Rho4 interaction with exocyst and septins regulates cell separation in fission yeast

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Rho GTPases are small proteins present in all eukaryotic cells, from yeast to mammals, with a function in actin organization and morphogenetic processes. Schizosaccharomyces pombe Rho4 is not essential but it displays a role during cell separation at high temperature. In fact, Rho4 is involved in the secretion of the hydrolytic enzymes that are required for cell septum degradation during this process. In rho4Δ cells, vesicles accumulate in the septum area and the glucanases Eng1 and Agn1 are not secreted to the culture medium. The localization of Eng1 and Agn1 depends on the exocyst and the septins. The exocyst is a conserved multiprotein complex important for the targeting and fusion of Golgi-derived vesicles with the plasma membrane. Septins are a family of GTP-binding proteins conserved in eukaryotes that function during cytokinesis. Here we show that Rho4 is required for the proper localization of the exocyst and septins at high temperature. Moreover, pull-down experiments demonstrate that Rho4 can interact with exocyst subunits, such as Sec8 and Exo70, and septin proteins, such as Spn3. We observe that Sec8 preferentially binds to activated GTP-Rho4, suggesting that Sec8 could be an effector of this GTPase. We propose that the interaction of Rho4 with the exocyst and septins confers a precise regulation for the secretion of glucanases at the appropriate place and time during the cell cycle.

INTRODUCTION

Cytokinesis is the final stage of the cell cycle during which the two daughter cells separate and become independent. In fungi, cell separation involves cell wall degradation and this process should be precisely regulated in space and time (Roncero & Sánchez, 2010). The cell wall is a structure external to the plasma membrane and is essential for the survival of fungal cells (Free, 2013). Glucan is the main component of the cell wall in fungi, and cell wall degradation requires specific glucanases. The fission yeast Schizosaccharomyces pombe is an established model system to study cytokinesis and cell separation (Goyal et al., 2011). Eng1 and Agn1 are the glucanases involved in the degradation of the β-glucan and α-glucan of the septum, respectively (García et al., 2005; Martín-Cuadrado et al., 2003). Precise localization of these glucanases is important for cell integrity. It has been reported that the exocyst and the septins complexes are required for this localization (Martín-Cuadrado et al., 2005; Santos et al., 2005).

The exocyst is a multiprotein complex highly conserved from yeast to humans. It is involved in late stages of exocytosis, promoting the tethering and fusion of post-Golgi secretory vesicles to the plasma membrane. The exocyst is composed of eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) (Heider & Munson, 2012). In S. pombe all subunits are essential, except Exo70. In fact, exocyst mutants, such as sec8-1, sec3-2 and exo70Δ, display cell separation defects at high temperatures, suggesting an important role for secretion during cell separation (Bendezú et al., 2012; Jourdain et al., 2012; Wang et al., 2002).

Septins are a family of GTP-binding proteins conserved from yeast to mammalian cells that function during cytokinesis (Longtine et al., 1996; Longtine & Bi, 2003). In fission yeast, five different septins have been described and named Spn1–5 (An et al., 2004). In contrast to other organisms, S. pombe septin mutants are viable, but they have cell separation defects and accumulate chains of cells (Tasto et al., 2003).

In yeast, Rho GTPases are responsible for the coordinated regulation of cell wall biosynthesis and the actin cytoskeleton, and they are required to maintain cell integrity and

Abbreviations: DIC, differential interference contrast; GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GEF, GDP–GTP exchange factor; GS beads, glutathione–Sepharose beads.

Four supplementary figures are available with the online Supplementary Material.
polarized growth (Pérez & Rincón, 2010). These small GTPases act as molecular switches that are turned on and off by binding to GTP or GDP. They are positively regulated by GTP–GDP exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (GAPs) and GDP dissociation inhibitors (GDIs) (García et al., 2006). The fission yeast contains six Rho GTPases, Cdc42 and Rho1–5; only Cdc42 and Rho1 are essential proteins. Rho4 GTPase localizes to the septum and is involved in cell separation during cytokinesis (Santos et al., 2003) and regulation of the actin cytoskeleton and cytoplasmic microtubules during cell growth (Nakano et al., 2003). Rho4Δ cells show defects in division at high temperature (Santos et al., 2003); in fact, Rho4 is required for the localization and secretion of glucanases Eng1 and Agn1, needed in turn for the separation of the two daughter cells (Santos et al., 2005). Although the precise regulators of Rho4 are not known, it has been described that Rho4 interacts with Rdi1, the unique Rho GDI in S. pombe (Nakano et al., 2003). In addition, Rga9 interacts with Rho4 and Cdc42 in a two-hybrid assay, suggesting that it may be a GAP for these GTPases (Nakano et al., 2003). As positive regulators of Rho4, it has been published that the Rho GEF Gef2 interacts with Rho1, Rho4 and Rho5 in pull-down assays. In fact, Rho4 localization to the division area is diminished in gef2Δ cells (Zhu et al., 2013). Additionally, an interaction with the Cdc42 GEF Scd1 has been also described (Iwaki et al., 2003), and recently, Gef3 has been identified as a Rho4 GEF (Wang et al., 2015).

Rho3 also participates in the secretion process during cytokinesis. Rho3 modulates the exocyst complex and overexpression of rho3Δ is able to suppress the lethality of the sec8-1 exocyst mutant (Wang et al., 2003). rho3Δ overexpression also suppresses the growth defect of cdc42 mutant alleles with various membrane traffic defects (Estravis et al., 2011). Additionally, Rho3 participates in Golgi/endosome trafficking through interaction with Apm1, a protein of the clathrin-associated adaptor complex AP-1 (Kita et al., 2011).

Rho3 and/or Rho4 orthologues have been described in other fungi, such as Saccharomyces cerevisiae, Candida albicans, Neurospora crassa, and Aspergillus nidulans (Elias & Klimes, 2012). S. cerevisiae Rho3 and Rho4 are partially redundant, and are involved in cell polarity through regulation of polarized exocytosis and actin cytoskeleton organization (Matsui & Toh-E, 1992b). They interact with the exocyst subunits Exo70 and Sec3, and with formins to activate actin cable formation (Dong et al., 2003; Robinson et al., 1999). In C. albicans, Rho3 and Rho4 fulfill separate cellular functions. RHO3 is an essential gene and experiments with cells in which RHO3 expression was shut down revealed a strong cell polarity defect and a partially depolarized actin cytoskeleton. On the other hand, deletion of RHO4 led to cell separation defects that could be suppressed by overexpression of the endoglucanase ENG1 gene, as in S. pombe rho4Δ cells (Santos et al., 2005, Dünkler & Wendland, 2007). Remarkably, Rho4 homologues in Neurospora and Aspergillus act earlier in the septation process. They are essential for cytokinesis and the absence of these genes results in the lack of actomyosin ring formation and septum construction (Kwon et al., 2011; Rasmussen & Glass, 2005). Phylogenetic studies divide fungal Rho4 GTPases in two groups, one specific for hemiascomycetes such as S. cerevisiae and the other for archiascomycyte and euascomycete including the group of filamentous fungi such as A. nidulans or N. crassa (Rasmussen & Glass, 2005).

We have reported before that Rho4 is required for secretion of the glucanases Eng1 and Agn1 (Santos et al., 2005). In this paper, we examine the relationship between Rho4, and the septins, and the exocyst complexes since Eng1 and Agn1 require these both exocyst and septins for their localization (Martín-Cuadrado et al., 2005). We show that Rho4 interacts with both the exocyst and the septins. The interaction of active Rho4 with the Sec8 subunit suggests that the exocyst could be a possible effector of the GTPase. By contrast, the Spn3 septin interacts with all forms of Rho4. Additionally, we compare Rho3 and Rho4 interactions with the exocyst. Our results suggest that both GTPases participate in secretion with different roles. We propose that active Rho4 regulates the exocyst function to promote glucanase secretion to the proper area during a specific time in the cell cycle.

**METHODS**

**Strains, growth conditions, and genetic methods.** Standard S. pombe media and genetic manipulations were used (Moreno et al., 1991). All the strains used were isogenic to WT strains 972 h+ and 975 h−, and they are described in Table 1. The strains were constructed by either tetrad dissection or random spore germination method. Cells were usually grown in either rich medium (YES) or minimal medium (EMM) with appropriate supplements. Escherichia coli DH5α was used as a host for propagation of plasmids. Bacteria were cultured in LB medium supplemented with 50 μg ml−1 ampicillin when needed. Solid media contained 2% agar.

S. pombe was transformed by the lithium acetate method (Ito et al., 1983). The nmt promoter-containing vector pREP3X (Forsburg & Sherman, 1997) was used for the overexpression of the rho4Δ gene, which was induced by growing the strains transformed with this plasmid on plates lacking thiamine.

**Pull-down assays and Western blotting.** Extracts from 5 × 10⁸ cells expressing different tagged proteins were obtained as described (Arelano et al., 1997), using 200 μl of lysis buffer (20 mM Tris/HCl pH 8.0, 100 mM NaCl, 0.5% NP-40 and protease inhibitors [2 μg ml⁻¹ aprotinin, 2 μg ml⁻¹ leupeptin, 2 μg ml⁻¹ pepstatin, 10 mM NaF, 1 mM PMSF]). Cell extracts (4 mg total protein) were incubated with glutathione–Sepharose beads for 2–4 h at 4 °C. The beads were washed four times with lysis buffer and resuspended in Laemmli sample buffer. Proteins were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and blotted to detect the GST-, Myc-, or GFP-fused epitopes with the corresponding antibodies and an ECL detection kit (GE Healthcare). Forty micrograms of protein was loaded for Western blot analysis of whole-cell extracts as a control for the amount of tagged protein present in the lysates.

**Microscopy techniques.** Cell samples were observed using a Leica DMXRA microscope equipped for Nomarski optics, and
photographed with a Photometrics Sensys camera. For epifluorescence, a Nikon Eclipse 98i microscope with ORCA ER camera was used and images were processed with Metamorph Premier software. For confocal images and 3D reconstructions, a Leica confocal TCS-SL was used.

### Other methods.
Plate assays for growth ability at different temperatures were performed by spotting the appropriate dilutions of exponential-phase growing cells on YES or EMM solid media. Exponentially growing cells (2 × 10⁷) were spotted at 1/4 dilutions. Plates were incubated for 2–4 days.

### Table 1. List of strains used in this study

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RESULTS

rho4Δ shows genetic interaction with the sec8-1 exocyst mutant

Rho4 plays a role in cell separation; rho4Δ cells accumulate vesicles in the septum area and glucanase secretion is impaired (Santos et al., 2003). On the other hand, the exocyst complex plays a key role in delivery of secretory vesicles in many organisms and, in S. pombe, exocyst mutants are defective in cell separation (Wang et al., 2002). To explore the possible relationship between Rho4 and the exocyst, we tested for genetic interactions between rho4Δ and some exocyst mutant strains. The sec8-1 mutant cells have a strong defect in secretion and cell separation, and exhibit a thermosensitive-lethal phenotype. This defect is partially rescued by the presence of an osmotic stabilizer such as sorbitol. We found that the growth defect of the sec8-1 rho4Δ double mutant was more drastic than that of any of the single mutants; it grew poorly at 32 °C and was non-viable at 35 °C. Moreover, whereas sorbitol was able to suppress the growth defect of the sec8-1 single mutant at 37 °C, it was only capable of partially rescuing the defect of the sec8-1 rho4Δ double mutant (Fig. 1a). Microscopic observation revealed that sec8-1 rho4Δ cells are multi-septated, show many branches and are frequently lysed (Fig. 1b). These results suggest a functional relationship between the GTPase Rho4 and the exocyst.

Exo70 is the nonessential exocyst component. Cells lacking Exo70 are defective in secretion and cell separation and show a severe growth defect at high temperature (Fig. 1a). The absence of Rho4 in exo70Δ cells only barely enhanced the defect; moreover, the morphology of exo70Δ rho4Δ cells was similar to that of the exo70Δ single mutant, implying no negative interaction between rho4Δ and exo70Δ (Fig. 1a, b). We also analysed the genetic interaction of rho4Δ with the sec3-2 exocyst mutant. We found that the sec3-2 rho4Δ double mutant behaved as the single sec3-2 and the growth defect was completely rescued by the addition of sorbitol to the medium (data not shown).

From these experiments we can conclude that Rho4 is functionally related to Sec8 but not to Exo70 or Sec3. This suggests that the relationship between the Rho4 GTPase and the exocyst complex could be different depending on the subunit involved.

Rho4 localization is independent of the exocyst

It has been described that Rho4 localizes to the septum after mitosis, when both nuclei are perfectly separated and the septum is forming, and remains in that location throughout cell separation (Santos et al., 2003). To further study the localization of Rho4, we used confocal microscopy. 3D reconstruction of the fluorescence signal in cells expressing...
**GFP–rho4** showed that Rho4 was initially localized as a ring that, as the septum is forming, spreads to form a disc (Fig. 2a). This localization indicates that Rho4 is incorporated into the new membrane formed around the septum as septation proceeds. To study the temporal regulation of the localization of Rho4 during septum formation, we analysed its localization compared with that of Rlc1 as a marker for actomyosin ring formation. The experiment demonstrated that Rho4 was always assembled after Rlc1, indicating that Rho4 arrives to the septum after the actomyosin ring is formed (Fig. 2b and data not shown).

It has been described that some GTPases follow the secretory pathway for localization (Wang *et al.*, 2003). To investigate whether the localization of Rho4 requires the function of the exocyst, we analysed GFP–Rho4 localization in *sec8-1* and *exo70Δ* mutants. GFP–Rho4 was able to localize to the septum in both exocyst mutants at either permissive or restrictive temperatures (Fig. 3). However, the cytoplasmic GFP–Rho4 signal was clearly increased in some *sec8-1* cells at the restrictive temperature (Fig. 3a).

**Rho4 is required for the exocyst localization at high temperature**

To explore whether Rho4 function is important for proper localization of the *S. pombe* exocyst, we also analysed the localization of different exocyst subunits, such as Sec6, Exo70 and Sec8, in *rho4Δ* cells. In both WT and *rho4Δ*, these exocyst components localized to regions of active secretion, poles, and septum at a temperature of 28 °C (Fig. 4a and data not shown). Although Sec6–GFP localization at the poles and septum was maintained in *rho4Δ*, many cells showed increased cytoplasmic staining when grown at 37 °C (Fig. 4a, b). Similarly, Sec8–GFP signal at the septum was much weaker and less symmetrical in the *rho4Δ* strain grown at high temperature (Fig. 4b and Fig. S1, available in the online Supplementary Material). Western blot analysis revealed that the global protein levels of the Sec6 and Sec8 exocyst subunits are not drastically altered in the *rho4Δ* mutant at 37 °C (data not shown). On the other hand, localization of Exo70 was not compromised at high temperature when the GTPase Rho4 was not present (Fig. 4c).

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**Fig. 2.** (a) Confocal images of cells with GFP–Rho4 at different phases of the cell cycle. DIC images of the cells are shown in the upper panels, a central plane of fluorescence in the middle panels and 3D reconstruction at the lower panels. (b) Localization of GFP–Rho4 and Rlc1-tomato in WT cells. Scale bar, 5 μm.

**Fig. 3.** Localization of GFP–Rho4 in WT (a), *sec8-1* (a) and *exo70Δ* mutant strains (b) growing at 28 °C (permissive temperature) and transferred to 37 °C (restrictive temperature) for 5 h. Scale bar, 5 μm.
From these studies we can conclude that Rho4 is required for the correct localization of some exocyst components, suggesting that exocyst function is compromised in cells lacking Rho4, probably explaining their cell separation defect. Two redundant morphogenetic pathways have been proposed in S. pombe, one of them acting through the formin For3 and the other one through the exocyst complex (Bendezú & Martin, 2011). Interestingly, we found that the for3Δ rho4Δ double mutant is rounder and shows a strong growth defect even at 32 °C compared to the single mutants (Fig. S2), further reinforcing the notion that exocyst function is impaired in rho4Δ cells.

The exocyst is an effector of Rho4 GTPase

The genetic interactions described above, and the fact that Rho4 is required for the correct localization of the exocyst, prompted us to check if these proteins physically interact. Pull-down of Sec8–GFP with glutathione–Sepharose (GS) beads from lysates of cells overproducing GST–Rho4 demonstrated that both proteins interact (Fig. 5a). We also observed interactions between Rhō4 and Exo70 proteins using the same approach in cells expressing exo70–13myc and overproducing GST–Rho4 (Fig. 5b).

Rho GTPases are proteins that, when activated, interact with their effectors. Hence, the exocyst could be an effector of the Rho4 GTPase. To explore this possibility we constructed strains carrying sec8–GFP and integrated GST fusions of WT rho4+, constitutively active rho4–G23V or inactive rho4–T28N alleles expressed under control of the nmt1 promoter. GS pull-down experiments in lysates of these cells clearly revealed that Sec8–GFP preferentially interacts with the Rho4–G23V hyperactive form and not with the inactive Rho4–T28N protein, suggesting that Sec8 could be an effector of Rho4 (Fig. 5a, c). In contrast, we found that Exo70–13myc interacts with all Rho4 versions: WT, hyperactive, and an inactive GDP-bound form (Fig. 5b, c). These data point out that although Rho4 can interact with different subunits of the exocyst, only some of them, such as Sec8, are effectors of this GTPase.

We described above that rho4Δ displays a negative genetic interaction with sec8–1, but not with exo70Δ. A possible explanation could be that the interaction of Rho4 with the exocyst is mediated through Exo70. However, pull-down experiments performed in a sec8–GFP exo70Δ GST–rho4 strain showed that Sec8 interacts with Rho4 even in the absence of Exo70 (Fig. 5d). This result indicates that Rho4 binds to the exocyst complex and that this interaction is not dependent on the Exo70 subunit.

Rho3 and Rho4 display different roles during cell separation

Rho3 GTPase modulates the exocyst function. It has been described that rho3Δ mutants are thermosensitive, are defective in acid phosphatase secretion, accumulate vesicles and are synthetically lethal with exocyst mutants such as sec8–1 and exo70Δ. Additionally, rho3+ overexpression is able to suppress the growth defect of exocyst mutants (Wang et al., 2003). To see if Rho4 has a function similar to that of Rho3, we analysed whether Rho4 overproduction can suppress the defects of exocyst mutants. However, rho4Δ overproduction could not rescue the thermosensitive phenotype of sec8–1 or exo70Δ (data not shown).

To further study the function of Rho3 and Rho4 during cytokinesis we analysed the defects of rho3Δ rho4Δ double mutant strain. As seen in Fig. 6(a), the thermosensitive growth of this double mutant was similar to that of rho3Δ single mutant strain. However, rho3Δ rho4Δ cells displayed a drastic defect in cell separation compared to WT cells. After 5 h at 36 °C, both WT and rho3Δ strains showed 5% of septated cells and rho4Δ showed 9% of septated cells. In contrast, in the rho3Δ rho4Δ double mutant, 40% of the cells had septa and 7% were multiseptated. These results suggest that Rho3 and Rho4 have different roles in cell separation.

![Fig. 4. Subcellular distribution of Sec6, but not Exo70, is impaired in the absence or Rho4 function.](image-url)
We have shown above that localization of exocyst subunits Sec8 and Sec6 is affected in rho4Δ cells grown at high temperature. Thus, we analysed the localization of Sec8–GFP and Exo70–GFP in rho3Δ rho4Δ double mutant strains, and observed that Sec8–GFP was not properly localized at the septum of rho3Δ rho4Δ cells (Fig. 6b). By contrast, Exo70–GFP localization was not affected by the lack of Rho4 GTPase, although there was a huge increase of fluorescence accumulated inside single rho3Δ and rho3Δ rho4Δ double mutant cells (Fig. 6c). It is known that rho3Δ accumulate abnormal electrodense membranes inside the cells and have secretion defects (Kita et al., 2011), so it is possible that Exo70 is retained in these internal membranes. In summary, rho3Δ rho4Δ double mutant displayed additive effects (Fig. 6b, c), showing defects in the localization of Sec8–GFP and Exo70–GFP. We also analysed whether Rho3 interacts with Sec8 or Exo70. GS-beads pull-down experiments with cells carrying GST–Rho3 and GST–Rho4G23V or GST–Rho4T28N showed that Rho3 interacts with Sec8–GFP and Exo70–GFP. GS-beads pull-down experiments with cells carrying Sec8–GFP and Exo70–myc showed that Sec8–GFP precipitates with GST–Rho4G23V or GST–Rho4T28N, but not with GST–Rho4. Sec8–GFP and Exo70–myc precipitates with GST–Rho4G23V or GST–Rho4T28N, but not with GST–Rho4. GS-beads pull-down experiments with cells carrying GST–Rho3 and GST–Rho4Δ showed that GST–Rho3 precipitates with Sec8–GFP and Exo70–myc. We also analysed whether Rho3 interacts with Sec8 or Exo70. GS-beads pull-down experiments with cells carrying GST–Rho3 and GST–Rho4Δ showed that GST–Rho3 precipitates with Sec8–GFP and Exo70–myc.
Sec8–GFP or Exo70–Myc showed interaction between the GTPase and these exocyst subunits (Fig. S3).

From these results, we can conclude that both Rho4 and Rho3 are involved in secretion, but play different roles in the regulation of exocyst function.

**rho4Δ and septin mutants display genetic interaction**

Localization of glucanases is dependent on both exocyst and septins (Martín-Cuadrado et al., 2005). In mammalian cells, it has been described that exocyst and septins function together for cell polarity processes during cytokinesis (Hsu et al., 1998). To study the possible relationship between Rho4 and the septins, we constructed spn1Δ rho4Δ, spn2Δ rho4Δ, spn3Δ rho4Δ and spn4Δ rho4Δ double mutants. All four of these strains showed stronger separation defects than the corresponding single mutant strains (Fig. 7a and data not shown), with a slightly increased proportion of septated and multiseptated cells in cultures even at 28 °C (Fig. 7b).

These additive effects suggest that either septins and Rho4 play different roles during cell separation, or that Rho4 collaborates in the organization of the septin ring.

We previously reported that overexpression of rho4Δ caused lysis and morphological defects (Santos et al., 2003). As can be seen in Fig. S4, rho4Δ overexpression is even more damaging when the septins are not present, corroborating that septins and Rho4 play different functions and suggesting that septins are critical for cell viability when Rho4 is in excess.
**Rho4 is required for proper septin ring formation at high temperature**

As mentioned above, the cell separation defects of rho4Δ cells may result from improper septin organization. To explore this possibility we analysed the localization of Spn3–GFP in rho4Δ cells. Spn3–GFP localized as in WT cells forming perfect rings at 28 °C; however, these rings were not properly assembled when rho4Δ cells were grown at 37 °C (Fig. 8a). During cytokinesis, septins localize to the separation area at the cell cortex; in rho4Δ cells, even at low temperatures, Spn3–GFP signal at the cortex was less intense and more spread than in WT cells. Additionally, confocal studies showed that at high temperatures, some septin rings were incomplete and some cells with completed septa still contained septins. Quantification is shown in Fig. 8(b). All of these defects could be explained if Rho4 is affecting protein levels or stability of Spn3–GFP. However, Western blot analysis indicated that Spn3–GFP levels were not affected in rho4Δ cells at 28 °C or 37 °C (Fig. 8c). These observations point to a Rho4 participation in the proper maintenance of the septin ring at high temperatures.

GFP–Rho4 localization in spn3Δ or spn4Δ was analysed to check if Rho4 and septins were interdependent. GFP–Rho4 localized correctly in septin mutants (data not shown) indicating that the septin complex is not required for its localization.

To further study the relationship between Rho4 and the septins, GS-beads pull-down studies were performed in spn3–GFP cells overproducing GST–Rho4. These experiments demonstrated that both proteins interact (Fig. 8d). Similar experiments performed with Spn1–GFP, another protein of the septin complex, also showed interaction with Rho4 (data not shown). Using the same approach, we analysed whether GST–Rho3 interacts with Spn3–GFP, and we could not detect interactions under our experimental conditions. These results suggest that although both Rho3 and Rho4 GTPases interact with the exocyst, only Rho4 interacts with the septin complex.

Rho4 interaction with the septins was also analysed in exo70Δ cells to see if it was dependent on the exocyst. However, the interaction was still present in the exo70Δ strain, suggesting that Exo70 is not required (data not shown). Additionally, we analysed the interaction of Rho4 and Sec8 in the spn3Δ and spn4Δ mutants and we observed that the interaction was maintained, suggesting that septins are not required for the association of Rho4 with the exocyst (data not shown).

To analyse if the septins are effectors of Rho4, we also used GST pull-down assays in spn3–GFP cells carrying the constitutively active rho4G23V or the inactive rho4T28N alleles (Fig. 8d). These experiments revealed that Spn3–GFP interacted with all forms of Rho4, indicating that septins are not effectors of Rho4 and this GTPase interacts with them independently of the activation state.

Fig. 8. (a) Localization of Spn3–GFP in WT (PPG37.21) and rho4Δ (PPG37.14) growing exponentially at 28 °C or 37 °C. Point arrows indicate localization of the Spn3 protein in separating cells. * indicates incomplete septin ring. Scale bar, 5 μm. (b) Quantification of the different localization patterns of Spn3–GFP shown in (a) at 37 °C. The categories, as depicted in the figure, are: Spn3–GFP localized to a ring in the early stage of cytokinesis, to one or two formed rings during cytokinesis, to the poles generated after separation, to incomplete rings and to the completed septum. Data are presented as percentages. – Indicates that no cell presented that localization. (c) Spn3–GFP levels analysed by Western blot in WT and rho4Δ mutant strains. Fifty micrograms of cell extracts were used and probed with anti-GFP antibodies. Tubulin was used as a loading control. * Indicates the specific band for Spn3-GFP. (d) Cells expressing endogenous Spn3–GFP and plasmids expressing Pnmt1–GST–rho4Δ, Pnmt1–GST–rho4G23V or Pnmt1–GST–rho4T28N were grown in the absence of thiamine for 15 h. Whole-cell extracts were pulled down with GS beads and the precipitates were probed with anti-GFP antibody. Total amounts of Spn3–GFP and GST–Rho4 alleles in the cell extracts were analysed by Western blot with anti-GFP and anti-GST antibodies, respectively.
**DISCUSSION**

**Rho4 and its relationship with the exocyst**

Rho4 is required for cell separation and is involved in the proper secretion of glucanases for cell wall degradation (Santos *et al.*, 2003, 2005). These hydrolytic enzymes depend on septin and exocyst complexes for the correct localization and secretion to the septum area. In this paper, we present evidence that Rho4 regulates the exocyst and interacts with the septins. First, we have shown a genetic interaction between rho4∆ and sec8-1. By contrast, rho4∆ and exo70∆ mutants show no interaction. The exocyst components can form subcomplexes with different functions; it has been described that Sec8 and Exo70 have different roles during the mating and sporulation process in *S. pombe* (Sharifnoghadam *et al.*, 2010). Exo70 is a nonessential component of the exocyst and it has been proposed that another subunit, Sec3, is redundant with Exo70 because the sec3-2 exo70A double mutant is inviable. Both subunits can act as exocyst tethers to the plasma membrane (Bendezú *et al.*, 2012). Similarly to rho4∆ and exo70∆, there is not genetic interaction between rho4∆ and sec3-2 mutant strains; therefore, Rho4 may have an overlapping role with some of the exocyst subcomplexes but not with the redundant tethering subunits.

Like the septins, the exocyst localizes to the poles and to the division site, forming a ring that doubles later on during the septation process (Jourdain *et al.*, 2012; Wang *et al.*, 2002). Additionally, Rho4 co-immunoprecipitates with different subunits of the exocyst complex, and the interaction with Sec8, but not with Exo70, seems to be stronger with active Rho4, suggesting that Sec8 could be an effector of this GTPase. To date no effectors of Rho4 are known, and only glucanases can be considered as direct or indirect targets of this GTPase. The interaction of active Rho4 with Sec8 may allow the fusion of vesicles containing hydrolytic activities, such as Eng1 and Agn1, at the specific septum area. It has been described that Sec8 and Exo70 also interact with the Cdc42 GTPase, but this interaction mainly occurs with the GDP form (Estravis *et al.*, 2011). The function of this interaction is not clear. Cdc42 regulates secretion and localization of the exocyst to the poles but it also regulates endocytosis and multiple traffic events in *S. pombe* (Estravis *et al.*, 2012). In the cdc42-L160S mutant, Sec8 and mainly Exo70 accumulate inside the cells, and rho3+ overexpression is able to suppress this defect (Estravis *et al.*, 2012). We demonstrate here that Exo70 is also accumulated in internal membranes in rho3Δ cells, but the localization of the Sec6 and Sec8 exocyst subunits is not affected (Wang *et al.*, 2003). Rho3 binds to different subunits of the AP-1 complex, which mediates transport from Golgi to endosomes. Thus, Rho3 interacts with the AP-1 accessory protein Pip1 in a nucleotide-independent way, but interacts with Apm1 preferentially in its active form (Kita *et al.*, 2011; Yu *et al.*, 2013). Taken together, these data indicate that exocyst localization might be precisely regulated in time and space and several GTPases such as Cdc42, Rho4 and Rho3 are involved in this process. In fact, we have shown that rho3Δ rho4Δ double mutant strain is viable but shows a stronger cell separation defect than the single mutants, indicating that both GTPases have different roles in this process. Both rho3Δ and rho4Δ display genetic interactions with exocyst mutants. However, they behave differently; whereas the phenotype of the rho3Δ exo70Δ double mutant is more drastic that any of the single mutants (Wang *et al.*, 2003), rho4Δ exo70A behaves as exo70A. Furthermore, Rho4, but not Rho3, regulates the precise localization of several exocyst subunits at high temperature. By contrast, Rho3 appears to preferentially affect Exo70 localization and not that of other exocyst subunits. On the other hand, overproduction of Rho3, but not that of Rho4, is able to rescue sec8-1 and exo70A mutants. Additionally, Rho3 localization to the poles and septum depends on a functional exocyst (Wang *et al.*, 2003) while we show here that the exocyst is not required for Rho4 localization to the septum.

In budding yeast, Rho3 and Rho4 display redundant functions and both interact with exocyst components such as Exo70 (Robinson *et al.*, 1999; Wu *et al.*, 2010). Here, we have shown that in fission yeast, Rho3 and Rho4 also interact with the exocyst, but they present different roles during cytokinesis. Similarly to *S. pombe*, *C. albicans* Rho3 and Rho4 are not redundant but no interaction with the exocyst has been reported (Dünkler & Wendland, 2007).

**Rho4 and its relationship with the septins**

Septins form a ring late in mitosis that does not constrict with the actomyosin ring and splits as the septum is formed. It has been described that septins are involved in the localization of Eng1 and Agn1 glucanases (Martín-Cuadrado *et al.*, 2005). Besides interaction with the exocyst, Rho4 also interacts with the septin complex. Spn3 interacts with all forms of Rho4, suggesting that Spn3 is not a Rho4 effector protein. However, septins require Rho4 for proper ring localization at high temperature. It has been proposed that septins define sites of cell growth by directing exocytosis towards them (Oh & Bi, 2011). According to this hypothesis, septins have been found associated with the exocyst in *C. albicans* or mammalian cells (Dünkler & Wendland, 2007; Hsu *et al.*, 1998). In *S. pombe*, this septin–exocyst interaction might be mediated by the GTPase Rho4.

It has been recently published that Gef3 is a GEF of Rho4 that interacts with septins (Wang *et al.*, 2015). Gef3 also interacts with Rho3 at the division site (Muñoz *et al.*, 2014). Interestingly, the gef3Δ strain displays cell separation defects similar to the rho4Δ strain and shows genetic interactions with exocyst and for3Δ mutants. Taken together, these results reinforce the fact that activation of Rho4 takes place at a precise location of the cell during the septation process.

With all these data, we propose a model whereby Rho4 colocalizes with both the exocyst and septins, forming a
ring at the septum edge. At late stages of cytokinesis, Gef3 arrives to the septation area through the septin ring and activates Rho4 (Muñoz et al., 2014; Wang et al., 2015). In turn, this GTPase activates the exocyst that targets the secretion of enzymes, such as Eng1 and Agn1, for septum cleavage at the end of cytokinesis. These enzymes lead to the separation of the two daughter cells.

Similar to S. pombe, in C. albicans rho-4 mutants form septa but cells have defects in the separation process (Dünkler & Wendland, 2007). Unlike yeasts, where cell separation takes place at the end of the cell cycle, the septum in filamentous fungi is not degraded and cells remain attached. In fact, Rho4 homologues in filamentous fungi such as Aspergillus or Neurospora display essential functions during septum formation (Kwon et al., 2011; Rasmussen & Glass, 2005). In A. nidulans, the interaction of Rho4 with its GEF, Bud3, mediates the recruitment of the formin to the site of the contractile actin ring assembly (Andrews & Stark, 2000). All these data point out that, in spite of their sequence homology, Rho4 GTPases can be classified in different functional and phylogenetic groups (a genetic tree with all the Rho4 superfamily genes are necessary for bud growth, and their defect is supported by Direcció´n General de Investigacio´n, MICINN, Spain (grant CSI037U14).

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