Role of Rho GTPases and Rho-GEFs in the regulation of cell shape and integrity in fission yeast

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Abstract

The Rho family of GTPases are highly conserved molecular switches that control some of the most fundamental processes of cell biology, including morphogenesis, vesicular transport, cell division and motility. Guanine nucleotide-exchange factors (GEFs) are directly responsible for the activation of Rho-family GTPases in response to extracellular stimuli. In fission yeast, there are 7 Dbl-related GEFs and they activate 6 Rho-type GTPases within a particular spatio-temporal context. The failure to do so might have consequences reflected in aberrant phenotypes and in some cases lead to cell death. In this review, we briefly summarize the role of Rho GTPases and Rho-GEFs in the establishment and maintenance of cell polarity and cell integrity in *Schizosaccharomyces pombe*.

Rho-GTPases and Rho-GEFs in fission yeast

Rho GTPases are key molecules in morphogenetic and polarity processes; approximately one per cent of the human genome encodes proteins that either regulate or are regulated by members of the Rho family of small GTPases (reviewed by [24, 43]). The guanine nucleotide-bound state of Rho GTPases determines the physiological activity of the protein. When bound to GDP, Rho GTPases are inactive, but when loaded with GTP, Rho proteins are conformationally primed to engage downstream effectors and influence cellular functions. The simplicity of this model contrasts with the complexity of the pathways regulated by these proteins. In fact, the proteins that control the nucleotide state of Rho GTPases are much larger and more complex than the

GTPases themselves and they contain multiple domains capable of protein-protein interactions [36, 74, 97, 105].

Rho GTPases activity is controlled by three types of proteins: (a) guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP, rendering the protein active [105]; (b) GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity turning off the GTPase [8]; (c) guanine nucleotide dissociation inhibitors (GDIs), whose role appears to be to block spontaneous activation [21] (Figure 1). These different possibilities are not mutually exclusive, and it is likely that full activation of GTPases requires activation of GEFs as well as inactivation of their GAPs and/or GDIs.

The fission yeast Rho family of small GTPases includes Cdc42p, Rho1p, Rho2p, Rho3p, Rho4p and Rho5p [2]. Fission yeast Cdc42p and Rho1p are functional homologues of human Cdc42p and RhoAp, respectively, and of budding yeast Cdc42p and Rho1p, respectively, and are essential for cell viability [72, 80]. Cdc42p has been reported to be involved in the establishment of cell polarity [72], while Rho1p is involved in the maintenance of cell integrity and polarization of the actin cytoskeleton [2-4, 80]. On the other hand, Rho2p, Rho3p, Rho4p and Rho5p are not essential for growth, although they also play important roles in morphogenesis. *rho2*$^+$ has been shown to be involved in the control of cell morphogenesis, probably by regulating the synthesis the α-1,3-glucan, via a Pck2p pathway [9, 40]. *rho3*$^+$ and *rho4*$^+$ participate in cell separation processes. Rho3p interacts with the formin For3p and modulates exocyst function [82, 119], and Rho4p is necessary for septum degradation during cytokinesis [83, 100, 101]. The function of Rho5p is still not well established; it is expressed under stress conditions (sporulation and stationary phase) and its overexpression can compensate Rho1p depletion [81, 96].

GEFs turn on Rho GTPases and are important for GTPase activation, localization, stabilization, and interaction with their effectors. Sequence analysis of the *S. pombe*

genome has revealed the existence of seven Rho-GEFs: *scd1*$^{+}$, *gef1*$^{+}$, *gef2*$^{+}$, *gef3*$^{+}$, *rgf1*$^{+}$, *rgf2*$^{+}$ and *rgf3*$^{+}$ (http://www.genedb.org/genedb/pombe/index.jsp), [42]. Of these, *scd1*$^{+}$ and *gef1*$^{+}$ are Cdc42p-specific GEFs and Rgf3p, Rgf2p and Rgf1p has been described to function as GEFs for Rho1p, while *gef2*$^{+}$ and *gef3*$^{+}$ have not yet been assigned to any known GTPase [11, 16, 29, 31, 41, 75, 78, 111]. Considering that there are four other Rho GTPases and at least two biochemically uncharacterized GEFs, it will take considerable effort in the future to sort out the biochemical specificities, cellular roles and regulation of each Rho-GEF.

GEFs for Rho GTPases contain a conserved domain in human Dbl and *S. cerevisiae CDC24*, known as the DH (Dbl homology) domain, which is necessary for GEF activity [25, 37]. Small GTPases contain nucleotide- and Mg^{2+}- binding pockets. The form of nucleotide (GDP or GTP) that is bound modulates the conformation of the switch region whereas Mg^{2+} is required for the high-affinity binding of guanine nucleotides. DH domains participate in the formation of the GTPase interaction pocket by promoting GTPase intermediates that are devoid of nucleotide and Mg^{2+}. Because of the high intracellular ratio of GTP:GDP, the released GDP is replaced with GTP, leading to activation (Figure 1).

DH domains, also called “Rho GEF domains”, share little sequence identity with each other. In *S. pombe*, the identity percentage between the deduced amino acid sequence of DH domain that belong to GEFs with the same substrate specificity is less than 20% upon comparing Rgf1p and Rgf3p, and Scd1p and Gef1p, respectively, while it rises to 63.4% upon comparing the DH domains of Rgf1p and Rgf2p, the closest related members among the GEF family. Despite this, crystallographic and NMR analyses of several DH domains have revealed a highly related three-dimensional structure [60, 110, 122]. DH domains have three conserved regions called (CR1, CR2 and CR3), each 10-30 amino acids long; two of those regions -CR1 and CR3- are exposed on the surface of the DH domain and pack to form the core domain. Amino-acid substitutions within

these regions adversely affect nucleotide exchange activity. In *S. pombe*, a point mutation located on helix H8 (CR3) of Rgf3p or deletion of 4 amino acids in the same region of the Rgf1p-DH-domain produce a lack-of-function phenotype [31, 111].

Almost all Rho-GEFs possess a pleckstrin homology domain (PH) adjacent and C-terminal to the DH domain (Figure 2), and in most cases the DH-PH tandem is the minimal structural unit that can promote nucleotide exchange in vivo. PH domains have been proposed to localize Rho proteins to plasma membranes, and to regulate their GEF activity through allosteric mechanisms [98]. A mutation between the PH and the CNH domains produces a novel allele of the *rgf3* gene [75]. The mutation prevents Rgf3p from localizing to the medial ring during cytokinesis and causes cell lysis, the same lack-of-function phenotype as a mutation within the GEF domain [111]. Moreover, the mutation is suppressed by a Rab GAP, which appears to stabilize Rgf3p and drives its recruitment to membranes, thus indicating that proper localization is also essential for function [75]. Interestingly, Gef1p, which lacks the PH domain, forms a ring structure at the cell division site that shrinks during cytokinesis [16, 41].

Apart from the DH-PH module, most GEFs contain additional functional domains, including: DEP (*Dishevelled*, *Egl-10*, and *Pleckstrin*), CNH (Citron and NIK1-like kinase homology domain), PB1 (Phox and Bem1p domain), and a Calponin homology (CH) domain, which in some proteins has been implicated in binding to actin [56, 118] (Figure 2). Their function is not clear, but in most cases they are likely to be involved in coupling GEFs to upstream receptors and signalling molecules. Here we shall discuss possible mechanisms of participation of Rho GTPases in cell polarity and cell wall biosynthesis, and the specific activation by its GEFs in the vicinity of the polarization point.

The role of Cdc42p, Scd1p and Gef1p in cell shape

In order to maintain intracellular osmolarity and to produce cell shapes other than spheres, cell expansion must be focused on particular regions. There are three switches in polarized cell growth patterns during the cell cycle [84]. First, selective and polar growth is initiated at the beginning of the cell cycle at the "old end" of the cell, the end that preexisted before cell division. This growth can be monitored by staining with the dye Calcofluor and by the presence of cortical actin dots [62, 69]. Second, during the G2 phase, in a transition called "new end take off" (NETO), cells initiate growth at the new end of the cell (the previous cell division site) [73]. This growth pattern is visualized by the appearance of both Calcofluor staining and cortical actin dots at the new end. Finally, when the cell reaches its maximal size, tip elongation ceases and mitosis occurs followed by cytokinesis. Following cell separation polarity must be re-established at the old end. Fission yeast uses both microtubules and the actin cytoskeleton for cell expansion, for reviews, see [14, 30, 125]. It has been proposed that it is the interaction of microtubules with the cell tips that allows the establishment of new sites of actin assembly (reviewed by [6, 13, 14, 38, 64, 109]).

Fission yeast Cdc42p is essential for cell proliferation and its inactivation probably affects many functions, the most readily observable of which is a change in cell morphology from elongated to round [72](Figure 3A). Overexpression of a constitutively active form of Cdc42p or a mutant that is slow to hydrolyze GTP produces large, round and misshapen cells, and the disruption of normal actin distribution [72]. These observations suggest that Cdc42p activation must be restricted temporally and spatially and that Cdc42p is involved in controlling polarized cell growth.

Cdc42p is a component of a multiprotein complex that functions downstream from Ras1p, the single Ras GTPase homolog in *S. pombe*. Like Cdc42p, Ras1p participates in the regulation of cell morphology and mating in *S. pombe* but, unlike Cdc42p, it is not essential for cell viability [28, 79]. To control cell morphology, Ras1p interacts with

Cdc42p via complex formation with the Cdc42p guanine nucleotide exchange factor Scd1p [11]. In addition Ras1p also activates the Byr2p protein kinase (a MEKK homolog) to mediate mating pheromone signalling [120].

How Cdc42p controls cell shape is anything but straightforward. Cdc42p is localized to the medial region of the cell in early cytokinesis and remains at the cell division site until cell separation; it also localizes to the cell periphery and internal membranes [70].

Downstream effectors of Cdc42p

Cdc42p can interact with multiple downstream effectors to regulate a diverse set of functions [23]. These effectors preferentially bind to GTP-bound Cdc42p and transduce the Cdc42p-dependent signals downstream to ultimately affect actin rearrangements, microtubule polarization, and other events. In *S. pombe*, the best known Cdc42p effectors are the PAKs (p21-activated kinases) Shk1p/Pak1p/Orb2p and Shk2p/Pak2p. *shk1* is an essential gene required for polarized growth and morphology, proper control of cell cycle progression, completion of cytokinesis, and the normal mating response [61, 87, 115], whereas $shk2^{+}$ is a non-essential gene that appears to be largely redundant with $shk1^{+}$ [71, 106, 123]. The function of Shk1p is positively modulated by three non-essential proteins -Scd2p [10, 11], Skb1p [33, 34] and Skb5p [124]- and negatively regulated by Skb15p, an essential WD repeat protein [47].

Insight into possible roles for Shk1p in regulating cell polarity has been gained from studies of *shk1* mutants. Cells of the *orb2-34* mutant (a hypomorphic allele of *shk1*) are unable to activate bipolar growth and grow only at one tip [115, 116]. Analysis of the actin cytoskeleton in *orb2* mutants reveals that actin is only localized at one growth pole. Moreover, it has been suggested that Shk1p may play a role in the process by which fission yeast cells recognize their ends as sites for growth [102]. Cells severely defective for Shk1p function, either due to deletion of the $shk1^{+}$ gene or to overexpression of a

kinase-defective Shk1p mutant protein, results in the formation of spheroidal cells that exhibit the cortical F-actin randomly distributed [61, 87].

With the knowledge that activation of Cdc42p leads to the accumulation of F-actin, the next question is reduced to understanding the link between the activated Cdc42p and actin polymerization. In *S. cerevisiae* a bifurcated signalling pathway downstream from Cdc42p recruits and activates the Arp2/3 complex. One branch, which requires formin homologues, mediates the recruitment of the Bee1p (the WASp orthologue) complex to the cortical site where the activated Cdc42p resides [53]. The other is mediated by p21-activated kinases (PAKs), which activate the motor activity of myosin-I through phosphorylation [53]. In *S. pombe*, patch assembly proceeds via two parallel pathways: one dependent on WASp Wsp1p and verprolin Vrp1p converges with another dependent on class 1 myosin, Myo1p, to activate the actin-related protein 2/3 (Arp2/3) complex [108]. In *S. pombe*, Cdc42p/Shk1p could promote polarized cell growth by controlling myosin phosphorylation. The Myo1p head contains the so-called TEDS rule phosphorylation site, which is indicative of regulation by PAK kinase and a mutation in that possible phosphorylation site slightly impairs the function of Myo1p [7, 114]. No interaction between Cdc42p and Wsp1p has been reported so far.

In addition to regulating F-actin cytoskeletal organization, Shk1p/Pak1p may also regulate the stability of the microtubule cytoskeleton [95]. Curiously, Tea1p is directly phosphorylated by Shk1p in vitro, suggesting that it is likely to be a direct substrate of Shk1p [48]. Tea1p is needed to establish polarized cell growth at cell tips that have not grown previously. Recently, it has been shown that Tea1p may regulate cell polarity by associating with large 'polarisome' complexes that include the formin For3p [27, 65]. Although the details remain to be elucidated, there is evidence that the interaction of the Ras1p/Cdc42p/Shk1p complex with Tea1p hints at a molecular pathway explaining how microtubules contribute to the proper spatial regulation of actin assembly and polarized cell growth.

Upstream modulators of Cdc42p

Cdc42p is activated by at least two GEFs, designated Scd1p or Ral1p [11, 29] and Gef1p [16, 41]. The *scd1*$^{+}$*/ral1*$^{+}$ gene was found in a search for round and sterile mutants (shape and conjugation deficiency). In the same study another regulator for Scd1p, Scd2p/Ral3p was selected [11, 29]. *S. pombe* Scd1p is 32% identical to Cdc24p, the only GEF for Cdc42p in *S. cerevisiae*. Moreover, this latter is able to rescue conjugation and weakly improve the cell shape of the *scd1-1* mutant. Scd1p interacts directly with Scd2p; however a complex with Cdc42p was observed only when Scd2p was co-expressed, suggesting that Scd2p may bridge and facilitate interactions between Cdc42p and its GEF [11]. Ras1p can also enhance the physical interaction between Scd1p and Cdc42p in the yeast two-hybrid system [11].

Disruption of *scd1*$^{+}$ causes deformation of cell shape and inability to mate [11, 29]. However, *scd1*Δ mutants can induce pheromones and sporulate efficiently. It is possible that Scd1p might contribute to mating by affecting functions such as cell polarity and cytoskeletal organization. Consistent with this hypothesis, Scd1p localizes to the cell ends in vegetative cells and to the tip of conjugation tubes in mating cells. Moreover, Scd1p also localizes to the cell equator, the nucleus and the spindle [57]. Such a dynamic localization suggests that Scd1p can engage a wide variety of activities such as the regulation of cell polarity, spindle formation and cytokinesis.

It has been established that Scd1p activates the Ras1p-Scd1p-Cdc42p-Shk1p signalling pathway for apical growth [10]. Interestingly, Scd1p also affects the functioning of microtubules. Inactivation of *scd1* renders cells hypersensitive to TBZ (thiabendazole), which promotes microtubule depolymerization, while mutations in *scd1*$^{+}$, together with tubulin mutations, block proper spindle formation [57]. Furthermore, *scd1*$^{+}$ mutation is synthetically lethal with the deletion of *tea1*$^{+}$ [89], which localizes to the tips of microtubules and to the cell ends [68].

Scd1p interacts with a conserved protein complex containing Yin6p and Moe1p to affect proper spindle formation and chromosome segregation [15, 126]. Deleting *yin6*$^+$, *moe1*$^+$ or both produces essentially the same phenotypes: slow growth in the cold and inefficient separation of sister chromatids. These abnormalities are exacerbated by *scd1*Δ or *ras1*Δ, causing severe chromosome missegregation and cell death. Yin6p, either alone or in cooperation with Ras1p/Scd1p, influences proteasome localization and assembly; in consequence, inactivation of Yin6p can lead to the accumulation of mitotic regulators affecting cell division and mitotic fidelity [127].

Another function of Ras1/Scd1p is to mediate cytokinesis. This is supported by the fact that the kinesins Klp5p and Klp6p can form a complex with both Scd1p and Cdc42p. Furthermore, inactivation of Klp5/6p, together with inactivation of the Ras/Scd1p pathway, leads to mispositioned or fragmented contractile rings [56].

Gef1p is another GEF for Cdc42p [16, 41]. *gef1*$^+$ deletion is viable but causes defects in bipolar growth and septum formation [16], and the protein is mainly localized to the cell division site, where Scd1p is also seen. Deletions of *gef1*$^+$ and *scd1*$^+$ are synthetically lethal, generating rounded cells that mimic the phenotype of *cdc42*$^+$ deletion. Therefore, the function of Gef1p and Scd1p to activate Cdc42p at the septation site may be indispensable for cell proliferation. Together with Scd1p, Gef1p forms a ring structure, which shrinks during cytokinesis [41]. Cdc42p is deposited at the shrinking Gef1p/Scd1p ring and is left behind, forming a plaque structure where the septum is being formed. It is possible that both GEFs play a key role not only in activating Cdc42p through GDP-GTP exchange but also in recruiting Cdc42p to the septation site through their affinity for it, although this remains to be confirmed. Accordingly, what is the relationship between Cdc42p and the construction of the septum? Cdc42p may activate vesicle transport to bring septum materials to the septation site, probably through reorganization of cytoskeletal F-actin. Related to the recruitment of Cdc42p at the division site, Gef1p also interacts with Hob3p, a protein that belongs to the BAR

(Bin-Amphiphysin-Rvs) family of adaptors (P. Coll and P. Perez, personal communication).

Gef1p and Scd1p cannot be substitute for one another in the morphogenetic role [16]. However, genetic experiments indicate that Gef1p may act in the same signalling pathway as Scd1p (Ras1p-Scd1p-Cdc42p-Shk1p), since overexpression of Gef1p suppresses the *orb2-34* phenotypes (a non-lethal thermosensitive mutation in the essential *shk1*$^{+}$ gene). This situation seems to be different from that of *S. cerevisiae*, where Cdc42p is regulated by a single GEF Cdc24p, which controls both apical growth and septation [36].

The role of Rho1p, Rgf3p, Rgf1p and Rgf2p in maintaining cell integrity

Cell growth in *S. pombe* is a process necessarily related to cell wall biogenesis, for reviews, see [14, 22, 109]. The cell wall is the essential cellular boundary controlling all communications with the extracellular world. Because of its mechanical strength, it allows cells to withstand turgor pressure and consequently prevents cell lysis. In fission yeast, the cell wall mainly consists of three polysaccharides -β(1,3)-glucan, α(1,3)-glucan, and galactomannoproteins- all of which form a large complex. Their coordinated synthesis and degradation is essential to ensure cell integrity during morphological changes [22].

Cell growth transitions are correlated with changes in the actin cytoskeleton. The growing ends of fission yeast contain polarized cables and actin patches [30, 63], while at cell division, actin patches disappear from the poles and the cytokinetic actomyosin ring (CAR) forms at the cell equator from a combination of the reorganization of interphase cables and de novo actin assembly [1, 90]. The relationship between actin and cell wall deposition at the cell poles and equator is still not well understood [109]. Actin cables are bundles of actin filaments nucleated by the formin For3p [26, 82].

Cables are thought to serve as tracks for the delivery of myosin V-driven vesicles towards the cell ends or the equator for cell growth. In the absence of Myo52 (type V myosin), the cell wall α–glucan synthase Mok1p is delocalized and cell wall formation is aberrant [76, 77, 121]. Accordingly, it has been shown recently that directionality of F-actin cables changes during the cell cycle; most F-actin barbed end faced the cell tip during interphase whereas most F-actins in the cables were oriented such that the barbed end faced the mid-region of the cell during mitosis. These orientations of F-actin would ensure proper transport of materials to growing sites [45]. Actin patches are sites of Arp2/3-mediated actin polymerization and are believed to mediate the internalization of endocytic vesicles moving inward from the cell tips [32, 91]. Whether such patches play any role in cell wall biosynthesis remains to be clarified. In regenerating protoplasts, patch localization coincides precisely with the active sites of cell wall deposition [50], and cortical actin is required for the localization of at least two classes of enzymes involved in cell wall synthesis: α-glucan synthase [46, 121] and β–glucan synthase [18]. Rho1p provides a link between polarized actin and the cell wall biosynthesis playing multifunctional roles upon interacting with its targets [3, 4, 80]. Rho1p functions downstream the polarity marker Tea1p [3], and localizes to places of active cell growth, both ends and the septum. Depletion for Rho1p activity in growing cells causes the disappearance of polymerized actin, while an increase in Rho1p expression produces larger actin dots, randomly distributed throughout the cell [3, 80]. A proper balance of Rho1 activity is important to regulate the actin cytoskeleton; however, to date no likely candidate to mediate changes in the actin cytoskeleton has been found. Formins are required for actin nucleation to form cables and are activated by binding to the Rho family GTPases [117]. Thus, they are likely candidates as mediators of changes in the actin cytoskeleton. *S. pombe* has three formins (Cdc12p, Fus1p and For3p), each of which nucleates a distinct actin structure [12, 26, 82, 93]. Other than the interaction between For3p with Rho3p, it is not yet clear whether any of the formins interact with

Rho1p [82]. No reports relating Rho1p and the Arp2/3 complex have been found in the literature so far [54, 108].

Downstream effectors of Rho1p

The final phenotype of cells devoid of Rho1p activity is lysis, mainly during cytokinesis but also at other stages of the morphogenetic cycle, indicating that Rho1p is required to maintain cell integrity (Figure 3B). In fact, the best characterize effectors of Rho1p are enzymes involved in cell wall synthesis: β(1,3)-glucan synthase (GS) [4] and the PKC-type proteins Pck1p and Pck2p [5, 103].

β(1,3)-glucan is the first polymer to be synthesized in *S. pombe* regenerating protoplasts [85, 86] and in the spore wall [67], and hence the regulation of this polysaccharide may be a key step in the sequential assembly of the other cell wall components. The enzymatic system that catalyzes the synthesis of this polymer is β(1,3)-glucan synthase (GS), a multimeric enzyme composed by at least two fractions: the catalytic moiety of the enzyme and the regulatory component. The catalytic subunit of GS is encoded by the family of the beta glucan synthase *bgs* genes (*bgs1*$^+$, *bgs2*$^+$, *bgs3*$^+$ and *bgs4*$^+$) all of them code for proteins essential at different stages in the cellular life cycle [17, 18, 52, 58, 59, 66, 67]. Rho1p directly stimulates GS and glucan synthesis in its GTP-bound prenylated form, providing a rationale for an understanding of the mechanism through which the cell can switch β(1,3)-glucan synthesis on and off by interconverting the GDP and GTP forms of Rho1p [4].

However, many questions remain unanswered. How does Rho1p regulate these 4 catalytic subunits? Rho1p travels to growth sites, the poles and the septum to meet Bgs1p, Bgs3p and Bgs4p. It is known that the three GS catalytic subunits localize to the poles during tip elongation at to the septum during cytokinesis. All of them are large integral membrane proteins whose levels do not fluctuate along the cell cycle [58, 66]. Bgs1p is a putative β(1,3)-glucan synthase required for the synthesis of the primary

septum and for constriction of the actomyosin ring (J.C. Ribas, personal communication, [58]), while Bgs3p and Bgs4p are good candidates for the synthesis of the β(1,3)-glucan of the surrounding cell wall and the secondary septum, which is similar in composition [17, 66]. Is Bgs regulation controlled by local or temporal activation of Rho1p or both? Does each GS need a different input to be activated?

GTP-bound Rho1p also interacts with both Pck1p and Pck2p. In this interaction, Rho1p seems to stabilise the kinases, increasing their concentration in the growing areas of the cell [2, 103]. *pck1*Δ mutants display a lytic phenotype and have a regular shape, while *pck2*Δ mutants appeared miss-shaped and bent, suggesting that these two protein kinases regulate cell morphology in different ways. Both mutants display slightly different cell wall defects; *pck1*Δ, but not *pck2*Δ, cells are hypersensitive to Echinocandin (Ech) [5] and *pck2*Δ mutants show hypersensitivity to lytic enzymes [112]. Pck2p is essential for the cortical localization of Mok1p (the α–glucan synthase) and is also necessary to regenerate the cell wall when protoplast are incubated in osmotically stabilized liquid medium [46, 49]. Moreover, Pck1p and Pck2p may be involved in reorganising the actin cytoskeleton, affecting polarity or secretion, i.e., processes in which Pkc1p, its counterpart in *S. cerevisiae*, has been implicated [19, 39]. Interestingly, the phenotype observed following combined depletion of both $pck1^{+}$ and $pck2^{+}$ is very similar to that observed in cells depleted for Rho1p [2].

Upstream modulators of Rho1p

Rho1p is activated by three GEFs called Rgf1p, Rgf2p and Rgf3p (for rho gef) [31, 42, 75, 78, 111]. All of these Rgfs have similar molecular structures to the budding yeast Rho1p-GEFs Rom1p and Rom2p [88, 104] (Figure 2).

rgf3$^+$ was first cloned by complementation of a mutant (*ehs2-1*) hypersensitive to drugs that interfere with cell wall biosynthesis [111], and was also found to complement a *lad1-1* mutation that undergoes cell lysis specifically at cell division [75].

rgf3$^+$ is essential for cell viability and depletion of Rgf3p causes cell lysis and elicits phenotypes very similar to those of cells devoid of Rho1 or Pck1/2 activity [5]. Rgf3p expression peaks during septation in an Ace2p-dependent manner [92, 99] and the protein exclusively localizes to the medial region of the cell. Early on in mitosis, Rgf3p forms a ring-like structure that contracts to a dot during the latest stages, while the Rho1p signal is delayed with respect to Rgf3p and accumulates at the cell division site, first as a ring and later on as a plate-like structure (our unpublished observation, [78]). In this context, there are two possible, not mutually exclusive mechanisms of action for Rgf3p. It has been shown that Rgf3p activates glucan synthase GS and raises the amount of cell wall β(1,3)-glucan [111]. Thus, it is probable that Rgf3p stimulates the Rho1p-mediated activation of a glucan synthase activity that would be crucial for proper septum functioning. Additionally, Rgf3p could be necessary to pull Rho1p, recruiting it to the first line of septum assembly.

The role of Rho proteins in cytokinesis has been clearly established [94]. In budding yeast, the Polo-like kinase Cdc5p is required for the recruitment of Rho GEFs to the division site that in turn is necessary for recruitment and activation of Rho1p [128]. Rho1p regulates formin-mediated contractile ring assembly [113], in consequence a failure in the mechanism of Rho1p regulation causes a profound defect in CAR assembly. In fission yeast, the formin Cdc12p is also required for contractile ring formation [1, 12]; however, Cdc12p does not have an RBD (Rho binding domain) and does not bind any of the Rho proteins [78].

Rgf3p localization to the medial ring requires actin polymerization and Cdc12p but seems to be independent of SIN [Septation Initiation Network] function (our unpublished observations, [75]). However, it is not yet resolved whether Rgf3p function

can be regulated by the SIN. The SIN pathway in *S. pombe* is required for actomyosin ring stability (reviewed by [35, 51, 107]). It has recently been shown that SIN might also function to regulate cell wall assembly at the septum [44]. This is based on the fact that several mutants of the SIN pathway lyse at low restrictive temperatures, with a phenotype similar to the one seen in *rho1*Δ and *rgf3* mutants. Lysis occurs after ring contraction and septum formation, during the process of septum cleavage, and can be rescued by overexpression of Rho1p. Thus, it is possible that the SIN targets Rho1p as one of its downstream effectors by activating Rgf3p [44].

The lysis caused by Rho1p depletion is not prevented by an osmotic stabilizer and occurs mainly after cytokinesis, probably because correct cell wall assembly is essential at that point of the cell cycle [3]. However, Rgf3p depletion is prevented by 1.2 M sorbitol, suggesting that in the absence of Rgf3p, but in the presence of an osmotic support, Rho1p could be activated by different stimuli. In fact, two other GEFs act on Rho1p: Rgf1 and Rgf2p [111].

Rgf1p specifically regulates Rho1p during polarized growth [31]. Rgf1p localizes to the cell tips in interphase cells and at the division septum in mitotic cells. During septum formation Rgf1p is distributed as a division plate that becomes double just before cell separation [31, 75, 78]. *rgf1*Δ cells are defective in cell integrity and lyse at one of the poles with a phenotype similar to that of cells devoid of Rho1p. Additionally, *rgf1*Δ cells show a defect in the actin reorganization required for the transition from monopolar to bipolar growth [31].

In *S. pombe,* Rho1p signalling is required to maintain cell integrity, regulating the biosynthesis of β(1,3)-glucan and the cell wall in general, and it is also required for actin polymerization. Rgf1p activates the β-GS complex containing the catalytic subunit Bgs4p and is involved in the activation of growth at the second end, a transition that requires actin reorganization [31]. Interestingly, while other mutants defective in bipolar growth, *tea1*Δ, *tea4*Δ, *bud6*Δ, grow at wild-type rates a novel aspect of the *rgf1*Δ is that

its growth rate and viability are compromised. This is probably because its failure to initiate bipolar growth coincides with cell lysis, thus coupling a growth polarity transition with cell wall biosynthesis [31].

Rgf1p localization to the poles depends on actin (our unpublished observations), and on phosphoinositides (phosphatidylinositol 4,5-bisphosphate) [20]. Moreover, Tea1p is also required for Rgf1p to be recruited to the new end [31]. During NETO, the formation of a protein complex that includes Tea1p, Tea4p and For3p, called the "polarisome" is necessary and sufficient for the establishment of cell polarity and localized actin assembly at the new ends. Whether Rgf1p is related to this complex will be a challenge for future investigation.

Depletion of Rgf3p in a haploid strain deleted for *rgf1*$^+$ produces viable cells in the presence of sorbitol, thus suggesting that Rgf2p or other activators may be acting on Rho1p (our unpublished observations). In fact, Mutoh *et al.* [77] have shown that Rgf2p also interacts with Rho1p. Although *rgf2*Δ cells are apparently very similar to wild-type cells, deletion of both genes, *rgf1*$^+$ and *rgf2*$^+$, is synthetically lethal [75, 78]. Moreover, the growth and morphology defects of *rgf1*Δ cells are suppressed by over-expression of *rgf2*$^+$, suggesting that both proteins are functionally redundant during vegetative growth [78]. In addition, Rgf2p may perform an essential function during the sporulation process. We found that *rgf2*Δ zygotes produced immature ascospores that were unable to germinate (our unpublished data). This phenotype is similar to the one seen in spores lacking *bgs2*$^+$, the sporulation-specific GS catalytic subunit [67], suggesting a role for Rgf2p in the spore wall maturation process.

Why does Rho1p have multiple GEFs? An attractive hypothesis is that each GEF could determine the downstream signalling specificity of Rho GTPases. This has been suggested for Ras1p signalling in fission yeast, where two GEFs, Ste6p and Efc25p, differentially regulate two Ras pathways [89]. Rgf1p would specifically activate the Rho1-GS complex during the transition from monopolar to bipolar growth. Rgf2p may

share an essential function with Rgf1p, although its contribution has not yet been defined. Rgf3p might be necessary to coordinate cell wall biosynthesis with the septation machinery in order to maintain cell integrity [111]. Double-mutant and phenotypic complementation results hint that Rgf1p and Rgf3p are not functionally exchangeable. Moreover, the localization patterns of Rgf1p and Rgf3p are very different, suggesting non-overlapping functions. The identification of Rho1-GEF interacting proteins will be necessary to understand how Rho1p regulates cell integrity. The genetic tools available in fission yeast and genome sequencing and deletion programs should be of great help in the near future.

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Figure legends

Figure.1. A biochemical model of Rho guanine nucleotide exchange factor (RhoGEF) function in the signalling scheme of Rho GTPases. The cycle between the active, GTP-bound, and the inactive, GDP-bound state of Rho GTPases is regulated by three classes of proteins. Guanine nucleotide-dissociation inhibitors (GDIs) mainly bind to the switch

regions and the C-terminal isoprenil moiety (black wavy line) of Rho GTPases to sequester them in the cytosol. GEFs also bind to the switch regions to stabilize nucleotide-depleted GTPases. However, owing to the relatively high concentration of intracellular GTP, nucleotide-depleted complexes rapidly dissociate into GTP-bound GTPases and free GEFs. When they are GTP-bound, Rho GTPases regulate the activity of their binding partners, or effectors, to promote cellular responses that usually influence the organization of the actin cytoskeleton or the expression levels of several genes. GTPase-activating proteins (GAPs) stimulate the intrinsic hydrolytic capacity of Rho GTPases to promote GDP-bound forms and terminate signalling. Pi inorganic phosphate.

Figure. 2. Multifunctional domain features of Rho guanine nucleotide exchange factors (GEFs) family member analysed by the SMART program ([55]; http://smart.embl.de/). Domains are indicated: CNH, citron homology domain. This acts as a regulatory domain and could be involved in macromolecular interactions; RhoGEF (DH domain), domain conserved among GEFs for Rho/Rac/Cdc42-like GTPases; PH, pleckstrin homology domain; DEP, domain of unknown function present in signalling proteins that also contain the PH, RasGEF, RhoGEF, RhoGAP, RGS, PDZ domains; PB1 domain, the Phox and Bem1p domain. PB1 domain function is the formation of PB1 domain heterodimers; CH, calponin homology domain, found as tandem repeats in proteins that cross-link actin filaments (such as fimbrin, spectrin and alpha-actinin) or link the actin cytoskeleton to intermediate filaments (such as plectin).

Figure.3. Roles of Rho GEFs in morphogenesis. (A) Fission yeast cell expansion is focused to particular regions. Cdc42p is recruited to the sites of polarized growth and is activated through its guanine nucleotide exchange factors Scd1p and Gef1p. Scd1p and

Gef1p activate the Ras1p-Scd1p-Cdc42p-Shk1p signalling pathway for apical growth and also to mediate cytokinesis. Mutations that render Cdc42p inactive produce round and misshaped cells, indicating that Cdc42p is involved in controlling polarized cell growth. (B) Once polarity has been established, Rho1p is recruited to the growth or the division site, where it is activated by three GEFs, Rgf3p, Rgf1p and Rgf2p, each with a different localization. Mutations in those GEFs or failure to activate Rho1p produces shrunk or lysed cells. Thus, the main role of the Rho1p GTPase is to preserve cell integrity, probably by coupling actin organization and secretion to cell wall synthesis.

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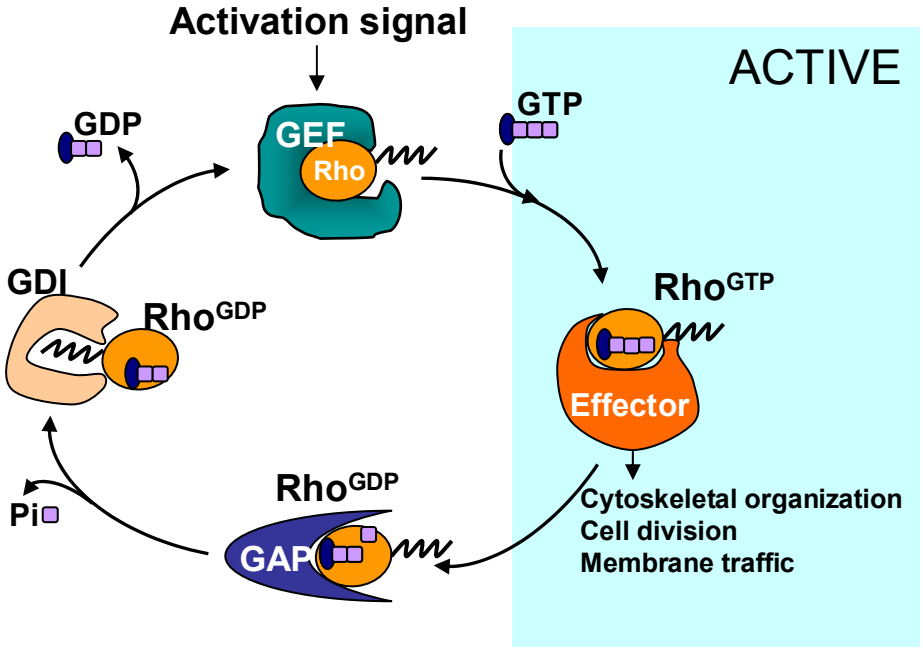
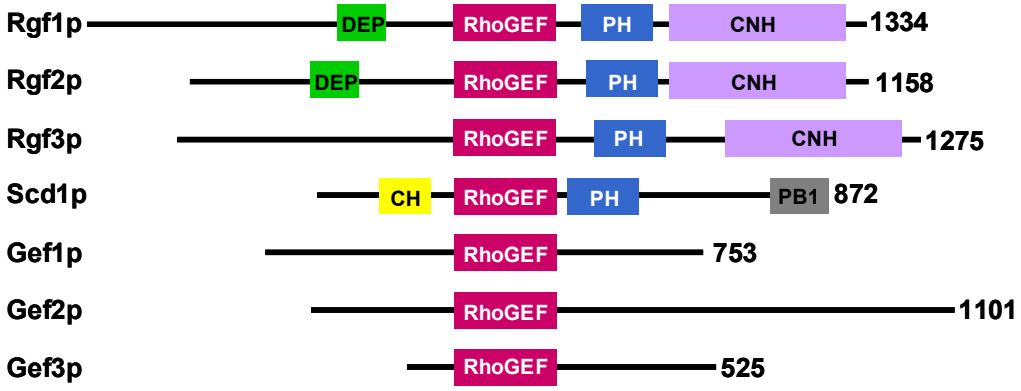
Garcia *et al*, 2006. Figure 1



Garcia *et al*, 2006. Figure 2



Garcia *et al*, 2006. Figure 3