Art1- Arrestin in cytokinesis

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ABSTRACT

Background: Cytokines partitions a mother cell into two daughter cells and is essential for cell proliferation and cell differentiation [1]. Septins, a family of conserved filamentous forming GTPases, are essential for cytokinesis in budding yeast and some animal cells. However, septin deletion mutants are viable in the fission yeast *S. pombe*, indicating the presence of a parallel pathway in *S. pombe* for maintenance of cell integrity during cytokinesis [2]. A synthetic lethal screen with septin deficient strain identified the arrestin Art1 as a component of this parallel pathway [3]. Arrestins are known to regulate cellular signaling in higher eukaryotes [4]. However, their functions in *S. pombe* are unknown.

Findings: Using combined biochemical, molecular, genetic, and cellular techniques, I found that in *art1* deletion cells, cell lysis occurred during cell-cell separation after mitosis. I showed that the *art1* deletion cells had a septal-wall defect that mimicked Rho guanine nucleotide exchange factor 3 (*rgf3*) mutants under electron microscopy. Rgf3 is a guanine nucleotide exchange (GEF) factor for the Rho GTPase Rho1. Rho1 and Rgf3 are essential in *S. pombe* and have homologs in mammalian cells. Rho GTPases are important regulators of cell growth and cytokinesis. They are activated by Rho guanine nucleotide exchange factor (GEF) mediated GTP loading during cell division [7]. Approximately 80% of *art1*

deletion cells showed a thin and defective septal cell wall at the site of division compared to wild type. This presumably leads to its cell lysis phenotype. Art1 and Rgf3 both concentrate to the contractile ring and septum at the same time and diffuse in the cytoplasm as discrete puncta. Art1 requires its C-terminus to localize to the contractile ring and carry out its function. Immunoprecipitation and yeast two-hybrid assays indicated that Art1 binds to Rgf3 at a domain adjacent to its catalytically active GEF domain. Moreover, protein quantification by measuring fluorescence intensity [6] revealed that loss of Art1 caused a decrease in cellular Rgf3 levels and adversely affected its contractile-ring localization. Conversely, Art1 overexpression led to an increase in cellular and contractile ring/septal levels of Rgf3. Interestingly, upon using an N-degron mutant of Rgf3 we discovered that Art1 could localize weakly to the contractile ring (20% of dividing cells), but its localization to the septum was abolished. Together, these data suggest that Art1 is involved in the recruitment of Rgf3 to the contractile ring but its retention in the septum is dependent on Rgf3. Thus Art1 and Rgf3 are partially interdependent on each other for their contractile ring and septum localization. In order to further confirm Art1's role in recruiting Rgf3, I will mislocalize Art1 in the cell and test if Rgf3 is also mislocalized to Art1's new location. Future studies will also investigate whether Art1 regulates GEF activity of Rgf3.

Conclusion and significance: The findings suggest that arrestin Art1 is involved in cytokinesis by regulating Rgf3 protein stability and partially affecting its localization. This could in turn affect Rgf3's GEF activity. Art1 is the first known *S. pombe* arrestin being characterized as having a role in cytokinesis. The results obtained imply a novel, previously unknown role for arrestins.

INTRODUCTION

Cytokinesis is the last step of the cell cycle that results in physical separation of two daughter cells. Most proteins involved in cytokinesis are evolutionarily conserved in all eukaryotes (Barr and Gruneberg, 2007; Pollard and Wu, 2010). The final step in cytokinesis for the fission yeast *Schizosaccharomyces pombe* (*S. pombe*) is the formation of the septum. The septum in *S. pombe* is a tri-laminar structure. The primary septum is synthesized in a centripetal manner as the acto-myosin contractile ring constricts and is sandwiched on either side by the secondary septa. The primary septum is eventually digested uniformly from both ends of the cell leading to the physical separation of the two daughter cells (Humbel et al., 2001; Sipiczki, 2007). Proper synthesis and maintenance of the septum is necessary for cell survival.

The *S. pombe* primary septum is composed predominantly of linear β-1,3-glucans and some branched β-1,3-glucans. The secondary septa is made up of branched β-1,3-glucans, α-1,3-glucans and galactomannans (Humbel et al., 2001) (Sipiczki, 2007). The enzymes involved in glucan synthesis are β-glucan synthases (Bgs) and α-glucan synthases (Ags). There are 4 β-glucan synthases (Bgs1-4). GTP-Rho1 acts as a regulatory subunit for these synthases and stimulates them leading to the formation of the septum (Arellano et al., 1999; Nakano et al., 1997). Rho1 belongs to a family of Rho1 GTPases that is evolutionarily conserved. In *S. pombe*, Rho1 family consists of Rho1 to Rho5 and Cdc42. This family of proteins can switch from an inactive GDP bound form to an active GTP bound form. The switch is catalyzed by Rho GEFs (guanine nucleotide exchange factors) that are critical activators that regulate Rho GTPase activity. *S. pombe* has 7 Rho GEFs-Rgf1-3, Gef1-3 and Scd1. Rho1 is activated by Rgf1-3 (Morrell-Falvey et al., 2005; Mutoh et al., 2005a; Tajadura et al., 2004). The inherent GTPases activity of Rho GTPases is accelerated by Rho GAP's (GTPase activating protein).

Rho GEF Rgf3, a homolog of eukaryotic Ect2 is the major GEF acting on Rho1. It is an essential protein. Cells with disrupted or deleted Rgf3 or Rho1 lyse during cell separation and appear shrunken. Rgf3 depleted cells had abnormal contractile ring formation and abnormal septal material deposition with cell separation occurring unevenly from one end of the cell. Rgf3 was found to interact with GDP-Rho1 and cells overexpressing Rgf3 were found to have increased glucan synthase activity further confirming that Rgf3 is a positive regulator of Rho1 function pertaining to cell wall synthesis (Morrell-Falvey et al., 2005; Mutoh et al., 2005b; Tajadura et al., 2004; Wu et al., 2010b). Like Rho GTPases, Rho GEF regulation is also tightly controlled. Disruption of Rho GEF activity is associated with developmental defects, cancer and neurological disorders in mice. Rho GEFs are known to be regulated by intramolecular binding, which prevents association with GTPases. They are also regulated by protein-protein interaction, localized activation, different recruiting adaptors and sometimes by GTPases themselves (Buchsbaum, 2007; Rossman et al., 2005). The mechanism of regulation of Rho GEF Rgf3 in *S. pombe* is unknown.

The arrestin *art1-s34* mutant was discovered in a synthetic lethal screen with septin deficient mutants (Wu et al., 2010b). Septins are conserved GTPases that are known to function as scaffolds and are essential for cytokinesis in budding yeast, mammalian and certain drosophila cell types. However unlike budding yeast, septins are not essential in *S. pombe*. The synthetic lethal screen identified Art1 along with Rgf3 to be part of a parallel pathway that is required to recognize and repair cell wall damage in the absence of septins. The *art1-s34* mutant was rescued by overexpression of Rho1 and Rgf3 indicating that Art1 and Rgf3 may function in the same cell integrity pathway parallel to septins (Wu et al., 2010b).

Arrestins are conserved proteins with a characteristic Ig- like beta- sandwich fold that are known for abrogating GPCR (G- protein coupled receptor) mediated signaling in eukaryotes. They

typically have an N-terminal and C-terminal domain involved in receptor binding along with a C terminal tail required for recruitment of the ubiquitination and endocytosis machinery (Aubry et al., 2009; Kendall and Luttrell, 2009; Premont and Gainetdinov, 2007). In budding yeast arrestin related proteins (with all or some of the domains of arrestin) are known to act endocytic adaptors and recycling of cell surface receptors (Herrador et al., 2010; Lin et al., 2008; Nakase et al., 2013; Nikko and Pelham, 2009) . There are at least 8 arrestin related proteins in *S. pombe*. They are not well studied except for one protein having a known role in meiosis and another in receptor endocytosis (Matsuyama et al., 2000; Nakase et al., 2013) .

This study examines the novel protein Arrestin- Art1, an arrestin-family protein that localizes to the site of cell division during cytokinesis in *S. pombe*. Art1 was found to have a role in regulating the function of the Rho1 GEF, Rgf3. We found Art1 to be important for Rgf3 stability and its localization to the division site during cytokinesis. Furthermore, the deletion mutant of Art1 had lower activity of Rho1 at the division site implying reduced activity of Rho GEF Rgf3. The results suggest a novel, previously unknown for arrestins in cytokinesis and the identification of a novel mechanism of Rho GEF regulation mediated by an arrestin related protein.

RESULTS

*art1*Δ is a late cytokinesis mutant.

The *art1-s34* mutant was isolated in a genetic screen to identify genes that were synthetic lethal with a septin deletion strain (Wu et al., 2010a). It was identified along with several genes that were known or predicted components of the cell integrity pathway. *The art1-s34* has a mild cell lysis defect when cultured in minimal media EMM5S at 30°C. About 5% of the cells undergo lysis (Wu et al., 2010b). However, when grown in nutrient rich YE5S media , in comparison to wild-type (wt) cells that had less than 1% cell lysis, 15% of *art1s-34* cells were found to undergo lysis (Figure 1B). To further study the role of Art1 in the cell integrity pathway we deleted the whole ORF and observed that the cell lysis percentage rose up to about 25% (Figure 1B). The lysis phenotype resembled the lysis seen in the previously described Rho-GEF *rgf3(lad1-1)* mutants (Morrell-Falvey et al., 2005). In *rgf3(lad1-1)* mutants the daughter cells remained attached to each other and the lysed doublets resembled the shape of 'boomerangs' (Figure 1A, arrowheads). Time lapse imaging of *art1*Δ cells showed us that the lysis occurred during cell separation (Figure 1C). Typically, as the cells are trying to separate after septum formation, one or both of the daughter cells will lyse. In a few cases, the daughter cells will shrink and seem to undergo lysis, but eventually recover.

To examine the defect seen in *art1*Δ mutants more closely, and determine if was indeed similar to *rgf3(lad1-1)* mutants, we examined cross-sections of wt and mutant cells via transmission electron microscopy (Figure 1D). We observed that in many mutant cells, the primary and secondary septa are wavy and have reduced thickness. The defect in cell wall is most noticeable during cell separation. In wt cells, cell separation occurs symmetrically, with the primary septum being digested evenly from both ends of the cell (Figure 1D, left). In the mutants

however, we observe that primary septum digestion occurs primarily from one end (Figure 1D, right). Also, in wt cells, the newly formed cell wall at the site of cytokinesis is evenly thick, (approximately 70% of the cell wall thickness found at other locations). In mutants however, the cell wall at the site of division is severely eroded (Figure 1D, right inset). This is similar to the *rgf3 (lad1-1)* mutant (Morrell-Falvey et al., 2005). Compared to wt cells, ≥70% of mutant cells have a thin newly formed cell wall and in approximately 50% of the cases it is thin and uneven leaving the membrane exposed (Figure 1E, inset).

Art1 localizes to the contractile ring and septum

To understand what function Art1plays during late cytokinesis, we tagged it at its C-terminus with the YFP variant, mECitrine and examined its location with the cell. Art1 localizes to the contractile ring (Figure 2A, B- arrowheads) during cytokinesis. After ring constriction, Art1 signal spreads unevenly around the septum (Figure 2A, B- arrows) and is present till the cells separate. Art1 is found diffused in the cytosol at all other cycle stages. We imaged Art1-mECitrine tagged cells with 0.2 μm Z- spacing and created a 3-D projection of the cells in Figure 2B. Art1 in the contractile ring appears as a hollow ring (Figure 2C-left) whereas Art1 in the septum appears as an uneven disk (Figure 2C- right). It is present at the division site till the cells separate. Art1 is abundant in the cell. Using quantitative fluorescence microscopy we determined that there are about 1740 ± 365 molecules of Art1 per cell. However only a small proportion, 102 ± 30 molecules are found in a mature contractile ring (Figure 5b, left).

Art1 appears in the ring when the SPB are about 3 μm apart which corresponds to late anaphase, about 10-11 minutes after SPB separation (Wu and Pollard, 2005) This is consistent with the late cytokinesis phenotype of *art1*Δ. Of interest is that Art1 is similar to Rgf3 in its timing of

localization to the ring, it too appears in the ring when the SPB are about 3 μm apart. Thus, based on phenotype, localization and time of arrival of Art1 to the ring, Art1 could be working with Rgf3 in septum/ cell wall formation (Tajadura et al., 2004).

Art1 interacts with Rgf3 and belongs to the cell integrity pathway with Rgf3 and Rho1

art1 and *rgf3* mutants have very similar defects in cell wall structure. Since Rgf3 is known be a RhoGEF for Rho1 which activates the cell wall synthesizing enzyme β-glucan synthase 1 (Bgs1), we hypothesized that Art1functions in the same pathway as Rgf3 and Rho1for cell wall synthesis. To support this hypothesis, a previous study from our lab has shown that the *art1-s34* mutant can be rescued by Rgf3 and Rho1 overexpression (Wu et al., 2010b). To confirm this hypothesis, we tested if *art1*Δ mutant could be rescued by Rgf3 and Rho1 over-expression. The percentage cell lysis of *art1*Δ cells decreases to ≤5% when rescued by Rho1 and Rgf3 overexpression (Fig. 3A). This suggests that Art1 may work with Rgf3 and Rho1 in the cell wall integrity pathway and that Rgf3 and Rho1 are upstream of Art1 in this pathway. Art1 localization in the cell is identical to that of Rgf3. Upon imaging Art1-mECitrine mCFP- myo2 strains we saw that the peak of fluorescence intensity of Art1-mECitrine and mCFP-myo2 in the ring does not overlap and Art1 signal lies outside of the peak for mCFP-myo2 (Figure 3B). Art1-mECitrine localization also lags behind Rlc1-mCFP (data not shown). This is similar to the reported localization of Rgf3 lagging behind Rlc1 and ahead of Rho1 in the ring (Mutoh et al., 2005a).

Based on the above data we hypothesized that Art1 and Rgf3 physically interact. To test this, we examined their interaction via Co-immunoprecipitation and yeast two hybrid assays. Art1-3YFP (yellow fluorescent protein) could pull down Rgf3-13Myc from cell lysates, suggesting that they are found in a complex in the cell (Figure 3D). Both Rgf3 and Art1 were found to be

phospho-proteins (Figure 3C) and so we simultaneously tested for phosphorylation dependency of their interaction. We found that after we treated Art1-3YFP pulled down from cell lysates with phosphatase for an hour, it could still bind to Rgf3-13Myc (Figure 3D). The interaction of Art1 with Rgf3 plays an important role in Art1 localization. When we used a temperature induced mutant of *rgf3* (*N-degron-rgf3* strain), we saw that Art1-mECitrine localization to the ring was severely affected.

Rgf3 depends on Art1 for its stability

Since Art1 and Rgf3 interact with each other, we wanted to determine what role Art1 plays in cytokinesis through Rgf3. We found that in *art1*Δ strains, Rgf3 localization to the ring was severely affected and Rgf3 localization in the cytoplasm was also reduced compared to wt. We could however detect Rgf3 in the septum (Figure 4A center panel). When art1 was over-expressed, Rgf3 levels in the cytoplasm and the ring were higher (Figure 4A right panel). We calculated the total number of Rgf3 molecules in the wt cell and the ring and compared it to levels in *art1*Δ and Art1 overexpression strains (Wu and Pollard, 2005). In wt cells, there are 618 ± 185 Rgf3 molecules in the cell and 187 ± 52 molecules of those are found in the ring. In the *art1*Δ strain however, the total number of Rgf3 molecules decreases to 266 ± 198. Rgf3 localization to the ring in *art1*Δ is severely reduced or absent and is not quantifiable. However, Rgf3 localizes weakly to the septum with a mere 41 ± 10 molecules. Finally, in Art1 overexpression cells, the number of Rgf3 molecules in the cell increases to 1447 ± 679 molecules and that in the ring also increases to 228 ± 50 molecules (Figure 4B). Thus, art1 protein levels can influence Rgf3 protein levels in the cell. We further confirmed this idea by carrying out a western to measure Rgf3 levels in *art1*Δ and art1 overexpression strains. We saw that in *art1*Δ strains the level of Rgf3 dropped to about 40% of

the original (Figure 4C, Top). Conversely, in art1 overexpression strains, the levels of Rgf3 seen via western were much higher than those seen in wt cells (Figure 4C, bottom). This data implies that art1 plays a role in Rgf3 stability. The effect of Art1 on Rgf3 function seems more modest however as loss of about 60% of Rgf3 causes only about 15-20% cell lysis and Art1 overexpression cannot stabilize Rgf3 levels enough to lead to a Rgf3 overexpression (multi-septate) phenotype.

Art1 plays a role in Rgf3 localization

In *art1*Δ mutants, we saw Rgf3 localize to the septum but not to the ring (Figure 4A, center). We hypothesized that besides playing a role in stability, art1 could play a role in recruiting Rgf3 to the ring. To test this hypothesis, we mislocalized Art1-mECitrine , to the interphase and cytokinesis nodes using Mid1-GBP (GFP binding protein) or to SPB using Cdc7-GBP (Rothbauer et al., 2008). Art1 was successfully mis-localized to the interphase nodes and the SPB. The mislocalization was only partial as native localization of Art1 to the ring and the septum were still seen. When these strains were crossed to Rgf3-mCFP, we saw Rgf3 successfully albeit weakly co-localize with Art1 at its new location. We saw Rgf3 mislocalize to bright interphase nodes/ clumps of nodes (Figure 5A, bottom panel in red boxes). Similar results were obtained with the Cdc7-GBP strains (Figure 5B). This data confirms that Rgf3 localization to the interphase nodes and SPB is real and that besides playing a role in Rgf3 stability, Art1 can recruit Rgf3 to the ring.

***art1*Δ mutants can influence Rho1 activity**

In this study, we have shown that *art1*Δ can be rescued by Rgf3 and Rho1 over expression and that Art1 can directly interact with Rgf3. RhoGTPase Rho1 and its GEF Rgf3 are known players in the

cell integrity pathway in *S.pombe* (Cruz et al., 2013; Viana et al., 2013; Wu et al., 2010b). Together they can activate cell wall synthesis by activating the cell wall synthesizing enzyme β-glucan synthase1 (bgs1) (Tajadura et al., 2004). Based on our data, we hypothesized that *art1*Δ would negatively affect the cell integrity pathway since it negatively affects Rgf3 levels and localization. Since art1 affects the localization and stability of Rgf3 which in turn is known to affect Rho1 activation, we decided to look at active Rho1 levels in the cell. Using the Rho1 biosensor (Rothbauer et al., 2008) we were look at GTP-Rho1 localization in the cells. We saw that whereas the overall localization and levels of the biosensor in the cell were similar in wt and mutant cells (Figure 6A,C), the amount of active Rho1at the division site- in growing and complete septum was lower in *art1Δ* mutants when compared to wt cells (Figure 6,B,D). We looked at the Rho1 biosensor levels at various stages of septum growth, measured as a fraction of the full sized septum (Figure 6B,D) and found that at every stage of septum growth, the amount of GTP-Rho1was reduced in the mutants when compared to wt cells (Figure 6B). This phenotype was similar to that seen in Rgf3 mutants (Figure 6E). Thus, we see that art1 mutants can negatively affect Rho1 activity at the division site.

CONCLUSION

In this study we found that Art1, a previously uncharacterized arrestin family protein plays an important role in cytokinesis. Art1 can physically bind to the Rho GEF Rgf3, a GEF for Rho GTPase Rho1, and recruit it to the contractile ring during cytokinesis. Moreover, we found that binding of Art1 to Rgf3 also increases its stability in the cell. Thus we have determined the role of Arrestin-Art1 as a positive regulator of Rho GEF Rgf3 function, in the absence of which Rho1 activity at the division site is reduced.

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Davidson et al., Figure 1

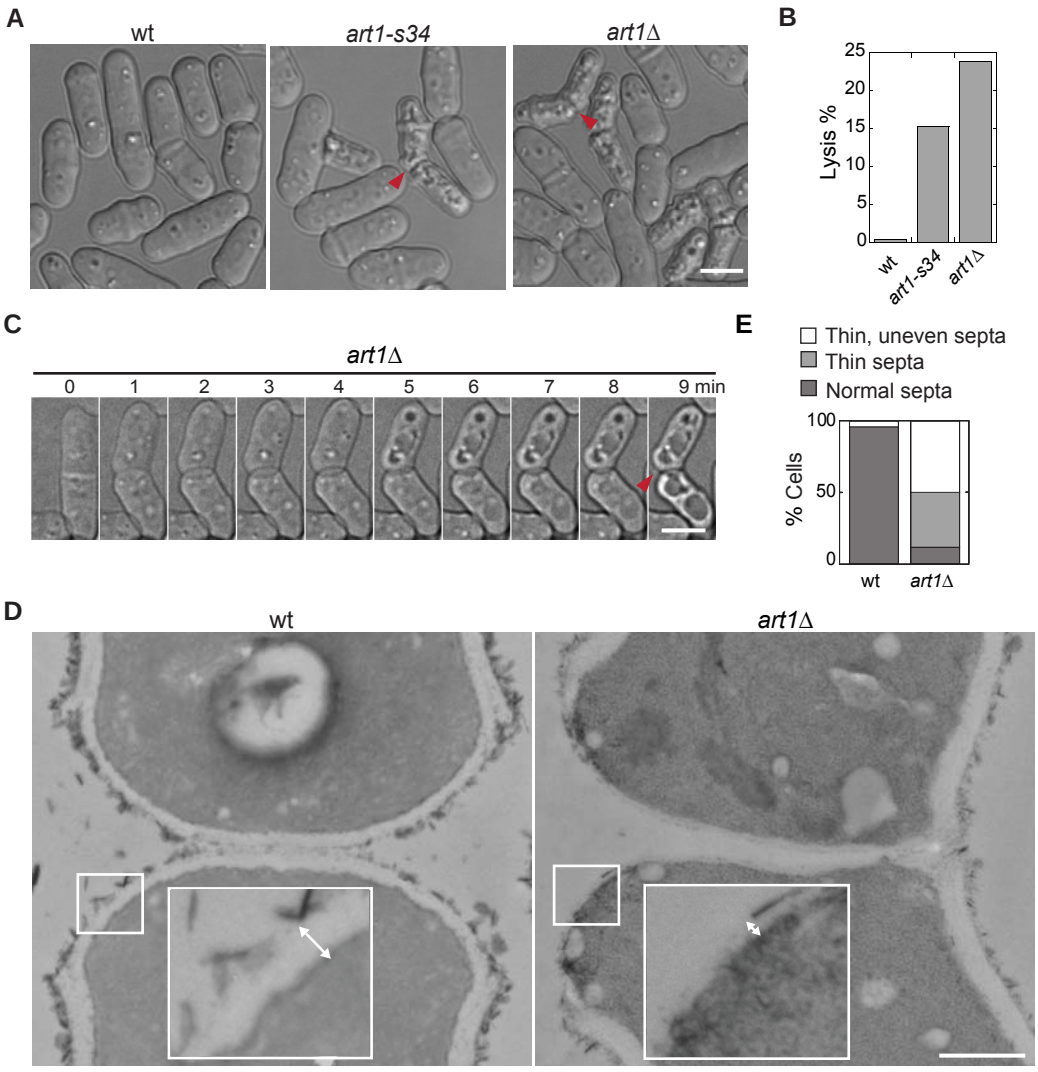

Figure 1- art1Δ is a late cytokinesis mutant. (A-C) art1Δ cells have a cell lysis phenotype. Differential interference contrast (DIC) images of wild-type (wt), art1-s34, art1Δ. (B) Percentage of cell lysis. (C) Lysis occurs during cell separation. Time lapse microscopy of art1Δ cells. Red arrowheads mark 'boomerang' shaped lysed doublets. Bars, A and C, 5μM. (D,E) Transmission electron microscopy (TEM) of wt and art1Δcells. (D) art1Δ cells have a defect in their newly formed cell wall. Inset- Magnification of the cell wall region in the small white box. Double- headed arrows mark the thickness of the cell wall. Bar, 0.5μm. (E) Qantification of cell wall defect. Cells with thin septa are defined as having ≤50% thinner septa than wt cells at the division site. Cells with uneven septa are defined as cells that were found to have regions with no cell wall along with regions with eroded cell wall.

Figure 2

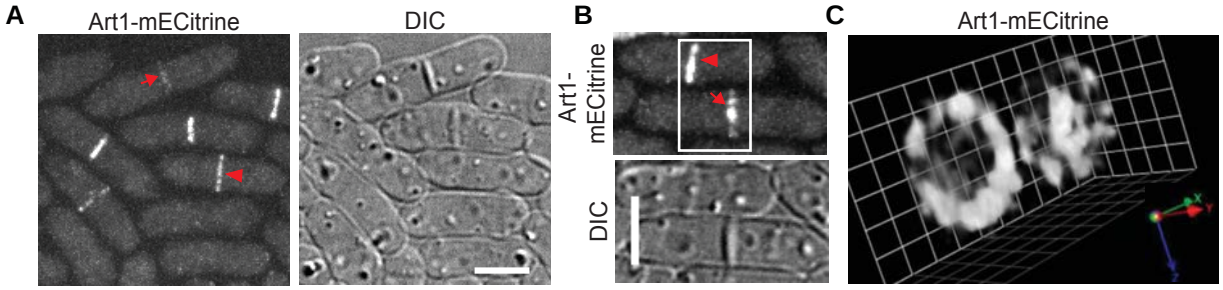

Figure 2- Art1 localizes to the AMR and septum independent of actin. (A) Fluorescence micrographs showing Art1-mECitrine localization at the acto-myosin ring (AMR) (red arrowheads) and septum (red arrows). (B) Magnified fluorescence micrograph of a cell with Art1-mECitrine in the ring and another with Art1-mECitrine in the septum. (C) 3-D projection of the boxed region shown in (B). Bar- 5μm. One unit=0.95μm.

Figure 3

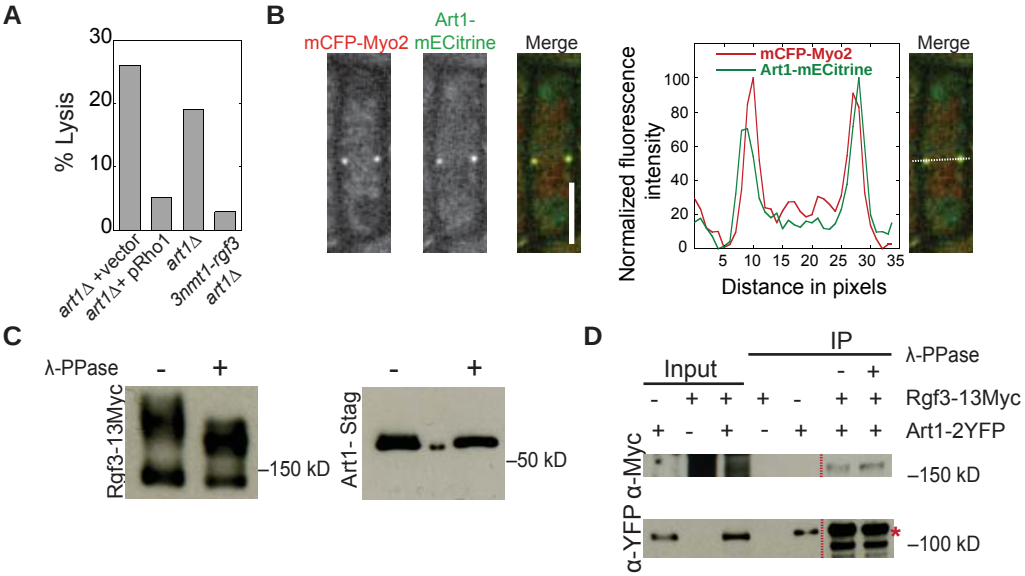

Figure 3- Art1 and Rgf3 interact. (A) Quantification of percentage lysed cells in art1Δ+ pRho1 and 3nmt1-rgf3 art1Δ strains. (B) Art1 and Rgf3 have similar localization. Images of Art1-mECitrine mCFP-Myo2 strain were collected. A single (central) slice was used to look at the signal from the two proteins. The composite of the two channels shows Art1-mECitrine (green) signal lagging behind mCFP- Myo2 signal. Right, fluorescence intensity profile of a line drawn through the ring showing Art1-mECitrine (green) lagging behind Art1-mECitrine.(C) Rgf3 and Art1 are phospho-proteins. λ -Phosphatase was used to treat Rgf3-13Myc (left) and Art1-Stag bound to beads. (D) Art1 and Rgf3 Co-IP with each other, independent of phosphorylation status. Cell lysates were prepared from Art1-2YFP, Rgf3-13Myc and Art12YFP Rgf313Myc double mutant strains. IP using antibodies against YFP were carried out in the above cell lysates.

Figure 4

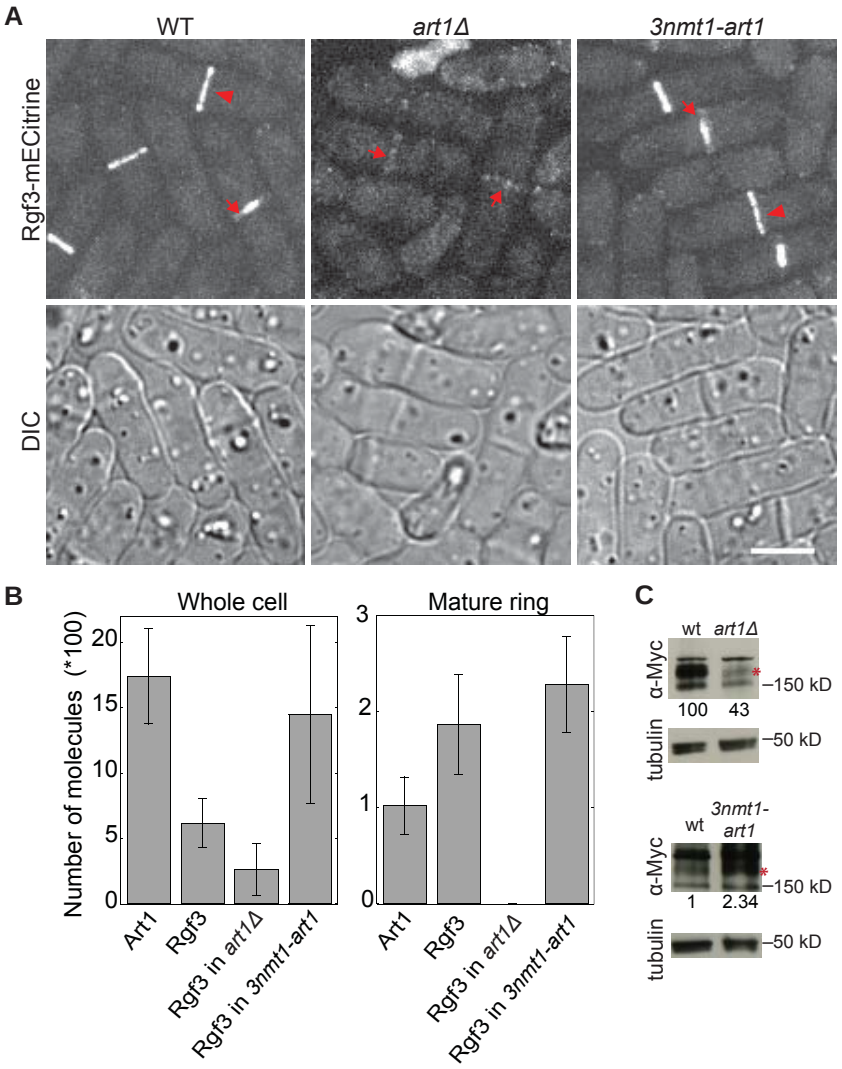

Figure 4- Rgf3 Is dependent on Art1 for its localization and stability. (A) Rgf3 localization to the contractile ring is partially dependent on Art1. Rgf3-mECitrine localization in wt, art1Δ and 3nmt1-art1 was examined. Arrows indicate septum localization and arrowheads point at the AMR ring. (B) Quantification of the total number of molecules of Art1 and Rgf3 in the globally in whole cell and in mature contractile rings. (C) Art1 plays a role in Rgf3 stability. TOP- Western using antibodies was carried out in cell extracts prepared from Rgf3-13Myc and Rgf3-13Myc art1Δ strains grown in YE5S. BOTTOM- Western using antibodies was carried out in cell extracts prepared from Rgf3-13Myc and Rgf3-13Myc 3nmt1-art1 strains induced in EMM5S. Bar, 5 μm.

Figure 5

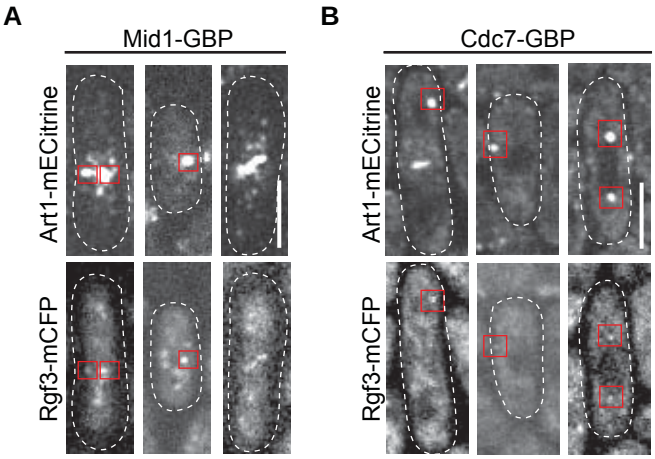

Figure 5- Art1 plays a role in Rgf3 localization. Rgf3-mCFP co-localizes with mis-localized Art1-mECitrine. Cells were grown and examined at 25°C. (A) Single plane images of Mid1-GBP Art1-mECitrine Rgf3-mCFP strain. Areas in red boxes show co-localization of Art1 and Rgf3. (B) Single plane images of Cdc7-GBP Art1-mECitrine Rgf3-mCFP strain. Areas in red boxes show co-localization of Art1 and Rgf3. Bars, 5 µm.

Figure 6

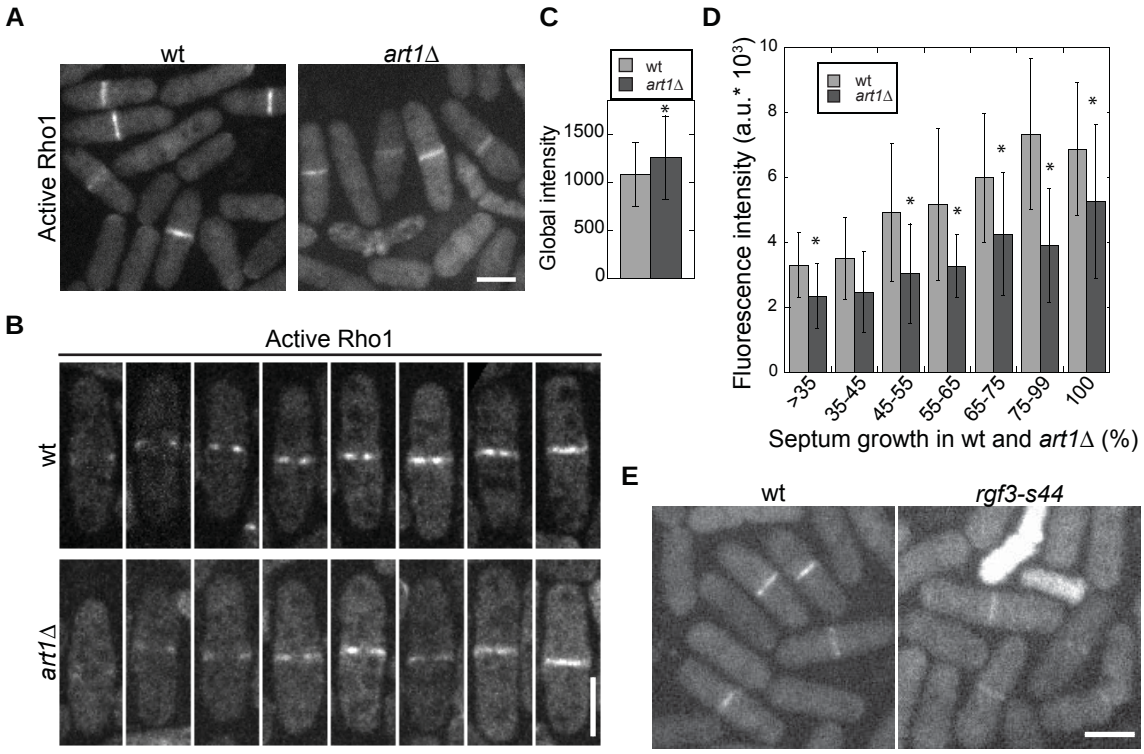

Figure 6- The role of Art1 in the cell integrity pathway. (A-B,D) Rho1 activation is inefficient at the division site in art1Δ cells. pkc1(HR-C2)- mECitrine was used as a biosensor to look at active Rho1-GTP localization. The biosensor was examined in wt and art1Δ strains. (A) Micrographs of wt and art1Δ strains with Rho1 biosensor at the division site (B) Individual cells representing various stages of septum growth in in wt and art1Δ strains.(C) Quantification of global Rho1 Biosensor intensity in wt (n=106) and art1Δ (n=113) cells. There is a small but significant increase in global cellular intensities between wt and art1Δ strains. (D) Active Rho1 biosensor intensity during septum growth was measured. Septum growth was measured as a percentage of the cell width. Active Rho1 levels are significantly reduced in many stages of septum growth in art1Δ strain (n=191) when compared to wt cells (n=219). Bar, 5µm. (D) Rho1 biosensor localization was examined in wt and rgf3 s-44 mutants.