Genetic suppression of defective profilin by attenuated Myosin II reveals a potential role for Myosin II in actin dynamics in vivo in fission yeast

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ABSTRACT The actin cytoskeleton plays a variety of roles in eukaryotic cell physiology, ranging from cell polarity and migration to cytokinesis. Key to the function of the actin cytoskeleton is the mechanisms that control its assembly, stability, and turnover. Through genetic analyses in Schizosaccharomyces pombe, we found that myo2-S1 (myo2-G515D), a Myosin II mutant allele, was capable of rescuing lethality caused by partial defects in actin nucleation/stability caused, for example, through compromised function of the actin-binding protein Cdc3-profilin. The mutation in myo2-S1 affects the activation loop of Myosin II, which is involved in physical interaction with subdomain 1 of actin and in stimulating the ATPase activity of Myosin. Consistently, actomyosin rings in myo2-S1 cell ghosts were unstable and severely compromised in contraction on ATP addition. These studies strongly suggest a role for Myo2 in actin cytoskeletal disassembly and turnover in vivo, and that compromise of this activity leads to genetic suppression of mutants defective in actin filament assembly/stability at the division site.

INTRODUCTION

Actin is a highly conserved cytoskeletal polymer that exists as monomers and filamentous structures that are important for a vast array of physiological processes in the living world. In eukaryotes, the actin cytoskeleton plays essential roles in cell polarity, migration, and cytokinesis (Pollard and Wu, 2010; Cheffings et al., 2016; Misu et al., 2017; Rottner et al., 2017; Skruber et al., 2018). A balance of factors that control filament nucleation and stability and those that promote its disassembly regulates the functions of the actin cytoskeleton (Lee and Dominguez, 2010). Over the past three decades, the fission yeast Schizosaccharomyces pombe has emerged as an attractive organism for the study of the actin cytoskeleton and its role in cell polarity and division (Pollard and Wu, 2010; Cheffings et al., 2016; Chiou et al., 2017). This is particularly due to the fact that many of the actin cytoskeletal proteins can be characterized through easily identifiable mutant phenotypes (Wertman et al., 1992; Holtzman et al., 1994; McCollum et al., 1996; Bezanilla et al., 1997; Kitayama et al., 1997; May et al., 1997; Motegi et al., 1997; Balasubramanian et al., 1998; Wu et al., 2001; Nakano and Mabuchi, 2006). Work in Saccharomyces cerevisiae has also helped define a number of phenotypes indicative of actin cytoskeletal defects (Wertman et al., 1992; Holtzman et al., 1994).

In fission yeast, actin exists in three types of structures: actin patches, which are nucleated by the Arp2/3 complex (Pelham and Chang, 2001; Sirotkin et al., 2010); linear interphase actin cables, nucleated by the formin-For3 (Feierbach and Chang, 2001); and the cytokinetic actomyosin ring (AMR), nucleated by the formin-Cdc12 (Chang et al., 1997) and the actin-binding protein Cdc3-profilin (Balasubramanian et al., 1994). The coiled-coil actin-binding protein Cdc8-tropomyosin ensures stability of actin cables and AMR (Liu and Bretscher, 1989; Balasubramanian et al., 1992; Gunning et al., 2015). The fission yeast actin cytoskeleton undergoes dramatic disassembly and turnover (Kovar et al., 2011). Treatment of cells with the actin polymerization inhibitor Latrunculin A (LatA) causes complete loss of actin cables, AMRs,
and actin patches (in that order) (Pelham and Chang, 2001, 2002).
While the actin severing protein Adf1 (cofilin) plays a key role in
actin cytoskeletal disassembly (Nakano and Mabuchi, 2006; Pav-
lov et al., 2007), the fact that the actin cables and rings still turn
over, albeit slowly, in adf1-1 mutants (Nakano and Mabuchi,
2006) suggests that other mechanisms should also exist to disas-
semble the actin cytoskeleton. The actin cytoskeleton is also
modulated by the Adf1-related Gmf1 protein in fission yeast (Na-
kano et al., 2010). Myosin-family proteins have also been shown
to destabilize the actin cytoskeleton in plants and metazoa
(Staiger et al., 2009; Murrell and Gardel, 2012; Henty-Ridilla et
al., 2013; Vogel et al., 2013; Cai et al., 2014), although genetic
evidence to this effect is scarce.

Myo2-S1 (Myo2 G515D) was isolated in a genetic screen for sup-
pressors of the high temperature lethality of cdc3-124 that led to
characterization of the Arp2/3 complex protein Sop2 (Balasubrama-
nian et al., 1996; Wong et al., 2000), although Myo2-S1 mutant has
not been previously characterized. Here we characterize the cellular
and molecular basis of the suppression of cdc3-124 and other
conditions that cause partial loss of the actin cytoskeleton. These
studies point to an in vivo role for Myosin II in actin filament
disassembly.

**RESULTS AND DISCUSSION**

myo2-S1 suppresses the ts lethality of cdc3-124

Previous work has shown that cdc3-124 is defective in AMR assembly
and colony formation above 32°C (Balasubramanian et al., 1996). To
investigate the suppression of cdc3-124 by myo2-S1, we freshly gen-
erated cdc3-124 myo2-S1 from a cross between a myo2-S1 parent
(obtained after three back crosses) and cdc3-124. This cdc3-124
myo2-S1 strain was compared with wild-type, cdc3-124, and myo2-
S1. As expected, wild-type formed colonies at all temperatures
tested, whereas cdc3-124 failed to form colonies at and above 32°C
(Figure 1A). myo2-S1 was able to form colonies at all temperatures
tested, although these cells displayed cytokinesis defects at all tem-
peratures (described in later sections) (Figure 1A). Importantly, cdc3-
124 myo2-S1 was able to form colonies at 34°C and did so poorly,
even at 36°C (Figure 1A). We found that cdc3-124 myo2-S1 was
sicker than myo2-S1, although this double mutant was healthier than
cdc3-124 and hence the observed genetic suppression. The genetic
screen (Balasubramanian et al., 1996) also identified a different sup-
pressor allele of myo2, myo2-S2 (Myo2 E679K) (Wong et al., 2000)
(Figure 1A). We recreated the cdc3-124 myo2-S2 and found that it
rescued cdc3-124 at 34°C, but barely at 36°C (Figure 1A). The well-
characterized myo2-E1 (Balasubramanian et al., 1998) did not rescue

**FIGURE 1:** myo2-S1 suppresses the lethality of cdc3-124 at the nonpermissive temperature. (A) Tenfold serial dilutions
of wild-type, cdc3-124, myo2-S1, cdc3-124 myo2-S1, myo2-S2, and
CDC3-124 myo2-S2 were spotted onto YE agar plates and
grown for 3 d at 24, 34, and 36°C. (B) CW staining was used to visualize the septum of wild-type, cdc3-124,
myo2-S1, and cdc3-124 myo2-S1 cells at 34°C. In the first column it is shown the acquired front view image of the
septum, while in the second column it is displayed the face-on view of the septum, which was generated with Fiji
software. Scale bar 3 µm. (C) Tenfold serial dilutions of wild-type, cdc8-110, cdc12-112, myo2-S1, cdc8-110 myo2-S1,
and cdc12-112 myo2-S1 were spotted onto YE agar plates and grown for 3 d at 24, 34, and 36°C. (D) Cells of the
indicated genotypes were grown at 24°C and shifted for 4 h at 34°C and stained with CF633-phalloidin and DAPI.
Arrows indicated normal AMRs while asterisks indicated abnormal rings. Scale bar 5 µm. (E) Quantification of CF633-
phalloidin and DAPI staining in D of three independent experiments (n = 3). Cells with two nuclei were classified
depending on the presence of no ring (2N/no ring), 1 normal ring (2N/1 ring), 1 abnormal ring (2N/abnormal ring), or
multiple nuclei and rings (MN/M rings).
cdc3-124. Since myo2-S1 better suppressed the cdc3-124 defect, we characterized this mutant further.

The strains described above were stained with Calcofluor white (CW) to visualize the division septa. Whereas normal (or weakly defective) division septa that appeared as a disk in tilted images were detected at 24°C in all strains, disk-like complete septa were found in wild-type, myo2-S1, and cdc3-124 myo2-S1, but not in cdc3-124, at 34°C (Figure 1B). Instead, patches of septum material that did not create a barrier between the two daughters were observed in cdc3-124 at 34°C (Figure 1B). Although the septa detected in cdc3-124 myo2-S1 and myo2-S1 were complete and spanned the entire division site, they were nearly all abnormal in that they did not have a flat disk-like morphology observed in wild-type septa. Nevertheless, the fact that these septa were complete facilitated successful completion of cytokinesis in cdc3-124 myo2-S1 and myo2-S1.

Given the roles of Cdc12-formin in linear actin filament nucleation during cytokinesis (Chang et al., 1997; Kovar et al., 2003) and Cdc8-tropomyosin in stabilization of actin filaments in the AMR (Balasubramanian et al., 1992; Gunning et al., 2015; Khaitlina, 2015), we tested if the observed suppression was specific to Cdc3-profilin defects or if myo2-S1 also suppressed defects in cdc12 and cdc8. To this end, we generated cdc8-S1 myo2-S1 and cdc12-112 myo2-S1. We found that cdc8-S1, but not cdc12-112, was suppressed at 34°C and tiny “pin-prick” colonies were observed even at 36°C (Figure 1C).

Next, we stained wild-type, cdc3-124, myo2-S1, and cdc3-124 myo2-S1 with CF633-phalloidin and DAPI to observe the actin cytoskeleton and nuclei (Figure 1, D and E). Whereas actin rings were not observed in cdc3-124, actin rings were observed in mitotic wild-type, myo2-S1, and cdc3-124 myo2-S1 (Figure 1D). The rings in myo2-S1 were more diffuse compared with those in wild type consistent with a role for Myo2 in AMR assembly. These experiments established that compromise of Myo2 function led to suppression of the defective AMR assembly, septum assembly, and colony formation in cdc3-124 (Figure 1E). The partial suppression of cdc8-S1 by myo2-S1 (Figure 1C) suggested that myo2-S1 suppression is not specific to cdc3-124.

To investigate the mechanism of suppression of cdc3-124 by myo2-S1, as well as to characterize the phenotypic consequences of myo2-S1, we generated wild-type, cdc3-124, myo2-S1, and cdc3-124 myo2-S1 expressing Rlc1-3GFP (AMR marker) and mCherry-Atb2 (mitotic spindle marker). At 24°C, AMR assembly and contraction were indistinguishable in wild-type and cdc3-124 myo2-S1 (Figure 1D). We tested if the observed suppression was specific to Cdc8 profilin defects or if myo2-S1 also suppressed defects in cdc12 and cdc8. To this end, we generated cdc8-S1 myo2-S1 and cdc12-112 myo2-S1. We found that cdc8-S1, but not cdc12-112, was suppressed at 34°C and tiny “pin-prick” colonies were observed even at 36°C (Figure 1C).

We prepared cell ghosts from wild-type, myo2-S1, and cdc3-124 myo2-S1 with CF633-phalloidin and DAPI to observe the actin cytoskeleton and nuclei (Figure 1, D and E). Whereas actin rings were not observed in cdc3-124, actin rings were observed in mitotic wild-type, myo2-S1, and cdc3-124 myo2-S1 (Figure 1D). The rings in myo2-S1 were more diffuse compared with those in wild type consistent with a role for Myo2 in AMR assembly. These experiments established that compromise of Myo2 function led to suppression of the defective AMR assembly, septum assembly, and colony formation in cdc3-124 (Figure 1E). The partial suppression of cdc8-S1 by myo2-S1 (Figure 1C) suggested that myo2-S1 suppression is not specific to cdc3-124.

To investigate the mechanism of suppression of cdc3-124 by myo2-S1, as well as to characterize the phenotypic consequences of myo2-S1, we generated wild-type, cdc3-124, myo2-S1, and cdc3-124 myo2-S1 expressing Rlc1-3GFP (AMR marker) and mCherry-Atb2 (mitotic spindle marker). At 24°C, AMR assembly and contraction were indistinguishable in wild-type and cdc3-124 cells, whereas both processes were slightly slower in myo2-S1 and cdc3-124 myo2-S1 cells (Supplemental Figure S1, A and B). Ring assembly in wild type at 34°C took 17 ± 6.8 min and ring contraction took 18.7 ± 2.1 min (Figure 2, B and C). As expected, AMRs did not assemble in cdc3-124 cells, although Rlc1-3GFP accumulated first in nodes and then in multiple spots and bar-like structures (Figure 2A, class II). myo2-S1 showed a range of phenotypes pertaining to cytokinesis. First, in ~50% of the cells, ring assembly was delayed and took ~1.8 times longer (30.2 ± 4 min) than in wild-type cells and fully compacted rings were observed only in late mitotic cells (Figure 2, A, class I and class III, and B). Second, the remaining ~50% of the cells made abnormal actomyosin structures that did not compact into a ring (Figure 2A, class IV). Ring contraction was uniformly slower in cells that appeared to have normal rings (38.3 ± 6.9 min) (Figure 2C). Furthermore, ring contraction was asymmetric in nearly two-thirds of cells that appeared to have a normal looking ring (Figure 2A, class III). cdc3-124 myo2-S1 largely resembled myo2-S1 and rings of normal appearance were detected in ~42% of the cells and these rings took 26.2 ± 1.9 min to assemble, while the rest of the cells assembled Rlc1-3GFP bundles that did not compact into a ring (Figure 2, A and B). Ring contraction in cdc3-124 myo2-S1 cells was almost always asymmetric and the process took ~three times the time taken in wild type (56.3 ± 26.8 min) (Figure 2, A and C). These experiments established that myo2-S1 was compromised for both known physiological roles of Myo2.

**ATP-dependent contraction is slowed in AMRs isolated from myo2-S1**

The mutated myosin allele, Myo2-S1, possesses a point mutation resulting in the replacement of Gly515 with an Asp in the L50 subdomain of Myo2 motor domain. G515D is located at the beginning of HR helix at the end of the activation loop (Varkuti et al., 2012). This glycine might play a role in the conformational flexibility of the activation loop, thereby contributing to the loop’s orientation that facilitates actin binding. To understand the effect of the mutation, we analyzed the position equivalent to G515 in various myosin structures. In the cryoEM structure of myosin bound to actin filaments (5JLH), G515 was observed to be at the beginning of the activation loop that interacted with N-terminus of an actin monomer (Figure 3A). Modeling of the G515D mutation on myosin motor domain structure (1VOM) showed a salt bridge between the mutated Asp515 and the Arg509 residues (data not shown). Arg520 in Caenorhabditis elegans muscle myosin, corresponding to Lys510 in S. pombe Myo2, has been shown to be crucial for binding with the N-terminus of actin (Varkuti et al., 2012). Furthermore, Arg520 mutation affected the ATPase activity of myosin indicating that Arg520 interaction with actin is essential for motor domain function (Varkuti et al., 2012). In a sequence alignment of 237 nonredundant myosin sequences, glycine is highly conserved and positively charged residue (at least one arginine or lysine) is a conserved feature of the activation loop (Figure 3B). Unavailability or misorientation of the positively charged side chain (K to R) to interact with actin in the G515D mutant (Myo2-S1) might weaken its interaction with actin, leading to abnormalities in ring assembly. Myo2-S1 may also affect the motor activity in the defective rings during contraction due to the absence of ATPase stimulation on actin interaction.

Given these considerations based on structural analysis of myo2-S1, we tested the biochemical properties of Myo2-S1, especially as it pertains with actin binding or motor activity. We were unable to purify this protein in sufficient quantities for biochemical studies. As an alternative, we used the permeabilized spheroplast assay, in which AMRs contract in an ATP- and Myosin II-dependent manner (Mishra et al., 2013). We prepared cell ghosts from wild-type, myo2-S1, and cdc3-124 myo2-S1 cells expressing Rlc1-3GFP that were grown at 24°C. We carried out two sets of experiments, one at 24°C (Supplemental Figure S2A) and the other at 34°C (Figure 4A, Supplemental Figure S2B, and Supplemental Movie S1). AMRs in wild-type ghosts contracted rapidly on ATP addition at 24°C (Supplemental Figure S2A and Figure 4C). However, AMRs became extremely unstable on introduction of the myo2-S1 mutation, suggesting that Myo2-S1 may interact weakly with actin causing the ring to be unstable (Supplemental Figure S2A). We therefore stabilized AMR by incubation of cell ghosts with the actin-stabilizer Jasplakinolide (Jasp) (Holzinger, 2009). We next treated Jasp-stabilized AMR in cell ghosts with ATP. As observed in the absence of Jasp, rings in wild-type ghosts contracted rapidly at 34°C (Figure 4, A and B and Supplemental Movie S1). However, even though AMRs were stably maintained in myo2-S1 and cdc3-124 myo2-S1, they either failed to contract or were very slow to contract (Figure 4, A and B, and Supplemental Movie S1). A similar trend was observed when
FIGURE 2: AMR assembly and contraction is partially restored in myo2-S1 and cdc3-124 myo2-S1 at 34°C. (A) Time-lapse series of strains of the indicated genotypes expressing Rlc1-3GFP and mCherry-Atb2. Cells were grown at 24°C and shifted 3 h at 34°C before being imaged at 34°C. More than 20 cells were imaged and quantified for each strain. On the side of each montage it is indicated the percentage of the different cytokinetic behaviors categorized into class I (normal AMR), class II (failed AMR assembly), class III (normal AMR assembly and asymmetrical AMR contraction), and class IV (abnormal AMR structures). Images shown are maximum-intensity projections of Z-stacks. Time indicated in minutes. Scale bars 3 µm. (B) Quantification of the time necessary for AMR assembly of class I and class III cells imaged in A. Statistical significance: Student’s test (****p < 0.0001). Error bars represent SD. (C) Quantification of the time necessary for AMR contraction of class I and class III cells imaged in A. Statistical significance: Student’s test (****p < 0.0001). Error bars represent SD.
Jasp-stabilized myo2-S1, and cdc3-124 myo2-S1 ghosts were incubated with ATP even at 24°C (Figure 4C), similar to previous observations with myo2-E1 ghosts (Mishra et al., 2013; Palani et al., 2017). In addition, we found that Jasp-stabilized AMRs in cdc3-124 myo2-S2 ghosts also underwent very slow ATP-dependent contraction at 34°C (Supplemental Figure S2, B and C) and at 24°C (Supplemental Figure S2D). These observations established that loss of Myo2 II motor activity strongly correlated with the suppression of AMR assembly/stability defects in cdc3-124.

**myo2-S1 suppresses direct pharmacological perturbation of the actin cytoskeleton**

Previous work has shown that Myosin II can break actin filaments in vitro both by stretching or by buckling (Murrell and Gardel, 2012; Vogel et al., 2013) due to its motor activity. We have shown that Myosin II mutants defective in ATP-dependent contraction suppress defects caused by partial loss of key proteins contributing to the assembly/stability of actin filaments in the AMR. We reasoned that shorter or unstable actin filaments may be protected for longer periods in the cdc3-124 myo2-S1, cdc8-110 myo2-S1, and cdc3-124 myo2-S2 mutants, contributing to the observed suppression. We therefore tested if direct pharmacological perturbation of actin cables and AMRs by treatment with low doses of LatA (Ayscough et al., 1997) can be reversed by compromise of Myo2.

Previously, we have shown that treatment of wild-type cells with low doses of LatA (~0.25 μM) leads to cytokinesis defects (Mishra et al., 2004). We plated serial dilutions of wild type, cdc3-124, myo2-S1, and cdc3-124 myo2-S1 with a series of doses of LatA and incubated these at 30 and 34°C (Figure 5A). Wild-type cells were capable of robust colony formation up to 0.25 μM LatA, but did not form colonies at 0.375 μM LatA (Figure 5A). Consistent with a role for Cdc3-profilin in actin filament assembly in the cytokinetic ring, cdc3-124 mutants were hypersensitive to 0.25 μM LatA. Interestingly, myo2-S1 was able to form colonies up to 0.375 μM LatA (Figure 5A), establishing that compromise of Myo2 function reverses the deleterious effects caused by actin perturbation. cdc3-124 myo2-S1 also formed colonies at 30°C at 0.375 μM LatA, although at 34°C, this strain only grew on plates containing 0.125 μM LatA (Figure 5A). The inability of the cdc3-124 to form colonies at and above 0.25 μM LatA potentially reflects an enhanced actin cytoskeletal defect due to the additive effect of loss of Cdc3 and LatA. CF633-Phalloidin staining of liquid cultures of the four strains showed that actin cables, rings, and patches were present at 0 time point in all four strains (Figure 5B and C). After a 3-h incubation in 0.375 μM LatA, actin patches were observed in all four strains. However, actin cables/rings were not detected in wild type or cdc3-124 but were clearly detected in myo2-S1 and cdc3-124 myo2-S1 (Figure 5, B and C). It is unclear if the observed cablelike structures in myo2-S1 and cdc3-124 myo2-S1 were interphase cables or improperly organized AMRs. Notwithstanding, collectively, these experiments established that partial genetic or pharmacological perturbation of formin-generated actin structures can be suppressed by motor activity-defective Myo2.

In this study we provide evidence for a role for Myosin II in actin filament disassembly through the use of genetic and in vitro analyses and structural modeling. Previous work has shown that single actin filaments and networks of actin filaments are broken and disassembled by Myosin II through buckling (Murrell and Gardel, 2012; Vogel et al., 2013). Previous work in *Schizosaccharomyces japonicus* has shown that actin filaments in isolated cytokinetic rings break into clusters in a Myosin II-dependent manner (Chew et al., 2017). Our work may provide in vivo evidence in fission yeast for a role of Myosin II in actin filament disassembly and turnover, consistent with the work in mammalian cells (Murthy and Wadsworth, 2005; Wilson et al., 2010). It is possible that formin-generated actin filaments are short and unstable in profilin and tropomyosin mutants and on exposure to low doses of LatA. In this case, compromise of Myosin II, which normally destabilizes actin filaments, rescues the instability of actin filaments and, in turn, the cytokinesis defects. It is possible that in the SCPR mechanism of cytokinesis (Vavylonis et al., 2008), actin filaments at the division site are broken by both cofillin and Myosin II and that our work provides evidence for Myosin II-based breakage of actin filaments at the division site.

**FIGURE 3:** Structural basis of *S. pombe* Myo2-S1 (G515D) mutation. (A) Gly515 in *S. pombe* Myo2 (equivalent to Gly549 of human myosin 14 in the actomyosin complex structure of PDB ID: 5JLH) is positioned at the boundary of the activation loop at the myosin-actin interface. Inset shows the zoomed view of the interaction between the activation loop and the N-terminus of actin protomer. Gly549 and Arg543, corresponding to Gly515 and Lys510 of *S. pombe* Myo2, are shown in stick representation. The figure was generated using PyMol. (B) Conservation of Gly515 and Lys510 depicted on a sequence logo representation. The figure was generated using WebLogo (Crooks et al., 2004) using a sequence alignment of 237 nonredundant myosin sequences aligned using ClustalO (Sievers et al., 2009).
MATERIALS AND METHODS

Yeast genetics and culture methods
Cells were grown and cultured at 24°C in yeast extract medium (YES) as described previously (Moreno et al., 1991). The presence of designated mutations on each used strain has been verified by PCR and DNA sequencing. Yeast strains used in the study are listed in Supplemental Table S1.

PFA fixation and fluorescence microscopy
S. pombe cells, growing at 24°C in YES medium, were either fixed in mid-log phases or shifted to 34°C for 3–4 h before fixation. Cells were fixed in a 4% paraformaldehyde solution for 12 min at room temperature and, after two washes with 1× phosphate-buffered saline (PBS), permeabilized with 1% Triton X-100 for 10 min. Cells were washed twice with 1× PBS and stained with either DAPI (4′,6-diamidino-2-phenylindole, from Life Technologies) for the visualization of the nucleus or CF633-Phalloidin (CF633, from Biotium) to visualize actin structures. To visualize the septa, fixed S. pombe cells were directly incubated with CW (Sigma-Aldrich). Still images were acquired using a spinning disk confocal microscope (Andor Revolution XD imaging system, equipped with a 100× oil immersion 1.45 NA Nikon Plan Apo lambda, and a confocal unit Yokogawa CSU-X1, EMCCD detector [Andor iXON] and Andor iQ acquisition software). Cells were imaged in a CellASIC microfluidic yeast plates (Y04C and D size), where 15 Z-stacks of 0.5-μm thickness images were acquired at 1-min intervals for Rlc1-3GFP (myosin regulatory light chain, used as AMR marker) and mCherry-Atb2 (alpha tubulin 2, used as cell cycle marker). PRISM 6.0 software (GraphPad) was used for quantification and the statistical significance was determined using Student’s t test (**p < 0.0001).

Isolation of AMRs and ATP-dependent contraction
AMRs were isolated as described previously (Mishra et al., 2013; Huang et al., 2016). Time-lapse imaging of Rlc1-3GFP was acquired as described before in CellASIC microfluidic yeast plates (Y04D), where 21 Z-stacks of 0.5-μm thickness images were acquired while treating the cells with 0.5 mM ATP, either at 24°C or after shifting 15 min the isolated ring at 34°C. Fiji imaging software was used to process the acquired images, obtaining the maximum intensity projection of the Z-stacks.

Drug treatments in cells and isolated AMRs
YES agar plates in Figure 5A were prepared by the addition of different concentrations of LatA (Enzo Life Sciences), where cells were successively being spotted at 10-fold serial dilutions. For the treatment in Figure 5B, cells were grown in liquid YES medium at 24°C and successively shifted at 34°C in the presence of 0.375 μM LatA in the medium for 3 h before fixation. Isolated AMRs were
treated, when indicated, with Jasp (Enzo Life Sciences) by the addition of the drug to the isolated rings at the final concentration of 20 μM.

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