosis regulates Cdc42 levels**

It has been reported that *Saccharomyces cerevisiae* Cdc42 dynamics depends on both the GDI that mediates membrane extraction, and on vesicle trafficking. The GDI mediates a fast recycling pathway, while actin patch-mediated endocytosis accounts for a slower one [19]. In contrast, *Schizosaccharomyces pombe* Cdc42 dynamics at the plasma membrane is largely independent of GDI-mediated
membrane extraction or endocytosis and plasma membrane recycling [21].

We analysed the localization of the different Schizosaccharomyces pombe Cdc42 forms in endocytosis mutants such as end4 at the semipermissive temperature of 32°C to determine if the constitutively active or dominant negative forms of Cdc42 are differentially regulated by endocytosis. There were no differences in the localization pattern of the Cdc42 forms, but a remarkable increase in GFP-Cdc42 fluorescence at both plasma and internal membranes (Fig. 5a). Moreover, Western blot analysis showed increased levels of the wild-type Cdc42 and the constitutively active GFP-Cdc42G12V form, suggesting that endocytosis could be a mechanism for degrading these forms of the GTPase (Fig. 5b, c). By contrast, the protein levels of the inactive GFP-Cdc42T17N were not altered in the end4 mutant compared to wild-type cells (Fig. 5b, c).

We also analysed whether Rdi1, the unique GDI in Schizosaccharomyces pombe, has an effect on the membrane localization or levels of the constitutively active or dominant negative Cdc42 forms. The localization of these forms in the rdi1 mutant was similar to that in wild-type cells, and the protein levels of GFP-Cdc42, GFP-Cdc42G12V and GFP-Cdc42T17N were not affected (Figs 5a and S4).

Cdc42 levels are altered in exocyst mutants

The exocyst is an essential conserved complex that functions in exocytosis and tethers the secretory vesicles to the plasma membrane [37]. Cdc42 likely follows the secretory pathway due to the prenylation signal that helps its insertion in the ER membrane [38]. We tested whether the exocyst participates in the localization of the different Cdc42 forms.

Exo70 is the only non-essential subunit of the exocyst complex [39]. In exo70 mutants, a general increase of protein levels of all Cdc42 forms was found (Fig. 6b, c), leading to increased levels of GFP-Cdc42, GFP-Cdc42G12V and to less extent of GFP-Cdc42T17N both at the plasma membrane and on internal membranes (Fig. 6a). The same results were observed by using the sec8-1 thermosensitive exocyst mutant even at the permissive temperature of 28°C (Fig. S5). These findings suggest that the exocyst could participate in Cdc42 removal from the plasma membrane. Another possibility is that, in the absence of a functional exocyst, alternative and more efficient routes are used for the delivery of Cdc42 to the membrane.

DISCUSSION

Cells promote polarized growth by activation of the Rho-family protein Cdc42 at the cell membrane (for a review see [40]). How is Cdc42 transported and anchored to the membrane? Cdc42 carries a C-terminal CAAX box that serves as a prenylation signal and allows the insertion of the protein into the plasma membrane [41]. As we show here, corroborating recent results [42], this post-translational modification is essential for Cdc42 localization.
Surprisingly, the constitutively active Cdc42G12V can still localize to the division area in a \textit{cwg2} mutant that impairs prenylation, suggesting an alternative mechanism for active Cdc42 localization. Although Hob3 is required for the early localization of Cdc42 and Gef1 to the division area [43], this mechanism is not dependent on Hob3 because, in its absence, Cdc42G12V localization to the septum still occurs. One possibility is that active Cdc42 uses other proteins such as the Pob1 scaffold [16] and/or the septins to localize to the ring during early septum formation.

Vesicle delivery through actin cables has been proposed as a positive feedback for generating and maintaining Cdc42 at the plasma membrane [44]. In \textit{Schizosaccharomyces pombe}, actin cables appear not to be the only mechanism for Cdc42 delivery to the membrane because localization of Cdc42 is not affected in a for3Δ mutant [21]. Cdc42 interacts with the exocyst complex [7, 45], but when exocyst function is compromised in \textit{exo70Δ} or \textit{sec8-1} mutants, Cdc42 protein levels and localization to the plasma membrane are unexpectedly increased. The exocyst could be involved in removing Cdc42 from the membrane, affecting, for example, the transport of a factor required for its dynamics at the membrane. In \textit{Saccharomyces cerevisiae}, it has been reported that defects in the exocyst might affect membrane trafficking balance and, as a consequence, endocytosis [46]. However, the \textit{Schizosaccharomyces pombe sec8-1} mutant does not have endocytosis defects [47]. Similarly to budding yeast, exocyst defects would affect the fusion of the Cdc42-containing secretory vesicles with the plasma membrane that may dilute the local surface density and negatively regulate the amount of Cdc42 protein in the plasma membrane [48]. Alternatively, exocyst-independent routes for Cdc42 delivery to the membrane could exist.

Cdc42 is activated by two GEFs, Scd1 and Gef1, which both localize to the cell poles and the septum, consistent with their role in promoting local Cdc42 activation [49, 50]. We have shown here that constitutively active Cdc42 is mainly localized at the plasma membrane. Similar results were reported using the colocalization of mCherry-Cdc42 at the membrane with the marker of active Cdc42, CRIB-GFP [21]. We have presented evidence indicating that active Cdc42 remains at the membrane longer than wild-type Cdc42 or inactive GDP-Cdc42, suggesting that activation slows down lateral diffusion and/or membrane–cytosol exchange. Recently, it has been reported that lateral diffusion is slowed down [21]. This slower mobility may be due to the formation of large complexes, the formation of microdomains, or multimerization [21]. Differential mobility of GDP-Cdc42 and GTP-Cdc42 may be sufficient to explain polarization without the requirement for directed

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\textbf{Fig. 5.} Cdc42 levels are affected in endocytosis mutants. (a) Fluorescence micrographs of \textit{Schizosaccharomyces pombe} cells grown at 32°C carrying a copy of GFP-cdc42, GFP-cdc42G12V or GFP-cdc42T17N integrated at the \textit{leu1} locus in wild-type (PPG98.05, PPG98.61 and PPG98.63, respectively), \textit{rdi1Δ} (PPG105.52, PPG105.54 and PPG105.56, respectively) and \textit{end4Δ} (PPG111.09, PPG111.11 and PPG111.13, respectively) mutants. Bar, 5 µm. N, nucleus; V, vacuole. (b) Levels of GFP-Cdc42, GFP-Cdc42G12V and GFP-Cdc42T17N in cell extracts of the \textit{end4Δ} mutant compared with wild-type were analysed by Western blot. Anti-Cdc2 was used as a loading control. (c) Quantification of relative protein levels is shown. Error bars correspond to standard deviation.
delivery to the tips. Local activation of Cdc42, and not local delivery, is necessary for polarization in budding yeast [12]. In Saccharomyces cerevisiae, a positive-feedback activation pathway requires a complex formed by Cdc24 (a GEF for Cdc42), Bem1 and the Cdc42 effector, PAK [11]. Negative-feedback pathways, where Cdc24 is inhibited by PAK or Cdc42 GAPs recruited by septins to the polarity site have also been reported [13, 14]. Similar to Saccharomyces cerevisiae, Cdc42, its GEF Scd1, and the adaptor Scd2, form an activation complex in Schizosaccharomyces pombe [44, 51] and negative-feedback pathways, mediated by PAK or Cdc42 GAPs have been also described [52, 53].

The lipid microenvironment can also contribute to Cdc42 turnover at the membrane; sites of exocytosis correlate with microdomains of higher concentration and slower diffusion of Cdc42 compared with surrounding regions [54]. In Saccharomyces cerevisiae, the cdc42-6 mutant can be rescued by overproduction of Mss4, a protein involved in the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP₂) [55]. In addition, phosphatidylinerine is co-enriched with Cdc42 in microdomains and required for Cdc42 localization [54, 56]. In Schizosaccharomyces pombe, Cdc42 localizes properly in the its3-1 mutant strain where the levels of PIP₂ are reduced ([21]; our unpublished results). In Candida albicans, PIP₂ is required for stabilization of active Rho1, but not for Cdc42 [57]. However, it has been recently described that phosphatidylinerine accumulates at cell tips and contributes to Cdc42 and Rho1 localization in fission yeast [58].

At least, two parallel mechanisms have been proposed for recycling of Saccharomyces cerevisiae Cdc42: (1) Rdi1-mediated membrane extraction and redelivery through the cytosol; and (2) endocytic uptake and redelivery on exocytic vesicles [19]. A third mechanism for recycling Cdc42 may exist, which is independent of both Rdi1 and endocytic/exocytic recycling [20, 48]. In Schizosaccharomyces pombe, the two recycling routes from and to the plasma membrane likely exist, but Rdi1 does not participate in control of the localization or levels of Cdc42 (21]; this work). By contrast, exocytosis and endocytosis seem to play a major role in the recycling of Cdc42 because this GTPase is accumulated at the membranes of exocyst and end4Δ mutants. Cdc42 can also be transported from endosomes to the vacuolar membrane where it could exert its function in vacuolar fusion [7]. Vacuoles might be a possible route for recycling and/or degradation.

In summary, correct Cdc42 localization and activation are important processes for cell survival and require complex regulation; however, the mechanisms behind this regulation are not fully conserved among organisms.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


25. Coll PM, Rincón SA, Izquierdo RA, Pérez P. Hob3p, the fission yeast ortholog of human BIN3, localizes Cdc42p to the division site and regulates cytokinesis. EMBO J 2007;26:1865–1877.


32. Coll PM, Rincón SA, Izquierdo RA, Pérez P. Hob3p, the fission yeast ortholog of human BIN3, localizes Cdc42p to the division site and regulates cytokinesis. EMBO J 2007;26:1865–1877.


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