

The Rho GDI Rdi1 Regulates Rho GTPases by Distinct Mechanisms

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The small guanosine triphosphate (GTP)-binding proteins of the Rho family are implicated in various cell functions, including establishment and maintenance of cell polarity. Activity of Rho guanosine triphosphatases (GTPases) is not only regulated by guanine nucleotide exchange factors and GTPase-activating proteins but also by guanine nucleotide dissociation inhibitors (GDIs). These proteins have the ability to extract Rho proteins from membranes and keep them in an inactive cytosolic complex. Here, we show that Rdi1, the sole Rho GDI of the yeast *Saccharomyces cerevisiae*, contributes to pseudohyphal growth and mitotic exit. Rdi1 interacts only with Cdc42, Rho1, and Rho4, and it regulates these Rho GTPases by distinct mechanisms. Binding between Rdi1 and Cdc42 as well as Rho1 is modulated by the Cdc42 effector and p21-activated kinase Cla4. After membrane extraction mediated by Rdi1, Rho4 is degraded by a novel mechanism, which includes the glycogen synthase kinase 3 β homologue Ygk3, vacuolar proteases, and the proteasome. Together, these results indicate that Rdi1 uses distinct modes of regulation for different Rho GTPases.

INTRODUCTION

Small guanosine triphosphatases (GTPases) of the Rho family control fundamental processes of cell biology common to all eukaryotes, such as morphogenesis, polarity, movement, and cell division (Jaffe and Hall, 2005). In the budding yeast *Saccharomyces cerevisiae*, which encodes six Rho GTPases (Cdc42 and Rho1 to Rho5), these proteins play a pivotal role in the establishment of cell polarity (Park and Bi, 2007). Budding yeast cells undergo polarized growth during various phases of their life cycle, including budding during vegetative growth, mating between haploid cells of opposite mating types, and filamentous growth upon nutrient limitation. Although Cdc42 and Rho1 are well characterized, little is known about the other Rho proteins. Membrane association of Rho-type GTPases is essential for their function, and it depends on a C-terminal prenyl moiety and an adjacent polybasic region. Cdc42 localizes to internal membranes and the entire plasma membrane, where it clusters at sites of polarized growth, including the tips of mating projections, incipient bud sites, the tips and sides of growing buds, and the bud neck region of large-budded cells (Ziman *et al.*, 1993; Richman *et al.*, 2002). In addition to membrane-bound Cdc42, a cytoplasmic pool has been found (Ziman *et al.*, 1993). Similar to Cdc42, Rho1 has been shown to localize at sites of polarized growth (Yamochi *et al.*, 1994).

Like other regulatory GTPases, they act as molecular switches, cycling between an active guanosine triphosphate

(GTP)-bound state and an inactive guanosine diphosphate (GDP)-bound state. This activity is highly regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The exchange of GDP to GTP, and thus the activation of Cdc42, is catalyzed by GEFs. In the active GTP-bound state, Rho GTPases perform their regulatory function through a conformation-specific interaction with effector proteins. GAPs stimulate the intrinsic GTPase activity, leading to inactivation. However, the role of GAPs might be more complex. For Cdc42, it has been suggested that GTP hydrolysis, and thus a cycling between GDP and GTP states, is necessary for the establishment of polarity, septin assembly, and mating (Gladfelter *et al.*, 2002; Caviston *et al.*, 2003; Irazoqui *et al.*, 2003; Barale *et al.*, 2006). Apart from GEFs and GAPs, Rho GTPases are also regulated by guanine nucleotide dissociation inhibitors (GDIs) (DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005; Dransart *et al.*, 2005). Three distinct biochemical activities have been attributed to Rho GDIs. First, they inhibit the dissociation of GDP from Rho GTPases. Second, they block intrinsic and GAP-stimulated GTPase activity. Third, Rho GDI extract Rho proteins from membranes and form high-affinity cytosolic complexes, in which the C-terminal prenyl group of the Rho protein inserts into the hydrophobic pocket formed by the immunoglobulin-like β sandwich of the GDI. The dissociation of the GTPase-GDI complex is regulated by various mechanisms. Several biologically relevant membrane lipids have been reported to decrease the affinity of Rho GDIs for GTPases (Chuang *et al.*, 1993). Complex formation and membrane extraction also depend on the phosphorylation status of the GDI, the GTPase, or both (Bourmeyster and Vignais, 1996; Mehta *et al.*, 2001; Forget *et al.*, 2002; DerMardirossian *et al.*, 2004). Furthermore, in higher eukaryotes some membrane-associated proteins, such as members of the ezrin/radixin/moesin family and the neurotrophin receptor p75^{NTR}, have the ability to disrupt the GTPase-GDI complex (Takahashi *et al.*, 1997; Yamashita and Tohyama, 2003).

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Abbreviations used: GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; PAK, p21-activated kinase; GSK-3 β , glycogen synthase kinase 3 β .

In *S. cerevisiae*, the role of the sole Rho GDI Rdi1 is unclear. Conflicting results have been reported for the overexpression of *RDII*. Masuda *et al.* (1994) show that high levels of *RDII* result in lethality, although the phenotype of these cells has not been characterized (Masuda *et al.*, 1994). In contrast, others claim that *RDII* overexpression causes only a slightly rounder cell morphology (Tcheperegine *et al.*, 2005). Deletion of *RDII* does not produce any detectable phenotypes in budding yeast (Masuda *et al.*, 1994), but in the human fungal pathogen *Candida albicans*, cells lacking *RDII* exhibit reduced polarized growth (Court and Sudbery, 2007).

Rdi1 is found in the cytoplasm, but it also localizes to the tips of small buds and the bud neck region, where it interacts with Cdc42 (Koch *et al.*, 1997; Richman *et al.*, 2004; Cole *et al.*, 2007). Rdi1 has the ability to extract Cdc42 and Rho1 from vacuolar membranes (Eitzen *et al.*, 2001), and it can also extract Cdc42 from the plasma membrane (Richman *et al.*, 2004; Tcheperegine *et al.*, 2005). However, it has not been tested whether Rdi1 interacts with other Rho GTPases.

Here, we show that Rdi1 interacts only with a subset of Rho GTPases. Whereas Cdc42, Rho1 and Rho4 form a complex with Rdi1 and can be extracted from membranes, Rdi1 does not bind to Rho2, Rho3, and Rho5. Furthermore, we demonstrate that the p21-activated kinase (PAK) Cla4 disrupts Rdi1–Rho1 and Rdi1–Cdc42 complexes. Finally, we show that Rdi1 and the glycogen synthase kinase 3 β (GSK-3 β) homologue Ygk3 promote Rho4 degradation by vacuolar proteases and the proteasome.

MATERIALS AND METHODS

Growth Conditions, Yeast Strains, and Plasmids

All yeast strains are listed in Supplemental Table 1. The strains used in this study were in the YPH499 background, with the exception of strains used for filamentous growth. For these experiments strains in the Σ 1278b background were used (CTY64, PC344, PYP966, THY697, THY705, and THY706). Yeast strains were grown in yeast extract, peptone, dextrose (YPD) or synthetic complete (SC) medium. For induction of the *GAL1* promoter, yeast cells were grown in yeast extract, peptone (YP) or SC medium with 3% raffinose instead of glucose. Galactose (final concentration 2%) was added to induce the *GAL1* promoter. SLAD medium contains 50 μ M ammonium sulfate, 2% glucose, 2% Bacto-agar, and 0.67% YNB. Yeast strains were constructed using polymerase chain reaction (PCR)-amplified cassettes (Longtine *et al.*, 1998; Knop *et al.*, 1999; Janke *et al.*, 2004).

All constructs used in this work are listed in Supplemental Table 2. For N-terminal tagging of Rho proteins, these genes were first amplified by PCR from genomic DNA and cloned into pRS315. A NotI site was introduced 3' of the start codon by site directed mutagenesis. The plasmid GTEPI, which contains a DNA fragment encoding three adjacent copies of the hemagglutinin (HA) epitope cloned into the NotI site in the polylinker of pBluescript II, was cut with NotI, and the liberated fragment was ligated in frame into the NotI site of the pRS315 plasmids carrying the *RHO* genes. 3HA-tagged *RHO* genes were transferred into pRS305. These genes were inserted at the genomic *LEU2* by transformation with EcoRV or KasI digested pRS305 carrying the 3HA-tagged *RHO* genes.

Immunoprecipitations and Membrane Extraction Assays

For immunoprecipitations and precipitations with glutathione-Sepharose (GE Healthcare, Chalfont St. Giles, United Kingdom) cells were disrupted with glass beads in lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 5% glycerol, 1% Triton X-100, and protease inhibitors) and clarified by centrifugation at 13,000 rpm for 5 min. Protein concentration was determined using Bradford protein assay solution (Roth, Karlsruhe, Germany). Epitope-tagged proteins were immunoprecipitated by adding the respective antibody and protein G-Sepharose (GE Healthcare). Resin was washed three times with lysis buffer, resuspended in 2 \times SDS sample buffer, and analyzed by immunoblotting. For membrane extraction assays, cells were treated as for immunoprecipitations but without Triton X-100. Cleared lysates were centrifuged for 1 h at 100,000 \times g. For dephosphorylation of 3HA-Rho4, the protein was precipitated with anti-HA antibodies, beads were washed three times with phosphatase buffer (20 mM Tris, pH 7.4, 10 mM NaCl, and 5% glycerol). Beads were then split into three and either no, 100 U of λ -phosphatase (New England Biolabs, Ipswich, MA), or 100 U of λ -phospha-

tase, which was heat-inactivated by treating it 30 min at 95°C, was added. Samples were incubated 30 min at 30°C, and proteins were eluted with SDS sample buffer.

Monoclonal mouse anti-HA (12CA5) was obtained from Roche Diagnostics (Mannheim, Germany), and goat anti-glutathione transferase (GST) was from GE Healthcare. Mouse monoclonal anti-Myc (9E10), anti-Cdc42, anti-Cdc11, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Microscopy

F-actin of yeast cells was stained with rhodamine-phalloidin (Invitrogen, Carlsbad, CA). One milliliter of cells was harvested and resuspended in phosphate-buffered saline (PBS), 0.1% Triton X-100, and 3.7% formaldehyde. After 30-min incubation at room temperature, cells were washed once with PBS and fixed for 2 h at 30°C with PBS, 3.7% formaldehyde. Cells were washed two times with PBS and resuspended in 90 μ l of PBS. Three units of rhodamine-phalloidin was added, cells were incubated for 2 h in the dark, and then they were washed three times with PBS. Cells were examined with an Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a 100 \times Plan oil-immersion objective, and images were captured using an AxioCam MRm charge-coupled device camera (Carl Zeiss).

Filamentous Growth Assays

For agar invasion assays, 10⁵ cells of an overnight culture were spotted on YPD and grown for 2 d at 30°C. Plates were photographed before and after being rinsed under a gentle stream of deionized water. For pseudohyphal growth assays, cells were grown overnight, and 100 cells were spread on solid SLAD medium. Plates were incubated for 4 d at 30°C, and images were taken using a 10 \times objective.

RESULTS

Rdi1 Has a Role in Pseudohyphal Growth and Mitotic Exit

To assess the function of Rdi1 in *S. cerevisiae*, we analyzed the phenotype of cells deleted for and overexpressing *RDII*, respectively. The *RDII* deletion strain was indistinguishable from wild-type cells in terms of growth, cell morphology, and mating (data not shown). It has been reported that in diploid *C. albicans* loss of the homologous gene *RDII* greatly reduces filamentous growth (Court and Sudbery, 2007). In addition, *RDII* deletion abrogates the enhanced filamentation of cells deleted for the Cdc42 GAPs *BEM3* and *RGA2* (Court and Sudbery, 2007). Therefore, we tested whether similar phenotypes can be observed for *S. cerevisiae*, where filamentous growth occurs in both, haploid (invasive growth) and diploid (pseudohyphal growth) cells (Palecek *et al.*, 2002; Park and Bi, 2007). Haploid cells penetrate agar in response to nutritional limitation. To analyze the role of *RDII* in haploid invasive growth, we deleted this gene in cells of the Σ 1278b background, which is necessary for tests of filamentous growth. The haploid Δ *rdi1* strain invaded agar like wild-type cells (Figure 1A). On nitrogen starvation, diploid cells assume an elongated morphology and generate chains of filamentous-form cells projecting from the main colony of yeast-form cells (Figure 1B). The homozygous Δ *rdi1*/ Δ *rdi1* diploid strain exhibited a defect in pseudohyphal differentiation when grown on low nitrogen medium (Figure 1B). Notably, overexpression of *CDC42* from a multicopy plasmid did not rescue this defect (Figure 1B). Thus, it seems unlikely that Rdi1 regulates Cdc42 activity like the GEF Cdc24. It is conceivable that the phenotype of the Δ *rdi1*/ Δ *rdi1* strain reflects elevated, deregulated Cdc42 activity. Possibly Cdc42 removal from membranes by Rdi1 is necessary to restrict active Cdc42 to the growing tip. In line with this model is our observation that the constitutively active *CDC42*^{G12V} mutant (Ziman *et al.*, 1991) displayed a severe pseudohyphal growth defect (Figure 1C).

Cdc42 and its effectors Ste20, Cla4, and Gic1 promote exit from mitosis via at least three independent mechanisms (Höfken and Schiebel, 2002; Jensen *et al.*, 2002; Seshan *et al.*,

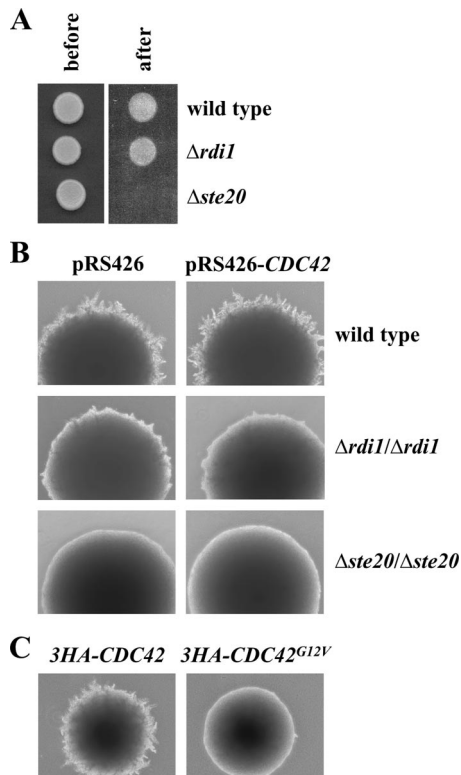


Figure 1. Rdi1 and filamentous growth. (A) *RDII* deletion does not affect haploid invasive growth. Cells (10^5) of the indicated strains were spotted on a YPD plate and incubated for 2 d at 30°C. Pictures were taken before and after gentle rinsing with water. *Δste20* cells, which fail to invade the agar, served as control. (B) *Δrdi1/Δrdi1* cells exhibit reduced diploid pseudohyphal growth. The indicated strains were grown on low nitrogen SLAD medium for 4 d at 30°C. *Δste20/Δste20* cells, which are defective in filamentous growth, were used as control. (C) Constitutively active Cdc42 impairs pseudohyphal growth. *3HA-CDC42* and *3HA-CDC42^{G12V}* under control of the *CDC42* promoter were integrated at the *URA3* locus by using an integrative plasmid. The indicated strains were treated as described in B. The results shown in this figure are representative of two independent experiments.

2002; Höfken and Schiebel, 2004; Bosl and Li 2005) (Figure 2A). The small GTPase Tem1 and its putative GEF Lte1 are the most upstream components of the mitotic exit network, the signaling cascade that controls exit from mitosis (Bosl and Li, 2005). Cells in which *LTE1* is deleted grow normally at 30°C, but arrest in anaphase with large buds, two separated 4,6-diamidino-2-phenylindole staining regions, and an elongated mitotic spindle when grown at low temperatures (Bosl and Li, 2005). We used this mitotic exit defect of *Δlte1* cells to assay the activity of Cdc42 regulators. Previously, it has been demonstrated that the deletion of *LTE1* in a temperature-sensitive mutant of *CDC24*, the sole GEF of Cdc42, is lethal at all temperatures (Höfken and Schiebel, 2002). Here, we show that the individual deletion of the Cdc42 GAPs *BEM3*, *RGA1*, and *RGA2* suppressed the cold-sensitive growth defect of *Δlte1* (Figure 2B). Interestingly, the double mutant *Δlte1Δrdi1* also grew like the wild-type strain at 10°C (Figure 2B). These results are consistent with a role for Cdc24 as a positive regulator and for Rdi1, Bem3, Rga1, and Rga2 as negative regulators of Cdc42. We then asked whether deletion of *RDII* and the GAPs rescued the mitotic exit defect of the *Δlte1* strain. Cells were arrested in G1 with α -factor and released at 10°C. In addition to the percentage

of large-budded cells, the release of Cdc14-green fluorescent protein (GFP) from the nucleolus was monitored during the cell cycle. Cdc14 is released from the nucleolus through anaphase and thus serves as a reliable marker for mitotic exit (Bosl and Li, 2005). At 10°C, wild-type cells released Cdc14 and underwent cytokinesis at ~6 h (Figure 2C). In contrast, most *Δlte1* cells arrested in anaphase with Cdc14-GFP trapped in the nucleolus (Figure 2C). *Δlte1* cells in which either *RDII* or the Cdc42 GAPs were deleted did not arrest in anaphase and released Cdc14 from the nucleolus with the same kinetics as wild-type cells (Figure 2C).

RDII overexpression has been reported to either result in lethality (Masuda *et al.*, 1994) or produce slightly rounder cells (Tcheperegine *et al.*, 2005). Therefore, we tested the consequences of higher *RDII* levels in our background. *RDII* under control of its own promoter on a multicopy plasmid had no effect on cell growth and morphology (Supplemental Figure S1A; data not shown). The same result was obtained for a strain in which a single copy of genomic *RDII* was under control of the strong *GALI* promoter (Supplemental Figure S1B). In contrast, *RDII* under control of the *GALI* promoter expressed from a 2 μ m-based multicopy plasmid (Masuda *et al.*, 1994) resulted in lethality (Supplemental Figure S1C). To examine the cause of this lethality, we monitored cell morphology over time after *RDII* overexpression. Four hours after induction of *GALI-RDII* from a multicopy plasmid, ~90% of cells arrested as slightly bigger round, unbudded cells (Supplemental Figure S1, D and E). The actin cytoskeleton was polarized in only 6% of these round cells, whereas 70% of unbudded cells with normal Rdi1 levels had an polarized actin cytoskeleton (Supplemental Figure S1E).

Rdi1 Specifically Interacts with Cdc42, Rho1, and Rho4

The most likely reason for the failure of cells to polarize after strong *RDII* overexpression is the removal of Rho GTPases from sites of polarized growth by Rdi1. It has been shown that Rdi1 is able to extract Cdc42 and Rho1 from membranes (Koch *et al.*, 1997; Eitzen *et al.*, 2001; Richman *et al.*, 2004; Tcheperegine *et al.*, 2005), but it has not been tested whether Rdi1 interacts with other Rho GTPases as well. Therefore, we performed immunoprecipitation experiments with strains in which Rho GTPases were N-terminally tagged with the HA and Rdi1 C-terminally with the myc epitope. Coimmunoprecipitation of Rho1 and Rho4, but not of Rho2, Rho3, and Rho5 with Rdi1 was observed (Figure 3A). The multiple bands observed for 3HA-Rho4 are due to phosphorylation of this protein (see below). In a similar immunoprecipitation experiment using antibodies against Cdc42, it was demonstrated that Cdc42 binds to Rdi1 (Figure 3B). Because all proteins were expressed from their own promoters for this experiment, Rho protein levels might not be sufficiently high for detection of a Rdi1-Rho complex. To circumvent this problem, *RHO* genes were fused with *GST* and placed under the control of the strong *GALI* promoter. Although all Rho GTPases were expressed at high levels upon induction, again only Cdc42, Rho1 and Rho4, but not the other Rho proteins, formed a complex with Rdi1 (Supplemental Figure S2).

To examine the amount of membrane-associated Rho GTPases, cell lysates were submitted to cell fractionation by high-speed centrifugation. Interestingly, the ratio between protein in soluble supernatant and the membrane pellet was quite different for the various Rho GTPases (Supplemental Figure S3). To characterize which Rho proteins could be extracted from membranes by Rdi1, lysates of cells with and without *RDII* overexpression, respectively, were fraction-

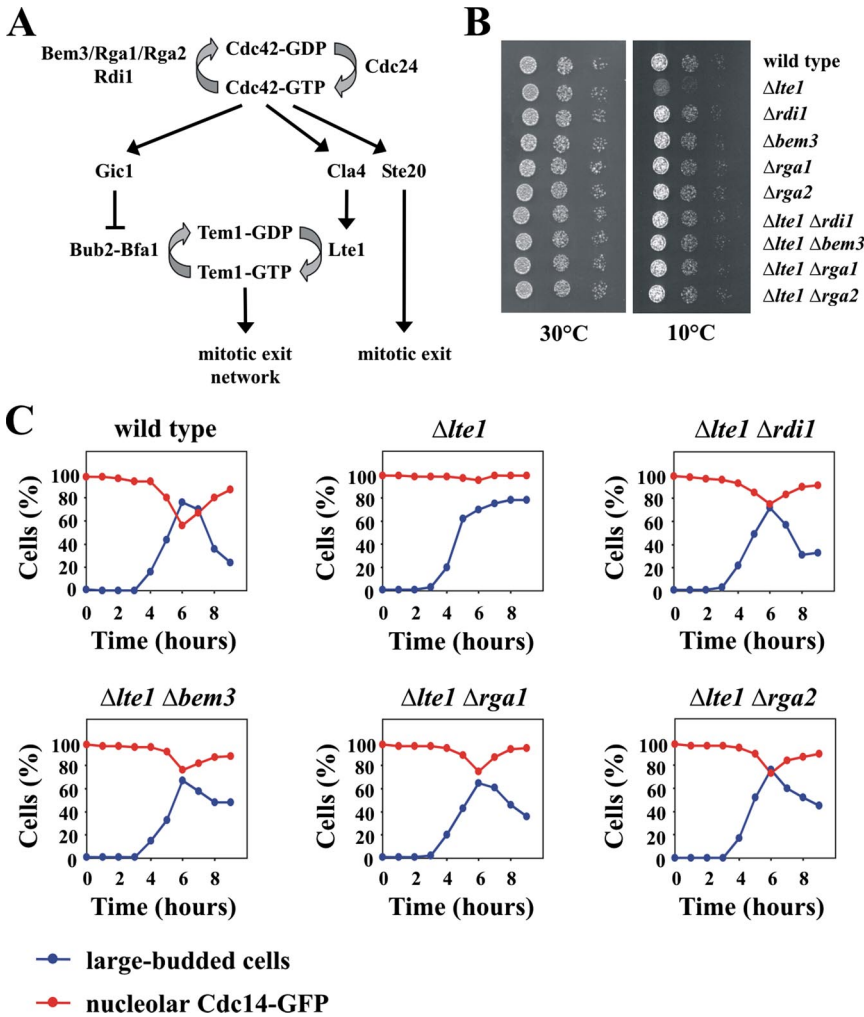


Figure 2. Deletion of *RDI1* suppresses the mitotic exit defect of $\Delta lte1$ cells. (A) The role of Cdc42 in mitotic exit. Cdc42 is activated by its GEF Cdc24, whereas the GAPs Bem3, Rga1, and Rga2 stimulate GTP hydrolysis. Activated Cdc42 triggers mitotic exit by three independent mechanisms. Gic1 disrupts the interaction between the small GTPase Tem1 and its two-component GAP Bub2-Bfa1, which results in the activation of Tem1. Cla4 stimulates Lte1, the putative GEF of Tem1, which causes Tem1 activation. Ste20 promotes mitotic exit independently of Gic1 and Cla4 by an unknown mechanism. (B) Suppression of the $\Delta lte1$ growth defect by deletion of either *RDI1* or Cdc42 GAPs. Serial dilutions (1:10) of the indicated strains grown on YPD at 10 and 30°C, respectively. (C) Deletion of either *RDI1* or Cdc42 GAPs suppresses the mitotic exit defect of $\Delta lte1$ cells. The indicated strains with *CDC14-GFP* were arrested in G1 with α -factor. Cells progressed synchronously through the cell cycle at 10°C upon removal of α -factor by washing with YPD medium. The number of cells with large buds and nucleolar Cdc14-GFP ($n > 100$ at each time point) was determined over time. Because *RDI1* and Cdc42 GAP single deletion strains grew like wild-type cells at 10°C (Figure 1B), these strains were not included for this analysis. The results shown in this figure are representative of two independent experiments.

ated and analyzed by immunoblotting. After overexpression of *RDI1*, the amount of cytosolic protein increased for Cdc42 and Rho1 (Figure 4A). Consistently, less Rho1 and Cdc42 protein was found in the membrane fraction of cells overexpressing *RDI1* (Figure 4A). However, because Rho proteins predominantly associate with membranes, these changes can be most easily observed in the cytosolic fraction. Higher levels of *RDI1* had no effect on the distribution of Rho2, Rho3, and Rho5 (Figure 4A). Interestingly, the total amount of Rho4 dropped following *RDI1* overexpression (Figure 5A). Because Rho4 protein level were only moderately reduced in cells lacking *PEP4* after *RDI1* overexpression (see below), the membrane extraction assay for Rho4 was performed in the $\Delta pep4$ background. Higher *RDI1* levels caused an increase of Rho4 in the supernatant and a reduction of membrane-associated Rho4 (Figure 4B). The ability of Rdi1 to extract Rho4 from membranes also was examined by fluorescence microscopy. GFP-Rho4 under control of its own promoter could be detected by immunoblotting, but it was too weak to be visualized by fluorescence microscopy. Therefore, we expressed *GFP-RHO4* from the inducible *GAL1* promoter. GFP-Rho4 was associated with internal membranes and the entire plasma membrane (Figure 6A). The protein clustered at the presumptive bud site and the bud neck of large buds. Unlike most other proteins that have a role in polarity, Rho4 was not or only weakly enriched at the tip of small buds and absent from mating

projections (Figure 6, A and B). As expected, *RDI1* overexpression resulted in the loss of membrane-bound Rho4 (Figure 6, C and D).

We next asked whether Rdi1 preferentially extracts the GDP- or GTP-bound form of Cdc42, Rho1, and Rho4 from membranes. The mutations *CDC42^{G12V}* and *CDC42^{D118A}* lock Cdc42 in the GTP- and GDP-bound state, respectively (Ziman et al., 1991). Therefore, we compared the intracellular distribution of these mutated proteins and also of the paradigmatic mutant proteins Rho1^{G19V}, Rho1^{D125A}, Rho4^{G81V}, and Rho4^{D197A} after *RDI1* overexpression. Unexpectedly, Rdi1 extracted the GTP-bound Cdc42^{G12V} and Rho1^{G19V} like wild-type proteins from membranes, whereas the cytosolic levels of GDP-bound Cdc42^{D118A} and Rho1^{D125A} did not increase in cells overexpressing *RDI1* (Figure 4, A and C). In contrast, wild type Rho4, Rho4^{G81V}, and Rho4^{D197A} seemed to be equally well extracted from membranes by Rdi1 (Figure 4, B and C). However, the effect of Rdi1 on Rho4 was less pronounced; therefore, it was more difficult to assess.

Because Rdi1 extracts Rho1 and Cdc42 from membranes, we examined whether these Rho proteins were present in the cytosol of cells deleted for *RDI1*. Unexpectedly, the cytoplasmic pool of Rho1 and Cdc42 was the same in wild type and $\Delta rdi1$ cells (Figure 4D).

In summary, our data indicate that Rdi1 only interacts with Cdc42, Rho1, and Rho4, but not with other Rho proteins.

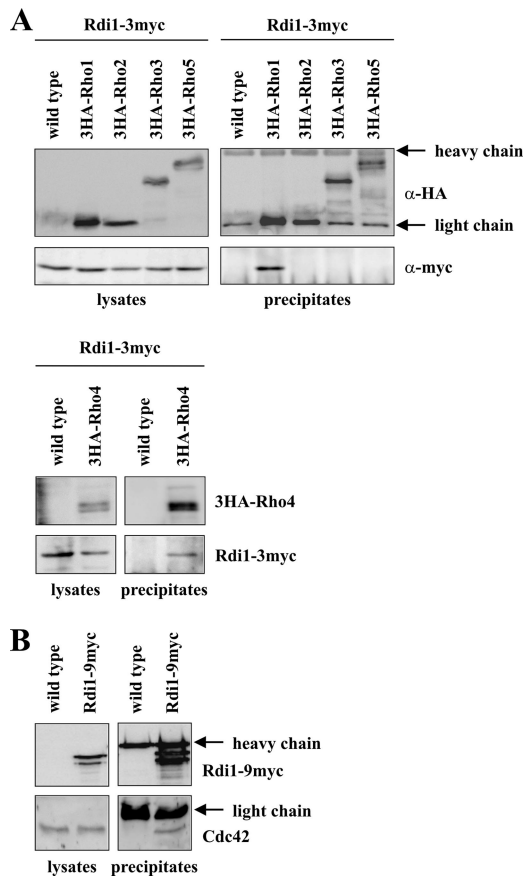


Figure 3. Rdi1 interacts with Cdc42, Rho1, and Rho4, but not with other Rho proteins. (A) Rdi1 coimmunoprecipitates with Rho1 and Rho4. Cells expressing *RDII-3myc*, and *RDII-3myc* with 3HA-tagged *RHO1*, *RHO2*, *RHO3*, *RHO4*, and *RHO5* were lysed, and equal amounts of protein extract were precipitated with anti-HA antibodies. Immunoprecipitates were analyzed by immunoblotting with antibodies against the myc and HA epitopes. 3HA-Rho1 and 3HA-Rho2 run at the same height as the light chain. However, a stronger signal indicates that these proteins were precipitated, compared with the heavy chain, which occurs with the same intensity in all samples. (B) Coimmunoprecipitation of Rdi1 with Cdc42. Cells expressing *RDII-9myc* and wild-type cells were lysed, and equal amounts of protein were precipitated with anti-myc antibodies. Precipitates were examined with antibodies raised against Cdc42 and the myc epitope, respectively. The results shown in this figure are representative of two independent experiments.

PAK-Family Kinase *ClA4* Regulates Association of Rdi1 with Cdc42 and Rho1

The association of Rho GTPases with Rho GDIs can be modulated by phosphorylation of these proteins (Bourmeyster and Vignais, 1996; Mehta *et al.*, 2001; Forget *et al.*, 2002; DerMardirossian *et al.*, 2004). Because the Cdc42 effectors *ClA4* and *Ste20* play a key role in cell polarity, we asked whether elevated levels of these kinases would reduce the efficiency of the coimmunoprecipitation of Cdc42 and Rdi1. Anti-myc epitope antibodies not only efficiently precipitated Rdi1-9myc but also Cdc42 (Figures 3B and 7A). Overexpression of *ClA4* abolished binding between Rdi1 and Cdc42 (Figure 7A). Importantly, higher expression of either *STE20* or *SKM1*, which are both Cdc42 effectors and PAK-family kinases as well, had no effect on the Cdc42-Rdi1 interaction (Figure 7, A and B). Thus, *ClA4*, but not related proteins, can specifically disrupt binding between Cdc42 and Rdi1. We

also tested, whether *ClA4* overexpression has the same effect on the Rho1-Rdi1 complex. However, although the coimmunoprecipitation of Rho1 and Rdi1 was highly reproducible, we came to varying results for the Rho1-Rdi1 complex after *ClA4* overexpression (data not shown). Therefore, using coimmunoprecipitations, it cannot conclusively be demonstrated whether *ClA4* has an effect on the Rho1-Rdi1 complex. To circumvent this problem, we tested whether high levels of *ClA4* change the intracellular distribution of Rho1 and Cdc42. Overexpression of *ClA4* decreased the amount of cytosolic Rho1 and Cdc42 (Figure 7C). Importantly, this effect was not observed in the absence of *RDII* (Figure 7C). These results suggest that *ClA4* disrupts the binding between Rdi1 and Cdc42 as well as between Rdi1 and Rho1. Consistently, in $\Delta clA4$ cells a slightly higher portion of Rho1 was found in the cytosol compared with the wild-type strain, whereas the cytosolic amount of Rho1 in the $\Delta clA4 \Delta rdi1$ double mutant was similar to the wild-type and $\Delta rdi1$ (Figure 4D). For Cdc42, there was only a weak or no effect for Cdc42 in the *ClA4* deletion strain (Figure 4D). Finally, using the membrane extraction assay it was tested whether *ClA4* antagonizes Rdi1. Whereas higher Rdi1 levels increased the cytosolic pool of Cdc42 and Rho1 (Figures 4A and 7D), simultaneous overexpression of *ClA4* and *RDII* reversed this effect (Figure 7D). Under these conditions, cytosolic amounts of Cdc42 and Rho1 are comparable with the wild-type situation. Next, we tested whether the kinase activity of *ClA4* is required for its effect on Rdi1 complexes. To this end, we examined the localization of 3HA-Cdc42 after overexpression of wild-type *ClA4* and the kinase-dead *ClA4*^{K594A}. As described above, overexpression of wild-type *ClA4* results in a reduced cytosolic pool of 3HA-Cdc42 compared with the wild-type strain (Figure 7, C and E). In contrast, this effect was much weaker in cells overexpressing the kinase-dead *ClA4* (Figure 7E).

Interestingly, *ClA4* did not affect the amount of Rho4 in the supernatant and pellet after high-speed centrifugation (Supplemental Figure S4, A and B). Thus, the regulatory role of *ClA4* is specific for Cdc42 and Rho1.

Rdi1 and the GSK-3 β Homologue *Ygk3* Promote Degradation of Rho4

As mentioned above, *RDII* overexpression reduces the total amount of Rho4 protein (Figure 5A). Conversely, higher protein levels of Rho4 were observed in $\Delta rdi1$ cells compared with the wild-type strain (Figure 5B). This could be achieved by two different mechanisms. Either Rdi1 targets Rho4 for degradation or, alternatively, Rdi1 regulates Rho4 protein levels through gene expression. Because Rho4 levels dropped markedly after only 2 h of *RDII* overexpression, a regulation at the transcriptional or translational level would only have an effect if Rho4 were a short-lived protein. To test this, we blocked protein synthesis with cycloheximide. Even after 2-h incubation with cycloheximide, Rho4 protein levels remained unchanged (Figure 5C), suggesting that Rho4 is a relatively stable protein. Thus, Rdi1 seems to regulate Rho4 levels through proteolysis. Because usually multiple bands were observed for 3HA-Rho4 in immunoblots, it was examined whether these are phospho-forms of 3HA-Rho4. In fact, slower migrating bands disappeared following phosphatase treatment (Figure 5D).

In a proteomic approach, phosphorylation of Rho4 by *Ygk3*, a homologue of GSK-3 β kinase, has been demonstrated previously (Ptacek *et al.*, 2005). Furthermore, *Ygk3* has a role in ubiquitin-dependent proteolysis (Andoh *et al.*, 2000). Therefore, we tested whether *Ygk3* is involved in the degradation of Rho4. In fact, *YGK3* overexpression resulted

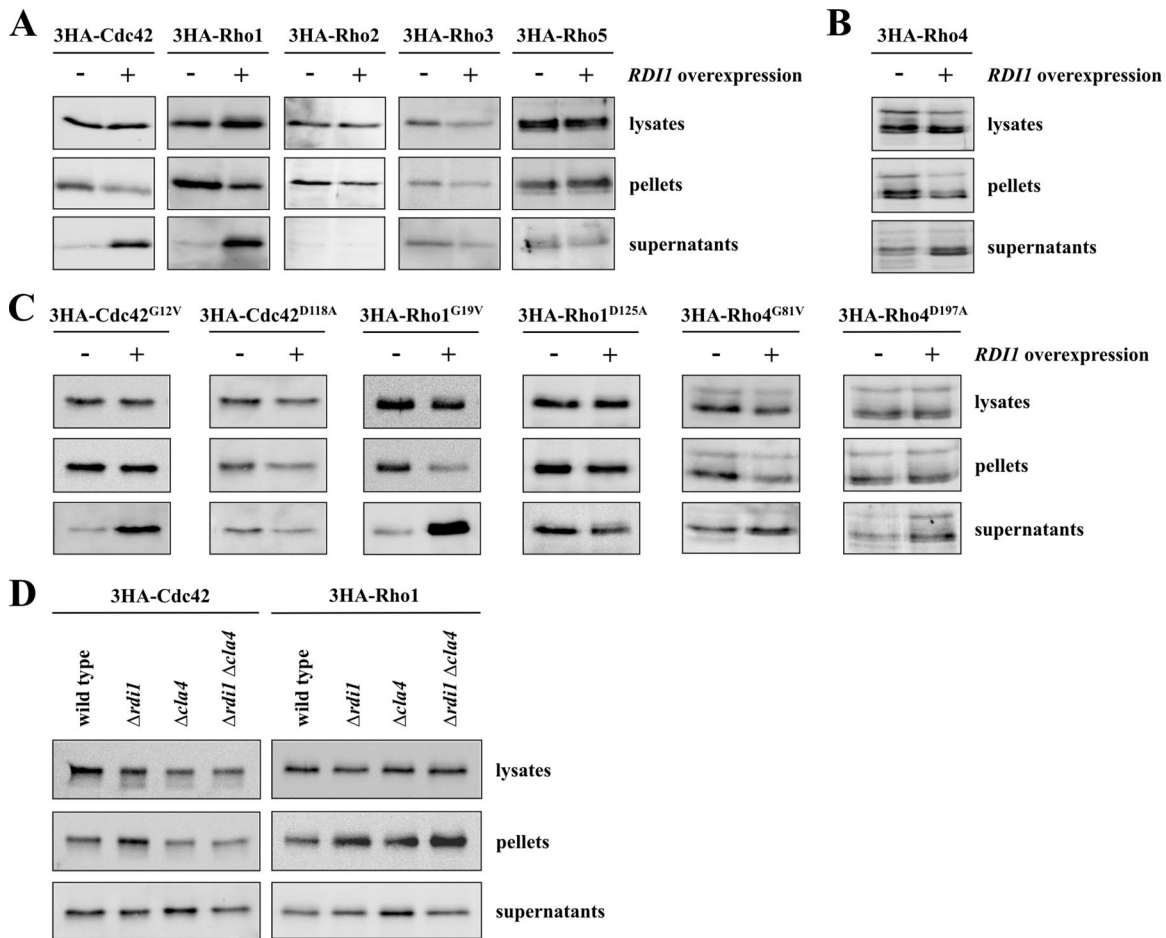


Figure 4. Rdi1 specifically extracts Rho1 and Cdc42 from membranes. (A) Rdi1 extracts Cdc42 and Rho1 from membranes. Cells expressing 3HA-tagged Rho proteins and carrying either *GALI-RDII* on a plasmid or the empty vector were grown in selective medium with 3% raffinose. *RDII* was overexpressed for 3 h by addition of galactose. Cells were lysed and equal amounts of protein extract were separated by centrifugation at $100,000 \times g$ to obtain the soluble supernatant and membrane pellet fractions. Rho proteins in different fractions were detected by Western blotting with antibodies against the HA epitope. (B) *RDII* extracts Rho4 from membranes. The experiment was performed as described in A but in a $\Delta pep4$ background to counteract Rho4 degradation caused by *RDII* overexpression (C). Rdi1 preferentially extracts GTP-bound forms of Rho GTPases from membranes. The experiment was carried out as described in A. For Rho4 proteins the assay was performed in cells lacking *PEP4*. (D) Effect of *RDII* and *CLA4* deletion on cytosolic levels of Cdc42 and Rho1. Cells of the indicated strains were lysed and protein extracts were fractionated by centrifugation at $100,000 \times g$. Cdc42 and Rho1 were detected by immunoblotting using anti-HA antibodies. The results shown in this figure are representative of two independent experiments.

in reduced Rho4 levels (Figure 8A), indicating that this protein, like Rdi1, promotes Rho4 proteolysis. Importantly, in the absence of *RDII* Ygk3 fails to degrade Rho4 (Figure 8A), suggesting that Rdi1 and Ygk3 act in the same pathway. In contrast *YGK3* overexpression had no effect on protein levels of Cdc42 and Rho1 (Figure 8B), which are also extracted from membranes by Rdi1. Because the ubiquitin ligase Rsp5 is involved in GSK-3 β -mediated proteolysis (Andoh *et al.*, 2000), we examined the role of Rsp5 in Rho4 degradation using a temperature-sensitive mutant. At both, the permissive (23°C) and restrictive (37°C) temperature, Rho4 degradation after *RDII* overexpression in *rsp5-1* cells was indistinguishable from the wild type (Supplemental Figure S5). Thus, the ubiquitin ligase Rsp5 is not required for Rho4 degradation. Eukaryotic cells contain two distinct systems for protein degradation: the vacuolar system and the proteasome. To find out whether Rho4 is degraded in a proteasome-dependent manner, Rho4 protein levels after *RDII* overexpression were examined in the presence and absence of the proteasome-specific inhibitor MG132 (Lee

and Goldberg, 1996). Treatment with MG132 stabilized Rho4 (Figure 8C), indicating an involvement of the proteasome in Rho4 degradation. We also examined whether the vacuolar system plays a role in Rho4 degradation. To this end, *RDII* was overexpressed in $\Delta pep4$ cells, which lack most vacuolar proteases (Ammerer *et al.*, 1986). Unexpectedly, Rho4 was not degraded in the absence of vacuolar proteases (Figure 8D), suggesting that Rho4 proteolysis depends on the vacuolar system as well as the proteasome.

Next, we examined whether Rho4 was degraded in a cell cycle-dependent manner. However, no major changes were observed at different stages of the cell cycle (Figure 9A). It is conceivable, that Rho4 degradation is necessary to restrict the protein to a certain membrane domain. Nevertheless, GFP-Rho4 is enriched at the presumptive bud site in wild-type cells and in the absence of *RDII* (Figure 6E). Therefore, it seems unlikely, that the polarized localization of Rho4 is achieved by its proteolysis. It is also possible, that only active Rho4 is extracted from the membrane and subsequently degraded to terminate its function. To test whether

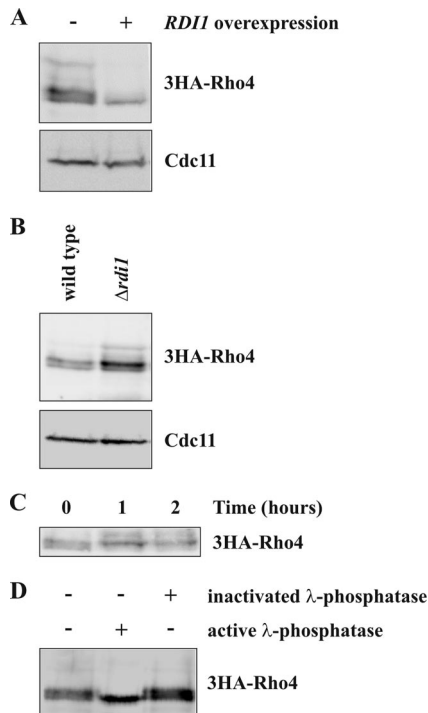


Figure 5. Rdi1 targets Rho4 for degradation. (A) *RDII* overexpression results in lower Rho4 protein levels. *3HA-RHO4* cells carrying either *GAL1-RDII* on a vector or the empty plasmid were grown in YP medium with 3% raffinose. *RDII* was overexpressed for 2 h by addition of galactose. Subsequently, cells were lysed, and equal amounts of protein extract were examined by immunoblotting using antibodies against the HA epitope and Cdc11 (loading control). (B) $\Delta rdi1$ cells have higher Rho4 protein levels. Cells expressing *3HA-RHO4* in a wild-type and $\Delta rdi1$ background were analyzed by immunoblotting with the indicated antibodies. (C) Rho4 is a relatively stable protein. Exponentially growing *3HA-Rho4* cells were incubated with 50 $\mu\text{g}/\text{ml}$ cycloheximide. At the indicated times, samples were taken and analyzed by immunoblotting. (D) Rho4 is a phosphoprotein. *3HA*-tagged Rho4 was immunoprecipitated with anti-HA antibodies and treated with buffer, active, or heat-inactivated λ -phosphatase. Samples were analyzed by immunoblotting. The results shown in this figure are representative of two independent experiments.

hyperactive Rho4 causes any damage to cells, we overexpressed *RHO4^{G81V}*. As described above, the paradigmatic *rho4^{G81V}* mutant is locked in the active GTP-bound state due to a defect in GTP hydrolysis. This constitutively active form of Rho4 reduces cell growth markedly (Figure 9B). Unfortunately, no morphological or other changes were observed in these cells (data not shown). Together, our data show that Rdi1 and Ygk3 promote Rho4 degradation, a process which depends on both, vacuolar proteases and the proteasome. Further, the degradation of Rho4 may be important to terminate its activity.

DISCUSSION

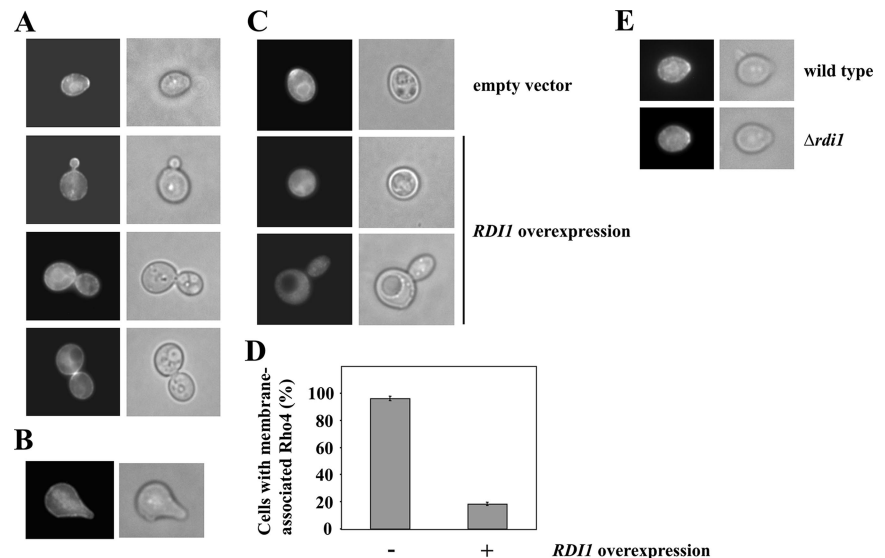
In this work, we show that the regulation of Rho proteins by the sole budding yeast Rho GDI Rdi1 is rather complex. Rdi1 interacts only with Cdc42, Rho1, and Rho4, but not with the other Rho proteins (summarized in Figure 10). We demonstrate that Rdi1 extracts Cdc42 and Rho1 from membranes and forms a soluble complex with these proteins, a process that is regulated by the PAK-family kinase Cla4. In contrast, membrane extraction of Rho4 by Rdi1 results in the degradation of this Rho protein. We found that this proteolytic pathway includes the proteasome, vacuolar proteases, and the GSK-3 β homologue Ygk3.

Functions of Rdi1

Very few phenotypes for the overexpression or deletion of *RDII* are known. Conflicting results have been reported for *RDII* overexpression. Tcheperegine *et al.* (2005) show that high levels of *RDII* cause only slightly rounder cell morphology, whereas others claim that *RDII* overexpression results in lethality (Masuda *et al.*, 1994). In our hands, effects of *RDII* overexpression were clearly dose dependent. Only *RDII* expressed from the *GAL1* promoter of a 2- μm plasmid was lethal. The actin cytoskeleton was depolarized in these cells and consequently buds were no longer present. This lack of polarization can be explained by the extraction of the essential Rho proteins Cdc42 and Rho1 from the plasma membrane by Rdi1.

To date, no phenotype has been described for $\Delta rdi1$ cells. Here, we show that deletion of *RDII* suppresses the mitotic exit defect of $\Delta lte1$ cells at low temperatures, possibly due to

Figure 6. Localization of Rho4. (A) Rho4 localization during budding. *GAL1-GFP-RHO4* of exponentially growing cells was induced for 120 min by the addition of galactose. Cells were fixed with formaldehyde and analyzed by fluorescence microscopy. (B) Rho4 is not enriched at the tip of mating projections. Logarithmically growing *GAL1-GFP-RHO4* cells were incubated with 1 $\mu\text{g}/\text{ml}$ α -factor and 2% galactose for 150 min. Fixed cells were examined by fluorescence microscopy. (C) Rdi1 extracts Rho4 from plasma membranes. Logarithmically growing *GAL1-GFP-RHO4* cells carrying either *GAL1-RDII* on a plasmid (pKT10-GAL-*RDII*) or the empty vector (pKT10-GAL) were induced with galactose for 120 min. (D) Quantification of C. The percentage of cells with membrane-associated Rho4 is given as the mean of three independent experiments with SD bars ($n > 100$ for each experiment). (E) *RDII* deletion does not affect polarized Rho4 localization. GFP-Rho4 of wild type and $\Delta rdi1$ cells was induced for 120 min.



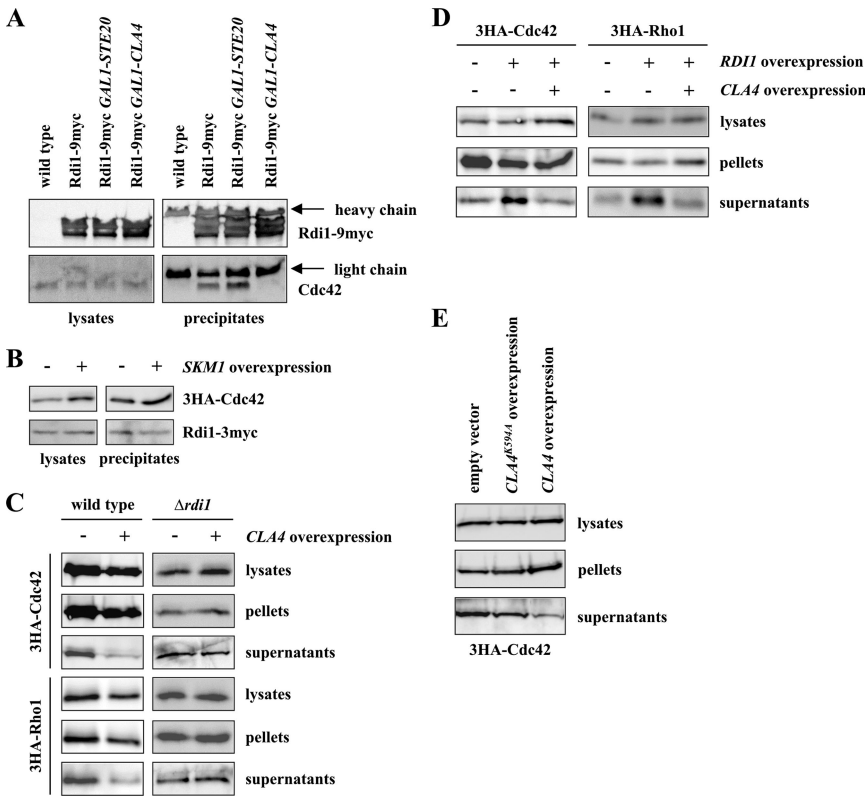


Figure 7. Cla4 regulates binding of Rdi1 to Cdc42 and Rho1. (A) *CLA4* overexpression inhibits complex formation between Cdc42 and Rdi1. The indicated strains were grown in YP medium with raffinose. We added 2% galactose to induce expression of *STE20* and *CLA4*, respectively. Cells were then lysed and equal amounts of protein were precipitated with anti-myc antibodies. Immunoblots were probed with antibodies raised against Cdc42 and the myc epitope. (B) *SKM1* overexpression has no effect on the Cdc42–Rdi1 complex. *3HA-CDC42 RDII-3myc* cells carrying either *GAL1-GST-SKM1* on a plasmid or the empty plasmid were induced with galactose for 120 min. Cells were subjected to immunoprecipitation with anti-HA antibodies. Immunoblots were analyzed with anti-HA and anti-myc antibodies. (C) *CLA4* overexpression decreases the cytoplasmic pool of Rho1 and Cdc42. Cells expressing the indicated 3HA-tagged Rho GTPase and carrying either *GAL1-myc-CLA4* on a plasmid or the empty plasmid, respectively, were induced for 120 min. Cells were lysed, and equal amounts of protein extract were separated by centrifugation at $100,000 \times g$. Rho proteins were detected by immunoblotting using antibodies against the HA epitope. (D) *CLA4* overexpression reverses *RDII*-dependent membrane extraction of Cdc42 and Rho1. *3HA-CDC42*, *3HA-CDC42 GAL1-3HA-RDII*, *3HA-RHO1*, and *3HA-RHO1 GAL1-3HA-RDII* cells carrying either an empty plasmid or a plasmid encoding *GAL1-myc-CLA4* were induced with galactose for 2 h. Subsequently, lysed cells were fractionated by centrifugation at $100,000 \times g$ and analyzed by immunoblotting. (E) Cla4 kinase activity is required for its effect on Cdc42–Rdi1. *3HA-CDC42* cells carrying either an empty plasmid, *GAL1-myc-CLA4*, or *GAL1-myc-CLA4^{K594A}* were treated as described in C. The results shown in this figure are representative of two independent experiments.

Cdc42 activation. Cdc42 regulates exit from mitosis via at least three independent pathways involving the Cdc42 effectors Cla4, Ste20, and Gic1 (Figure 2A) (Höfken and Schiebel, 2002; Jensen *et al.*, 2002; Seshan *et al.*, 2002; Höfken and Schiebel, 2004). Previously, we could demonstrate that a temperature-sensitive allele of the Cdc42 GEF *CDC24* in a *Δlte1* background is lethal at all temperatures (Höfken and Schiebel, 2002). Here, we observed that deletion of either *RDII* or of one of the Cdc42 GAPs results in suppression of the mitotic exit defect of *Δlte1* cells. Consistently, overexpression of either *STE20*, *GIC1*, or *GIC2* has the same effect on cells deleted for *LTE1* (Höfken and Schiebel, 2002; Höfken and Schiebel, 2004). This suggests that deletion of the GAP genes or *RDII* leads to a slight activation of Cdc42 and its effectors, which trigger exit from mitosis independently of Lte1 (Figure 2A).

Interestingly, we observed a pseudohyphal growth defect for the diploid homozygous *Δrdi1* deletion strain. Because *CDC42* overexpression does not rescue this phenotype, it is unlikely that Rdi1 activates Cdc42. Because the deregulated *CDC42^{G12V}* mutant exhibited a similar filamentation defect, Rdi1 may rather be necessary to restrict active Cdc42 to the growing tip. At first sight removal of Rho GTPases from the plasma membrane seems to suggest that Rdi1 acts as an inhibitor. However, Rho proteins could be targeted back to the membrane, where they are required, e.g., to sites of polarized growth. Furthermore, the extraction of Rho GTPases by Rdi1 from the membrane could play an important role in the maintenance of polarized distribution of these molecules. Cdc42 and other polarity proteins are not immobilized via scaffolds, but they are instead maintained in

dynamically polarized states (Wedlich-Soldner *et al.*, 2004). Endocytosis is required for the maintenance of cell polarization, e.g., by counteracting lateral diffusion of polarized proteins within the membrane (Valdez-Taubas and Pelham, 2003; Marco *et al.*, 2007). Therefore, Rdi1-dependent extraction of Rho GTPases from membranes and return transport could be equally important for their polarized localization.

It has been reported that the expression of the constitutively active *CDC42^{G12V}* or *CDC42^{Q61L}* results in enhanced pseudohyphal growth (Mösch *et al.*, 1996; Roberts *et al.*, 1997). For these experiments, the *CDC42* alleles were overexpressed from the inducible *GAL1* promoter, whereas in our analysis *CDC42* was placed under the control of its own promoter. In both cases, the endogenous copies of *CDC42* were also present in the cell. Therefore, the protein levels seem to be critical. Possibly, physiological levels of *Cdc42^{G12V}* may interfere with Cdc42 signaling pathways, causing a defect in pseudohyphal growth, whereas high amounts due to overexpression of constitutively active forms of Cdc42 cause enhanced pseudohyphal growth. Similarly, dose dependent polarity phenotypes were observed for other Cdc42 mutants which were attributed to differences in the capacity for GTP hydrolysis by Cdc42 of yeast cells after expression of physiological or supraphysiological levels (Iraozqui *et al.*, 2004). Further work is needed to analyze whether molecular mechanisms along this line play a role for *Cdc42^{G12V}* effects on pseudohyphal growth.

In contrast to the diploid homozygous *Δrdi1/Δrdi1* strain, which exhibited a marked filamentation defect, haploid *Δrdi1* cells invaded agar like the wild-type strain. The reason for this difference is unclear. Notably, *RDII* deletion in the

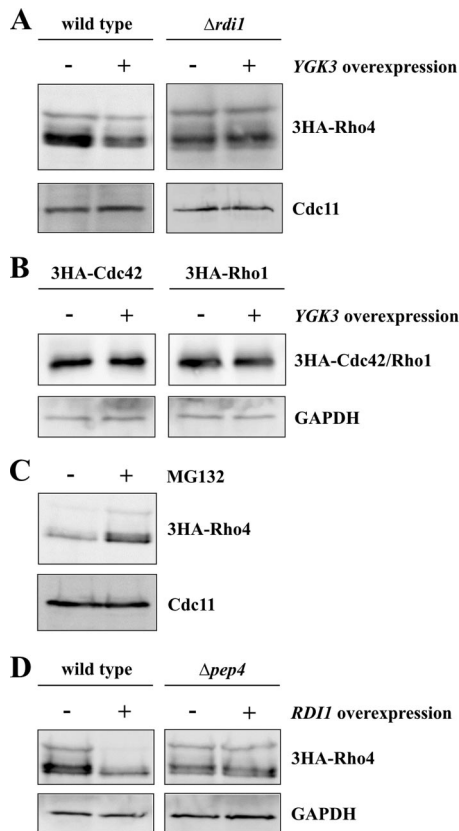


Figure 8. Degradation of Rho4. (A) *YGK3* overexpression leads to Rho4 degradation. Cells in the wild-type and $\Delta rdi1$ background, respectively, expressing *3HA-RHO4* and carrying either an empty plasmid or a plasmid encoding *GAL1-GST-YGK3* were induced for 2 h. Equal amounts of proteins were separated by SDS-PAGE, and Rho4 was detected by antibodies against the HA epitope. Cdc11 was used as loading control. (B) Overexpression of *YGK3* has no effect on protein levels of Cdc42 and Rho1. The experiment was performed as described in A. (C) Rho4 degradation depends on the proteasome. *GAL1-RDI1* was overexpressed from a 2- μ m plasmid (pKT10-*GAL-RDI1*) in *3HA-RHO4* cells in the presence of the proteasome-specific inhibitor MG132 (50 μ M) or with dimethyl sulfoxide alone. The experiment was carried out in a $\Delta erg6$ background, because these cells have enhanced permeability for MG132 (Lee and Goldberg, 1996). (D) Rho4 is degraded by vacuolar proteases. Wild-type and $\Delta pep4$ cells with HA-tagged Rho4 carrying an empty plasmid and a plasmid with *GAL1-RDI1*, respectively, were incubated for 2 h with galactose to overexpress *RDI1*. Rho4 was detected by immunoblotting using anti-HA antibodies. GAPDH served as loading control. The results shown in this figure are representative of two independent experiments.

diploid fungal pathogen *C. albicans* shows a similar defect in filamentation, when grown on solid medium (Court and Sudbery, 2007). Therefore, the role of Rdi1 might be well conserved.

Complex formation between Rdi1 and Rho GTPases

Although the association of Rdi1 with Cdc42 and Rho1 has been demonstrated (Koch *et al.*, 1997; Eitzen *et al.*, 2001; Richman *et al.*, 2004; Tcheperegine *et al.*, 2005), it remained unclear whether Rdi1 also interacts with other Rho GTPases. Therefore, we systematically examined the binding specificity of Rdi1. By using coimmunoprecipitations and membrane extraction assays, we could demonstrate that Rdi1 only binds to Cdc42, Rho1, and Rho4. Even after strong

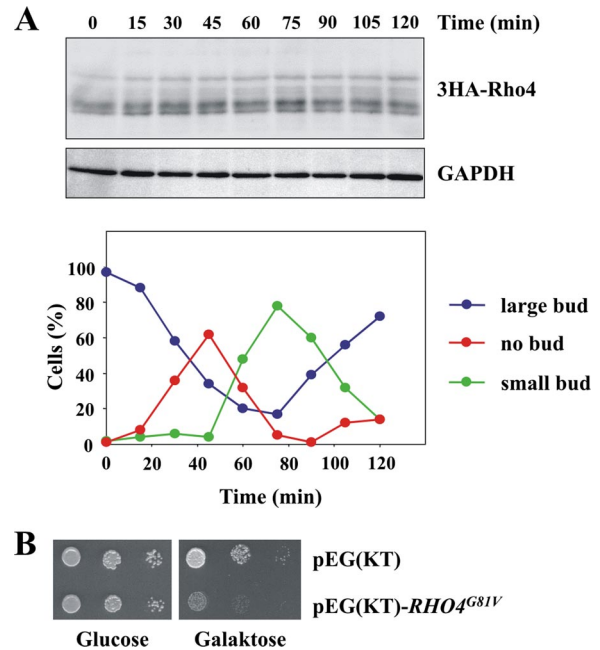


Figure 9. Rho4 protein levels do not change during the cell cycle. (A) Rho4 protein levels remain constant throughout the cell cycle. *GAL1-CDC20 3HA-RHO4* cells were arrested in metaphase by incubating cells in YP raffinose medium (no expression of *CDC20*). Galactose was added to the synchronized cells to induce *CDC20* expression and to trigger anaphase onset. At the indicated times, samples were taken, cells were lysed, and equal amounts of protein were analyzed by immunoblotting with antibodies against the HA epitope and GAPDH (loading control). Cell cycle progression was determined by following the number of cells without, with a small and with a large bud by microscopy ($n > 100$ at each time point). (B) Overexpression of constitutively active *RHO4* inhibits growth. Cells carrying either *GAL1-GST-RHO4^{G81V}* or the corresponding empty plasmid pEG(KT) were spotted on SC-Ura plates with glucose and galactose, respectively, and they were grown for 2 d at 30°C. The results shown in this figure are representative of two independent experiments.

overexpression of *RHO2*, *RHO3*, and *RHO5*, the corresponding proteins did not form a complex with Rdi1. In line with these data, Rdi1 failed to extract the Rho GTPases Rho2, -3, and -5 from membranes. It seems likely that the specific association between Rdi1 and Rho GTPases depends on protein-protein interactions. X-ray crystallography revealed the binding of Rho GDIs to the switch I and II regions of Rho proteins (Hoffman *et al.*, 2000; Scheffzek *et al.*, 2000). Specific residues in these regions might determine whether Rdi1 binds to a Rho protein. Furthermore, Rho2 and Rho3 not only carry a prenyl moiety but also were identified as being palmitoylated (Roth *et al.*, 2006). This additional lipid group could possibly prohibit the release of Rho2 and Rho3 from membranes by Rdi1.

Unexpectedly, we found that Rdi1 preferentially extracts the GTP-bound state of Cdc42 and Rho1 from membranes. In contrast, Rdi1 extracted comparable amounts of wild-type, GDP-, and GTP-associated Rho4 from membranes. However, it cannot be excluded that the point mutations, that were introduced to lock the Rho proteins either in the GDP- or GTP-bound state, also affect the interaction with Rdi1. Whether Rho GDIs form a complex with the GDP- or GTP-bound form of Rho GTPases is still controversial. Using fluorescence spectroscopy, it has been demonstrated that RhoGDI binds both forms of human Cdc42 equally well

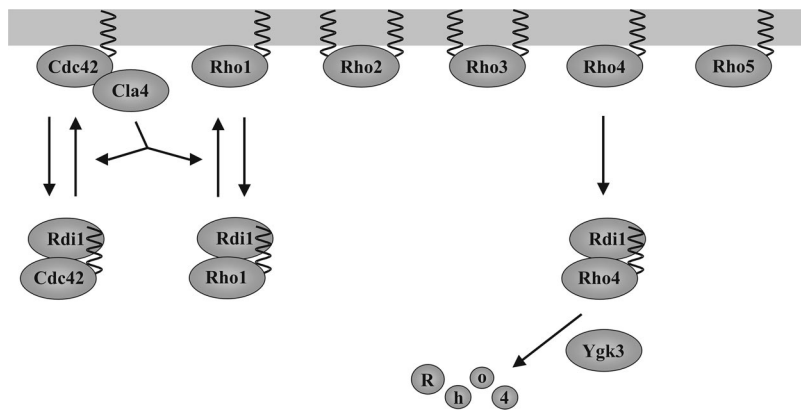


Figure 10. Model for the regulation of Rho GTPases by Rdi1. Rdi1 selectively extracts Cdc42, Rho1, and Rho4 from membranes and forms a complex with these proteins. Rho2 and Rho3 not only attach to the membrane through a prenyl group but also are palmitoylated. Cla4 disrupts binding between Rdi1 and Cdc42 and between Rdi1 and Rho1. Because Cla4 acts as a downstream effector of Cdc42, both proteins may constitute a positive feedback loop involved in the establishment of cell polarity. Furthermore, Cdc42 may regulate Rho1 activity via Cla4. The GSK-3 β homologue Ygk3 and Rdi1 target Rho4 for degradation by vacuolar proteases and the proteasome after membrane extraction.

(Nomanbhoy and Cerione, 1996). In contrast, budding yeast Rho1 exclusively binds in the GDP-bound state to Rdi1 (Koch *et al.*, 1997). The reason for these discrepancies is unclear, and further experiments are needed to reconcile these conflicting results.

We and Koch *et al.* (1997) found Cdc42 and Rho1 in the cytosol of $\Delta rdi1$ cells to a similar extent as in the wild-type strain. Possibly another protein can bind to the prenyl group of these Rho GTPases and forms a soluble complex. In addition, the observed cytosolic protein might be newly synthesized, which is not yet prenylated and therefore remains in the cytosol.

We also presented data, which suggest that the PAK-family kinase and Cdc42 effector Cla4 negatively regulates binding between Rdi1 and Cdc42 as well as Rho1 (Figure 10). Several lines argue against the idea that Cla4 simply competes with Rdi1 for binding with Rho proteins. First, this effect is specific for Cla4. Skm1 and Ste20 belong to the same kinase family as Cla4, and they act as direct downstream effectors of Cdc42 as well (Park and Bi, 2007). However, these related proteins did not interfere with Rdi1. Second, Cla4 is an effector of Cdc42 but not of Rho1 (Park and Bi, 2007). Nevertheless, we could show that Cla4 efficiently regulates binding between Rdi1 and Rho1 and between Rdi1 and Cdc42. Third, a kinase-dead mutant of Cla4 had almost no effect on Rdi1 complexes. Because Cla4 kinase activity is required for its effect on Rdi1, it is tempting to speculate that complex formation is regulated through phosphorylation of either Rdi1 or the Rho GTPases by Cla4. However, we could not demonstrate such a phosphorylation event (data not shown). Thus, it is conceivable that the regulation of Rdi1 by Cla4 is mediated via an unknown protein. It is unclear how Cla4 interferes with Rdi1 function. Cla4 could either inhibit the formation of a Rho GTPase–Rdi1 complex or alternatively disrupt an already existing complex.

There is some evidence that kinases modulate the interaction between RhoGDIs and Rho GTPases (Bourmeyster and Vignais, 1996; Mehta *et al.*, 2001; Forget *et al.*, 2002; DerMardirossian *et al.*, 2004). In mammalian cells, Pak1 phosphorylates RhoGDI at Ser101 and Ser174, which results in the disruption of Rac1–RhoGDI complex (DerMardirossian *et al.*, 2004). Notably, these serine residues are not conserved in budding yeast Rdi1.

Because Cla4 acts downstream of Cdc42, but also has a function in the disruption of the Rdi1–Cdc42 complex, Cla4 and Cdc42 may constitute a positive feedback loop involved in the establishment of cell polarity. According to this model, GTP-bound Cdc42 might recruit and activate Cla4, which in turn disrupts binding between Rdi1 and Cdc42. As

a consequence, more Cdc42 is targeted to the plasma membrane at sites of polarized growth, where it is activated by its GEF Cdc24. Interestingly, Cla4 also phosphorylates Cdc24 (Gulli *et al.*, 2000; Bose *et al.*, 2001), and it was suggested that both proteins restrict Cdc42 activation via a negative feedback loop (Gulli *et al.*, 2000). Together, Cla4 might have a dual role in the regulation of Cdc42 activity. In the early stages Cla4 may be involved in the plasma membrane recruitment and subsequent activation of Cdc42, whereas at later stages Cla4 terminates polarity via Cdc24. Importantly, Cla4 also has an effect on Rho1. Thus, Rho1 activity is indirectly regulated by Cdc42. A similar cross talk between Rho GTPases has been proposed for Pxl1. This paxillin-like protein may coordinate the function of Rho1 and Cdc42 during bud formation (Gao *et al.*, 2004; Mackin *et al.*, 2004). We observed that Cla4 has no effect on the interaction between Rho4 and Rdi1. Thus, Cla4 is highly specific for Cdc42 and Rho1.

Degradation of Rho4

Here, we observed that Rho4 protein levels depend on Rdi1. Lower amounts of Rho4 were found in cells with high levels of Rdi1 and vice versa. Because Rho4 is a relatively stable protein and a marked reduction of Rho4 levels took place after only 2 h of *RDI1* overexpression, it is unlikely that this reduction is due to diminished protein synthesis. Thus, Rdi1 promotes Rho4 proteolysis. Further analysis revealed that Rho4 degradation was almost completely blocked in the absence of vacuolar proteases. In addition, using the proteasome inhibitor MG132, we could demonstrate that Rho4 proteolysis, at least to some extent, also depends on the proteasome. Finally, we observed that Ygk3, which phosphorylates Rho4 (Ptacek *et al.*, 2005), promotes Rho4 degradation. Very little is known about Ygk3. It is one of four yeast homologues of GSK-3 β , and it probably has a role in ubiquitin-dependent protein degradation (Andoh *et al.*, 2000; Kassir *et al.*, 2006). GSK-3 β has a wide range of functions in higher eukaryotes, including the establishment of cell polarity (Kim and Kimmel, 2006). Notably, in the Hedgehog and the canonical Wnt pathway, GSK-3 β mediates degradation of a transcription factor through phosphorylation of these proteins (Kim and Kimmel, 2006). Together, we propose a model, in which Rdi1 and Ygk3 coordinately regulate Rho4 degradation by vacuolar proteases and the proteasome (Figure 10).

Very few examples for the regulation of small GTPases through degradation have been reported. In fibroblasts, the HECT domain E3 ubiquitin ligase Smurf1 promotes the local degradation of RhoA and regulates cell polarity and protrusion.

sion (Wang *et al.*, 2003). Similarly, the related Smurf2 triggers Rap1B proteolysis during neuronal differentiation (Schwamborn *et al.*, 2007). There are several important differences between these examples and our observations. RhoA and Rap1B degradation depends on HECT domain E3 ubiquitin ligases. In contrast, Rsp5, the sole yeast ubiquitin ligase with a HECT domain (Rotin *et al.*, 2000), is not involved in Rho4 degradation. Furthermore, Rho4 is degraded by vacuolar proteases, which has not been reported for RhoA and Rap1B. Finally, we demonstrate the involvement of the Rho GDI Rdi1 and the GSK-3 β homologue Ygk3 in Rho4 degradation. Thus, Rho4 is likely to be degraded by a distinct mechanism.

It seems unusual that Rho4 degradation depends on the proteasome and the vacuolar system, but this has been reported for other proteins as well. For example, a mutated version of the plasma membrane H⁺-ATPase Pma1 misfolds, and it is subsequently degraded by the proteasome and the autophagy pathway (Mazón *et al.*, 2007). However, it remains unclear why some proteins are degraded by both systems.

The physiological relevance of Rho4 proteolysis is not clear. RhoA and Rap1B are both degraded to restrict these proteins to a defined membrane region (Wang *et al.*, 2003; Schwamborn *et al.*, 2007). This does not seem to be the case for Rho4, which is localized to the entire plasma membrane and enriched at the presumptive bud site. If this polarized localization was achieved by Rho4 degradation, major changes in Rho4 protein levels during the cell cycle would be expected, but they were not observed in this study. Alternatively, it is conceivable that Rdi1 removes only active Rho4 from the membrane and targets it for destruction to terminate Rho4 activity. Thus, only a relatively small portion of Rho4 would be degraded; consequently, cell cycle-dependent changes may not be detectable by immunoblotting. In support of this model, we could also demonstrate that overexpression of constitutively active *RHO4* is deleterious for cells.

Importantly, Rho GTPases, Rho GDIs and GSK-3 β can be found in higher eukaryotes, where they also play a pivotal role in cell polarity. Therefore, the proposed regulatory link may be conserved from yeast to mammalian cells.

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