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The Function of Fission Yeast Rho1-GEFs in the Control of Cell Growth and Division

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Abstract

Guanine nucleotide exchange factors (GEFs) are directly responsible for the activation of Rho-family GTPases in response to physical and chemical stimuli and ultimately regulate numerous cellular responses such as polarized growth, morphogenesis, and movement. The GEF proteins are characterized by a Dbl-homology (DH) domain that contacts the Rho GTPases, to catalyzing nucleotide exchange, and an associated Pleckstrin homology (PH) domain, which fine-tunes the exchange process by a variety of mechanisms related to the binding of phosphoinositides. Most GEFs are divergent in regions outside the DH/PH module and contain additional protein-protein or lipid-protein interaction domains that presumably dictate unique cellular functions. Fission yeast Rho1-GEFs act as a link between growth processes and the cell cycle machinery. In this chapter, we focus on the recent leaps in our understanding of how Rho1-GEFs control interphase and cytokinesis in fission yeast. Furthermore, we will go beyond mitosis and highlight the unexpected roles of Rho1-GEFs in the DNA damage response.

Keywords: guanine nucleotide exchange factor (GEF), small GTPases, morphogenesis, fission yeast, genome integrity

1. Introduction: fission yeast Rho1p regulates actin dynamics and cell integrity

Rho GTPases are key regulators of the actin cytoskeleton dynamics in eukaryotic cells. Moreover, they also regulate diverse cellular functions including cell cycle, gene expression, vesicle trafficking, and cell polarity [1–3]. In response to physical and chemical stimuli, most Rho GTPases switch between an active GTP-bound conformation, which interacts with downstream

effectors, and an inactive GDP-bound conformation. Because GDP is in general tightly bound and GTP is hydrolyzed very slowly, small GTPases require the helping hand of guanine nucleotide exchange factors (GEFs) that facilitate GDP dissociation, as well as the help of GTPase-activating proteins (GAPs) that stimulate GTP hydrolysis [4, 5]. For certain small GTPases that carry a farnesyl or a geranylgeranyl group in their C-terminus, GDP/GTP alternation combines with cytosol/membrane alternation, which is mediated by guanine dissociation inhibitors (GDIs) that sequester the GTPase within the cytosol in an inactive conformation by shielding their lipid moiety. In addition, the fine-tuning of Rho GTPases is achieved at the posttranscriptional level by microRNA (miRNA) and at posttranslational level by covalent modifications that affect its intracellular distribution, stability, and turnover, among others [6].

Fission yeast Rho GTPase Rho1p is essential and is a functional homolog of human RhoA and budding yeast Rho1p [7]. Rho1p is present on the plasma membrane (PM) and at internal membranes (unpublished results). Prior to the septum invagination, the protein slightly concentrates to the middle cortex of the cell. As the actomyosin ring shrinks, Rho1p signals continue to invaginate and finally split into two closely associated discs [7, 8].

Depletion for Rho1p activity in growing cells causes cells to lyse, and the cells shrink and die in a kind of “apoptosis” accompanied by the disappearance of polymerized actin. An increase in Rho1p expression produces larger actin dots, randomly distributed throughout the cell [7, 9] and a thick cell wall [10]. Thus, a proper balance of Rho1p activity is important for regulating the actin cytoskeleton and the cell wall polymers. To date, there is no likely effector(s) of Rho1p in the regulation of the actin cytoskeleton. However, the protein regulates cell integrity through its interaction with at least three different targets: the $\beta(1,3)$ -glucan synthase, which is responsible for the synthesis of β -glucan, the major cell wall component [11–14], and the kinases Pck1p and Pck2p (the orthologs of *Saccharomyces cerevisiae* Pkc1p and human PKC). Pck1p and Pck2p operate in a redundant fashion to control essential functions in morphogenesis and cell wall biosynthesis [15–17]. Rho1p, Pck1p, and Pck2p function upstream of the mitogen-activated protein kinase (MAPK) module (Mkh1p, Skh1p/Pek1p, and Pmk1p/Spm1p) of the cell integrity signaling pathway (CIP) [18–21]. This signaling cascade becomes activated under adverse conditions and regulates cell separation, morphogenesis, cell wall construction, or ionic homeostasis [22, 23]. Pck2p elicits the activation of the MAP kinase Pmk1p in response to most environmental stimuli, whereas Pck1p plays a minor role as a positive regulator of Pmk1p during vegetative growth and cell wall stress [21, 24].

Regarding upstream components of Rho1p signaling, two proteins Mtl2p (Mid two like 2) and Wsc1p, with the characteristics of cell wall stress sensor-like proteins, act by turning on the GTPase. Both proteins are required to maintain the physiological levels of Rho1-GTP under the chronic cell wall stress produced by antifungal agents [25]. Interestingly, signaling through the MAPK Pmk1p remained active in the *mtl2* Δ and *wsc1* Δ disruptants exposed to cell wall stress.

2. Structure and features of fission yeast Rho1p-GEFs

Fission yeast Rho1p acts as a hub for the integration of different signals, and only recently have conditional mutants been described for studying its central role in cell integrity signaling

[26]. In fact, much of what is known about the function of Rho1p comes from studying its regulators, GEFs and GAPs. Rho1p activity is regulated by three GEFs: Rgf1p, Rgf2p, and Rgf3p [8, 27–30]. Other members of the Rho-GEF family in *S. pombe* are the Cdc42p-specific GEFs: Scd1p and Gef1p [31, 32], and Gef3p specific for Rho4p [33, 34]. Gef2p [35, 36] and Mug10p have not yet been assigned to any known GTPase (<https://www.pombase.org>).

Rho1p-GEFs (Rgf1-3), like most Rho-GEFs, are multidomain proteins and contain a Dbl-homology (DH) domain, which contacts the Rho GTPase followed by a Pleckstrin homology (PH) domain (reviewed in Ref. [37]). The DH domain stabilizes GTP-free Rho intermediates, leading to GTP loading, owing to high levels of intracellular GTP [38, 39]. The nature of this interaction has emerged from crystallography or nuclear magnetic resonance studies of DH domain-containing GEF constructs in complex with their cognate GTPase [5, 40]. DH binding induces conformational changes in the switch regions and the P loop of the GTPase, while leaving the remainder of the structure largely unperturbed [4, 39]. DH domains contain three conserved regions (CR1, CR2, and CR3) and form structures similar to elongated bundles of α -helices arranged in a “chaise longue” shape. Amino acid substitutions within these conserved regions adversely affect nucleotide exchange activity. In *S. pombe*, a point mutation located on helix H8 (CR3) of Rgf3p or the deletion of four amino acids in the same region of the Rgf1p- and Rgf2p-DH domain produces a lack-of-function phenotype [27, 30]. PH domains in DH-PH RhoGEFs are endowed with a variety of regulatory functions and can be autoinhibitory, assist in the exchange reaction, and influence the targeting of RhoGEFs to phosphoinositide-containing membranes [41]. In *S. pombe*, *rgf3⁺*, a mutation that falls between the PH and Citron and NIK1-like kinase homology (CNH) domains (*lad1-1* mutant), prevents Rgf3p from localizing to the medial ring during cytokinesis [29]. Similarly, in the Rgf1p Δ PH mutant, the normal localization of Rgf1p at the two tips is disrupted, and the signal becomes mainly monopolar [42]. Both mutations, *lad1-1* and the Rgf1p Δ PH, phenocopy the lack-of-function phenotype.

Apart from the DH-PH module, Rgf1p, Rgf2p, and Rgf3p contain protein-protein interaction domains. Rgf1p and Rgf2p hold a DEP domain that was first discovered in flies (*Disheveled*), worms (*EGL-10*), and mammals (*Pleckstrin*). The DEP domain is a globular domain of about 100 residues that is present in a limited number of protein families with diverse functions related to signal transduction. The best-known function of the DEP domain is plasma membrane anchoring, but DEP domains are also involved in signal termination, intradomain binding, and in dimerization [43–45]. It is worth noting that Rgf1p and Rgf2p, the two proteins containing a DEP domain, localize to both poles and the septum, while Rgf3p, which lacks the DEP domain, localizes exclusively to the septum [8, 29, 30]. Accordingly, in the Rgf1p Δ DEP mutant, which lacks 26 internal amino acids of the DEP domain, the protein partially disappears from the poles. However, in this mutant, Rgf1p strongly accumulated inside the nucleus [42]. This finding suggests that the DEP domain of Rgf1p could mediate membrane anchoring and suggests a function for DEP domains in the intramolecular interactions that drive changes in localization (see next section).

The three Rho1p GEFs bear a C-terminal regulatory domain termed the citron homology domain or CNH. Structurally, the CNH domain belongs to the super-family of β -propellers [46] and is present near the C-terminus of several kinases implicated in the regulation of the actin cytoskeleton [e.g., citron, nck-interacting kinase (NIK) and TNIK (traf-2 and nck-interacting kinase)] and

in the regulation of Rom1p and Rom2p (the *S. cerevisiae* orthologous of *S. pombe* Rgf1p/2). This CNH domain is of unknown function but might be a protein-protein interaction domain [47, 48]. The CNH domain is essential for Rgf1p function in cell integrity and cell polarity [42]. Moreover, cells carrying mutations in the CNH domain of Rgf3p are inviable, and swapping of the CNH domain of Rgf3p for its counterpart in Rgf1p did not rescue the lethality in the *rgf3Δ/rgf3⁺* diploid (unpublished data). These observations suggest that the CNH domain of Rgf1p and Rgf3p may mediate its interaction with different proteins, thus providing signal specificity.

3. Recruitment of Rgf1p, Rgf2p, and Rgf3p to different subcellular sites

The essential localization of Rho1p to the cellular membranes makes it difficult to understand the specific tasks of this protein in polarized growth, secretion, and gene expression. In many cases, it is the specific localization of the corresponding GEFs and GAPs that activate/inactivate the GTPase in time and space, allowing the GTPase to function in different signaling pathways [49]. Most Rho-GEFs localize either to the cytoplasm or to the plasma membrane (PM), and only a few of them are seen in the nucleus.

In *S. pombe*, Rgf1p shows a dynamic localization during an unperturbed cell cycle. Its distribution mirrors that of the cortical actin patches that accumulate at actively growing tips during interphase and relocate to a central ring during mitosis (**Figure 1**) [8, 27, 29, 30]. Accordingly, the localization of Rgf1p-GFP to cell tips was strongly affected by the disruption of the actin filaments with Latrunculin A (an actin-depolymerizing agent), but was unaffected by acute disruption of the microtubules with methyl benzimidazole carbamate (MBC). In fission yeast, the actomyosin ring is assembled before anaphase A, whereas its constriction occurs after completion of anaphase B [50]. Rgf1p-GFP appears in the midzone membrane in early anaphase. As the ring constricts, Rgf1p is detected near the actomyosin ring as well as in the developing membrane forming a double-filled disc.

Rgf2p localizes uniformly at the periphery of the spore, probably associated with the fore-spore inner membrane. Rgf2p-GFP fluorescence appears in the fraction of cells that have already undergone meiosis I and II, where the spore outline is perfectly defined [51]. The fluorescence signal is hardly seen in vegetative wild-type cells. However, when expressed in a multicopy plasmid with its own promoter, Rgf2p fluorescence localizes to the growing ends, the septum, and across the whole cell surface [8, 29, 51].

Rgf3p-GFP localizes exclusively to the contractile ring (**Figure 1**) [8, 29, 30]. Rgf3p appears in the contractile ring when SPBs are ~3 μm apart and contracts with the ring until the signal reaches the center of the cell and then fades. Rgf3p fluorescence is at the trailing edge of the myosin II-regulatory light-chain Rlc1p, which may indicate that Rgf3p is closer to the plasma membrane than myosin II [8]. Recently, super-resolution microscopy was used to determine the spatial localization of contractile ring components relative to the membrane. These experiments have showed that Rgf3p localizes to an intermediate layer of the ring that

Figure 1. A schematic representation of Rgf1p and Rgf3p localization along the fission yeast cell cycle. See text for details.

includes Pxl1p, Fic1p, Spa2p, Pck1p, Clp1p, Pom1p, and Cyk3. This layer is sandwiched by the membrane-bound scaffolds Mid1p, Cdc15p, and Imp2p on the outer side and by F-actin and motor proteins on the inner side [52]. Interestingly, Cdc15p and Imp2p recruit Rgf3p, Pxl1p, Fic1p, and Cyk3p to prestriction CRs [53–55]. Rgf3p localization also depends on the CR-localized arrestin Art1p [56]. Art1p and Rgf3p physically interact and are interdependent for localization to the division site. Moreover, both proteins are involved in the maintenance of active Rho1p levels at the division site [56].

Many signaling pathways are activated under stress conditions, and a change in the localization of the GEFs may be crucial for inhibiting or redirecting polarized growth under the new situation. For instance, Rgf1p is released from the cellular poles and enriched in the cytoplasm under osmotic stress (sorbitol and KCl 1.2 M). This situation is transient, and the protein returns to the cell tips 2 h after treatment, even in the presence of stress (unpublished observations). On the contrary, cell wall stress induced by caspofungin, an antifungal agent that inhibits β -glucan biosynthesis, increases the level of Rgf1p at the cell tips at least three-fold. Unexpectedly, Rgf1p accumulates in the nucleus in response to DNA replication damage caused by hydroxyurea (HU, an inhibitor of the ribonucleotide reductase that blocks DNA replication). This is characteristic of Rgf1p, since neither Rgf2p nor Rgf3p is observed to undergo altered cellular localization under DNA replication-stressed cells [42]. During a normal cell cycle, Rgf1p continuously shuttles between the nucleus and the cytoplasm. Import to the nucleus is mediated by a nuclear localization sequence (NLS) at the N-terminus, whereas release into the cytoplasm requires two leucine-rich nuclear export sequences (NES1 and NES2) at the C-terminus of the protein. When cells are subject to replication stress, the nuclear accumulation of Rgf1p depends on the DNA replication checkpoint kinase Cds1p and the 14-3-3 chaperone Rad24p. Both proteins control the nuclear accumulation of Rgf1p by inhibiting its nuclear export [42].

4. Rho1p-GEFs at the cell tips

Rgf1p localizes to the growing ends and the septum, where Rho1p and its effectors Pcks and the GSs are known to function. Rgf1p and Rho1p interact by co-immunoprecipitation, and deletion of Rgf1p greatly decreases the amount of GTP-bound Rho1p, suggesting that Rgf1p is responsible for most of the GTP-bound Rho1p available in the cell [19, 27]. Approximately 15% of the *rgf1Δ* cells lyse and the mutants are hypersensitive to cell wall-damaging agents and other types of stress [19, 27, 42, 57]. The lysis phenotype of *rgf1* null cells is similar to that seen after depletion of Rho1p. However, while *rho1⁺*-depleted cells die in pairs (Λ) that lose their integrity mainly during the division process, in *rgf1Δ* cells lysis occurs mainly in single cells and in pairs of lengthy cells (Λ). These observations indicate that *rgf1Δ* cells do not lose their integrity during septation.

Rgf1p regulates cell integrity directly through Rho1p by activating the β-GS complex containing the catalytic subunit Bgs4p [27] and indirectly (also through Rho1p) by signaling upstream from the Pmk1p mitogen-activated protein kinase pathway (CIP, cell integrity pathway) [19]. Rgf1p positively regulates the activation of the CIP in cells stressed by cell wall damage and osmotic shock. Moreover, Rgf1p mainly acts alone in this process since Pmk1p activation was largely independent of the other two Rho1p-GEFs, Rgf2p and Rgf3p [19]. Thus, Rgf1p is important for cell wall remodeling at the cellular poles during an unperturbed cycle, acting through Rho1p-Bgs4p, and under stress conditions through Pck2p-Pmk1p.

Another characteristic of the *rgf1Δ* cells is that they are monopolar because after mitosis they fail to activate bipolar growth. In *S. pombe*, both cell ends are different at least in terms of the time of growth activation [58–60]. In wild-type cells, it is always the old end (the one that preexisted before cell division) that initiates growth after cell division. Then, after a period of approximately 50 min, each cell initiates bipolar growth in a process called New End Take-Off (NETO) [61]. This growth transition is triggered by the activation of CDK1 on spindle pole bodies at mid-G2 phase [62] and requires correct completion of the last stages of cytokinesis to render the new cell pole growth competent [63]. Moreover, transient depolymerization of actin has been shown to promote NETO in G1-arrested cells, suggesting that the reorganization of actin may be sufficient to initiate NETO [64]. How the cell cycle signal is transmitted to the cytoskeletal proteins inducing growth initiation at the second cell pole is unknown.

NETO is directed by specific polarity proteins, the kelch-repeat protein Tea1p, the SH3 domain-containing protein Tea4p, and the DYRK (dual-specificity tyrosine phosphorylation-regulated) kinase, Pom1p [65–67]. Tea1p and Tea4p are deposited at cell poles by microtubules where they form protein complexes that recruit and activate the GTPase Cdc42p, a key protein for actin reorganization [68–70]. Similar to Tea1p, Tea4p, and Pom1p, Rgf1p is required for NETO. In the absence of Rgf1p, Cdc42p and the actin patches localize exclusively to the growing end (data not shown and [27]). Thus, Rgf1p could be a good candidate to promote the actin reorganization required to initiate growth at the second end. In line with this, it has been recently shown that Rgf1p is phosphorylated by the MARK/PAR-1 family kinase Kin1p [71]. Kin1p regulates cell polarity and cell wall biosynthesis through unknown mechanisms [72–74]. Moreover, the same authors have shown that Kin1p is a substrate of the CaMKK-like

(Ca²⁺/calmodulin-dependent protein kinase) Ssp1p [71], also known to contribute to NETO through its function in actin remodeling [64, 75]. Additional substrates for Kin1p are Tea4p, Mod5p, Rga2p, Rng10p, and Chr4p [71]. Thus, Rgf1p could form part of the Ssp1p-Kin1p-signaling pathway for cell polarity and cytokinesis (see subsequent text).

Rgf2p localizes at the cell poles and the septum and plays a minor role in β -glucan biosynthesis during vegetative growth [8, 51]. *rgf2* Δ cells grow like wild-type cells at high and low temperatures and in the presence of heat, osmotic, and genotoxic stresses. However, the disruption of *rgf1*⁺ in an *rgf2* Δ background is lethal, suggesting that Rgf2p shares with Rgf1p an essential function during vegetative growth [8, 51]. Mild overexpression of *rgf2*⁺ (driven by its own promoter or the *rgf1*⁺ promoter in a multicopy plasmid) fully rescues the lysis of *rgf1* Δ cells and partially rescues their bipolar growth defect [51]. The overexpression of the lack of function allele, *rgf2-PTTR* Δ [51], under the control of the *nmt1* promoter increases the percentage of lysis and monopolar cells in the wild-type strain (unpublished results). Therefore, a high level of Rgf2p phenocopies the absence of Rgf1p and suggests that both proteins may compete for the same substrates. In the absence of Rgf1p, Rgf2p takes over the essential functions for Rho1p during vegetative growth.

5. Rho1p-GEFs in cell separation (mitosis and cytokinesis)

S. pombe cells divide similar to mammalian cells, utilizing an actin- and myosin-based contractile ring (CAR) [50, 76]. However, as cell-walled organisms, cytokinesis in *S. pombe* also requires the synthesis of a septum between daughter cells [77, 78]. This septum is composed of three layers. The central layer is the primary septum (PS) that is synthesized in a centripetal manner as the actomyosin contractile ring constricts and is sandwiched on both sides by the secondary septa (SS) [79, 80]. After septum formation, glucanases are secreted to break down the inner primary septum which splits the daughter cells [81, 82].

Septum synthesis is carried out by the GS complex, which includes a regulatory subunit (Rho1p) [83] and three essential catalytic subunits, Bgs1p, Bgs3p, and Bgs4p. It is known that Bgs1p forms linear β -glucans and is essential for PS formation [11] and Bgs4p forms branched β -glucans and is responsible for SS [14]. The function of Bgs3p in β -glucan biosynthesis is unknown. However, cells depleted for Bgs3p are shorter and rounder than wild-type cells and do not lyse, suggesting that the protein must be important for cell polarity and not directly involved in the preservation of cell integrity [84].

Among the Rho1p GEFs, Rgf3p localizes to the CAR and is the main candidate for the role of a positive regulator of Rho1p function during cell separation [8, 30, 85]. First, *lad1-1* cells (a mutant allele of *rgf3*) undergo cell lysis specifically at cell division; electron microscopy analysis indicates that lysis occurs only as the primary septum begins to be degraded [29]. Second, echinocandin hypersensitivity in *ehs2-1* (a mutant allele of *rgf3*) cells is suppressed by mild overexpression of Bgs1p, Bgs2p, and Bgs3p in multi-copy plasmids [30, 84] and third, Rgf3p interacts with GDP-Rho1p, and cells overexpressing Rgf3p have increased GS activity [30].

Ring maturation and constriction also takes longer in *rgf3* mutants [8, 56]. It is possible that Rgf3p acts as a physical link between components of the CAR and the membrane-bound Bgs-mediated septum growth [52]. As previously pointed out, CAR-localized proteins, such as Cdc15p, Imp2p, and Art1p, recruit Rgf3p, which activates the regulatory subunit of the β -GS [53, 56]. The same proteins may also have a role in the trafficking of the catalytic subunits, the Bgs enzymes (β -GS). For instance, Cdc15p participates in the transport of Bgs1p from the late Golgi to the septum membrane, while Rga7p (a Rho GAP) contributes to the transfer of the Bgs4p to the same area [86, 87]. Therefore, cell lysis in *rgf3* cells could be due to a defect in the newly formed cell wall, but whether it is involved in PS or SS biosynthesis is not yet known.

Another point that remains uncertain is the relationship between Rgf3p-Rho1p and the septation initiation network (SIN), the signaling pathway that coordinates mitosis with cytokinesis. SIN signaling requires three protein kinases Cdc7p, Sid1p and Sid2p, and the GTPase Spg1p, and is required for CAR constriction and for septum formation [88, 89]. Overexpression of Rho1p or Rgf3p, but not Rgf1p, partially rescues the lethality of *sid2* mutants at a low restrictive temperature [90, 91]. Based on these results, it has been proposed that SIN activates Rho1p, which in turn activates the Bgs enzymes [91]. However, the SIN target(s) remains unknown. A systematic search for Sid2p substrates has exploited the fact that phosphorylation of the Sid2p consensus site, RxxS [92], creates a binding site for 14-3-3 proteins. The comparison of the proteins that associate with Rad24p when the SIN is switched on or off has generated a list of potential Sid2p targets [93]. Although both Rgf1p and Rgf3p contain several RxxS consensus sites (10 and 7, respectively), only Rgf1p and not Rgf3p appeared in the search [93]. Rgf3p could be directly phosphorylated by Sid2p or another NDR kinase, but that has not been tested. Finally, Rho1p is essential for the feedback activation of Spg1p during actomyosin ring constriction [90].

In animal cells that enter mitosis, RhoA (Rho1p in yeast) and Ect2p (RhoA GEF) play important roles in the remodeling of the actomyosin cortex critical for accurate cell division [94, 95]. In addition, several RhoGEFs have been implicated in the process of chromosome segregation. ARHGEF10 controls centrosome duplication by activation of RhoA [96]. More recently, Net1p, the closest homolog of Rgf1p in mammals, has been shown to be required for chromosome alignment during metaphase and for the generation of stable kinetochore-microtubule attachments; its inhibition results in SAC activation. However, these functions are independent of its nucleotide exchange activity [97].

In *S. cerevisiae*, Cdc5p (polo-like kinase) regulates contractile ring formation via Tus1p and Rom2p, two Rho-GEF proteins and orthologs of Rgf3p and Rgf1p, respectively, that activate the GTPase Rho1p [98]. Rho1p regulates formin-mediated contractile ring assembly [99].

In *S. pombe*, the lytic phenotype displayed by *rho1* and *rgf3* mutants has proven problematic for identifying a possible role for Rho1p in the early stages of cell division. In addition, Rgf1p and Rgf2p also appear at the division site. Rgf1p accumulates in the nucleus of cells treated with HU, except in those cells that have already entered mitosis [42]. However, little is known about a possible role for Rgf1p and Rgf2p in cell division. Double-mutant and phenotypic complementation results suggest that Rgf1p and Rgf3p are not functionally exchangeable. Disruption of *rgf1*⁺ in an *rgf3* mutant (*ehs2-1*) produced viable cells at 28°C but not at 37°C,

a temperature which allows both mutants to grow on plates [27]. In addition, the moderate expression of *rgf1*⁺ does not suppress the lysis of *ehs2-1* cells at 37°C [30]. However, it still needs to be tested whether cell death in the *rgf1Δehs2-1* occurs during cell separation and it is not a consequence of the sum of tip growth defects plus septation defects. Regarding Rgf2p, cells of the double *rgf2Δehs2-1* mutant are viable at all temperatures and phenotypically similar to *ehs2-1* cells.

6. Role of Rho1-GEFs in the maintenance of genome integrity

Besides their classical role as membrane-bound signal-transducing molecules, it has recently been shown that Rho GEFs, Rho GTPases, and downstream components are found in the nucleus, suggesting that Rho-related-signaling processes may also take place in this cellular compartment [100, 101]. Nuclear Rho GEFs, Net1p and Ect2p, regulate, respectively, RhoA- and RhoB-mediated cell death after DNA damage [102–104]. Net1p-knockdown cells fail to activate the nuclear RhoA fraction in response to ionizing radiation [105], and Ect2p regulates epigenetic centromere maintenance by stabilizing newly incorporated CENP-A (a histone H3 variant that acts as the epigenetic mark defining centromere loci [106]).

As pointed out earlier, Rgf1p is accumulated in the cell nucleus during the stalled replication caused by hydroxyurea and is important for tolerance to chronic exposure to the drug [42]. HU causes deoxyribonucleoside triphosphate starvation by inactivating ribonucleotide reductase and blocks the progression of replication forks from early firing origins, activating the DNA replication checkpoint pathway [107]. The central sensor of the DNA replication checkpoint pathway is Rad3p, the fission yeast homolog of human ATR. Rad3p phosphorylates and activates the checkpoint kinases Cds1p or Chk1p, depending on the stage of the cell cycle and the nature of the upstream signal. DNA damage inflicted during S phase leads to the activation of Cds1p, whereas DNA damage activates Chk1p during the G2 phase. Once activated, Cds1p and Chk1p phosphorylate downstream targets to slow down cell-cycle progression and implement DNA repair mechanisms [108, 109].

Nuclear accumulation of Rgf1p after replication stress depends on the replication checkpoint kinases, Rad3p and Cds1p, and on the chaperone Rad24p that belongs to the 14-3-3 family. In the proposed model, when cells are subject to replication stress, Cds1p activation recruits Rgf1p through phosphorylation priming its interaction with Rad24p. This interaction would hide the NES sequence, reducing its association with the exportin Crm1p and thus blocking its exit from the nucleus [42].

While the mechanism for Rgf1p nuclear accumulation is outlined, much less is known about the function of Rgf1p in replication stress, as both processes seem to be directly related. An Rgf1p mutant, Rgf1p-9A, which substitutes nine serine potential phosphorylation Cds1p sites for alanines, (1) does not interact with endogenous Rad24p, (2) fails to accumulate in the nucleus in response to replication stress, and (3) displays a severe defect in survival in the presence of HU. Moreover, the Rgf1p-9A cells do not show the phenotypes characteristic of the *rgf1Δ* cells such as monopolar growth, sensitivity to caspofungin, and the *vic* phenotype

(viable in the presence of immunosuppressant and chlorine ion) [19, 27]. These results suggest that the interaction with Cds1p-Rad24p is required specifically for tolerance to replication stress. Thus, Rgf1p could be part of the mechanism by which Cds1p and Rad24p promote survival in the presence of chronic replication stress [42].

Rgf1p is also involved in tolerance to genotoxic agents other than HU [57]. *rgf1Δ* cells are sensitive to camptothecin (CPT, a topoisomerase inhibitor) and highly sensitive to exposure to phleomycin (Phl, a derivative of bleomycin); both agents induce DNA double-strand breaks (DSBs) [110–112].

DSBs are repaired by two major pathways non-homologous end joining (NHEJ) in G1 and homologous recombination (HR) in S and G2, when the sister chromatid is accessible for use as a template for repair. HR is largely error-free and is the preferred method in yeast. HR initiates when the DSB is resected by nucleases and helicases, generating 3' single-stranded DNA (ssDNA) overhangs onto which the Rad51p recombinase, with the help of Rad52p, assembles as a nucleoprotein filament [113]. This structure can invade homologous duplex DNA, which is used as a template for DNA synthesis [114, 115].

It has been recently shown that Rgf1p is involved in the repair of DNA double-strand breaks induced by Phl treatment [57]. The deletion of Rgf1p does not prevent the imposition of the checkpoint, but it does prevent recovery from DNA damage, resulting in permanent activation of Chk1p and permanent arrest of the cells in G2/M. This phenotype correlates with the inability of *rgf1Δ* cells to efficiently repair fragmented chromosomes after Phl treatment and with the presence of long-lasting, unrepaired, Phl-induced Rad52p foci in *rgf1Δ* cells.

Moreover, Rho1p and some of the proteins involved in Rho1p signaling also function in the recovery from a DNA-damage G2-induced arrest induced by Phl. Similar to the *rgf1Δ* mutant cells, the *rho1-596* mutant [26] is sensitive to CPT and Phl. The dissolution rate of Phl-induced Rad52p-YFP foci in *rho1-596* and *rgf1Δ rho1-596* cells at 28°C (permissive temperature) is very similar to that of the *rgf1Δ* cells, suggesting that Rho1p functions in DSB repair [57]. Future studies defining the interaction of Rgf1p/Rho1p with other DSB repair proteins at Rad52p factories may help to delineate its role in completing DSB repair.

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Conflict of interest

The authors declare that there is no conflict of interest.

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