Counting actin in contractile rings reveals novel contributions of cofillin and type II myosins to fission yeast cytokinesis

Mamata Malla*, Thomas D. Pollardbc,d, and Qian Chenab,*
department of Biological Sciences, The University of Toledo, Toledo, OH 43606; Departments of Molecular Cellular and Developmental Biology, Molecular Biophysics and Biochemistry, and Cell Biology, Yale University, New Haven, CT 06520-8103

Abstract Cytokinesis by animals, fungi, and amoebas depends on actomyosin contractile rings, which are stabilized by continuous turnover of actin filaments. Remarkably little is known about the amount of polymerized actin in contractile rings, so we used low concentrations of GFP-Lifeact to count total polymerized actin molecules in the contractile rings of live fission yeast cells. Contractile rings of wild-type cells accumulated polymerized actin molecules at 4900/min to a peak number of ~198,000 followed by a loss of actin at 5400/min throughout ring constriction. In adfl-M3 mutant cells with cofillin that severs actin filaments poorly, contractile rings accumulated polymerized actin at twice the normal rate and eventually had almost twofold more actin along with a proportional increase in type II myosins Myo2, Myp2, and formin Cdc12. Although 30% of adfl-M3 mutant cells failed to constrict their rings fully, the rest lost actin from the rings at the wild-type rates. Mutations of type II myosins Myo2 and Myp2 reduced contractile ring actin filaments by half and slowed the rate of actin loss from the rings.

Introduction Cytokinesis separates daughter cells during the last stage of the cell cycle. Amoebas, fungi, and animal cells assemble an actomyosin ring to provide force to form a cleavage furrow (for review see Pollard and O’Shaughnessy, 2019). Contractile rings consist of actin filaments, actin-binding proteins including α-actinin, capping protein, cofillin, formins, and type II myosins (Wu et al., 2003). In fission yeast, two type II myosins contribute about equally to the rate of ring constriction (Bezanilla et al., 2000; Laplante et al., 2015). Myo2 is essential for viability, while the unconventional myosin-II called Myp2 is not.

Although actin is the most abundant protein in contractile rings, much less is known about its dynamics than that of the myosins or actin-binding proteins owing to difficulty in tracking actin in live cells. Fluorescent phalloidin is widely used to stain actin in fixed cells, but this provides only a snapshot. SIR-actin, jasplakinolide conjugated to silicon rhodamine, can stain actin filaments in live cells (Lukinavicius et al., 2014), but jasplakinolide will alter actin dynamics. Microinjection of fluorescently labeled actin is an option for some animal cells (Cao and Wang, 1990a) but has not been exploited for quantitative measurements and may not be feasible for some cells including fungi. Unfortunately, the genetically encoded fluorescent tags tested to date compromise the function of actin during cytokinesis. For example, the formins that nucleate and elongate actin filaments for the contractile ring in fission yeast filter out all the actin fused to either fluorescent proteins such as GFP or small tetracysteine peptide tags (Wu and Pollard, 2005; Chen et al., 2012).

Consequently, only indirect labeling of actin filaments in the contractile ring has been successful. Although less versatile than direct labeling, we measured about 190,000 actin molecules, equal to 500 μm of actin filaments, in the fission yeast contractile ring by titration with Lifeact (Courtemanche et al., 2016), a small peptide that binds actin filaments (Riedl et al., 2008). We also estimated the average length of the filaments from the ratio of polymerized actin to formin...
Here we expanded our previous study (Courtemanche et al., 2016) to count polymerized actin in contractile rings of wild-type fission yeast and strains with mutations in cofilin, formin, and type II myosins. The cofilin mutant *adf1-M3* with half of wild-type severing activity had strong effects on both contractile ring assembly and disassembly. The contractile rings of the cofilin mutant cells have about twice the normal numbers of actin, formin Cdc12, Myo2, and Myp2 molecules, while contractile rings of cells with a hypomorphic mutation of formin Cdc12 accumulated actin slowly. Remarkably, mutations of either type II myosin, Myo2 or Myp2, reduced contractile ring actin by half.

**RESULTS**

**Measurement of actin turnover in contractile rings using GFP-Lifeact**

To measure the number of polymerized actin molecules in contractile rings of live fission yeast over time, we expressed GFP-Lifeact (GFP-LA) constitutively from the endogenous *leu1* locus, driven by a constitutive *Padf1* promoter of the endogenous cofilin gene. All yeast strains used in this study expressed GFP-LA at similar levels (Supplemental Figure S1), which saturates only 6% of polymerized actin molecules and avoids artifacts during cytokinesis and endocytosis caused by higher concentrations of GFP-Lifeact (Courtemanche et al., 2016). Using a calibrated fluorescence microscope (Wu and Pollard, 2005), we converted the fluorescence intensity of GFP-Lifeact into the numbers of actin molecules in subcellular structures (Courtemanche et al., 2016). Additionally, we expressed from the endogenous *Rlc1*, the regulatory light chain for both Myo2 and Myp2, tagged with tdTomato (Rlc1-tdTomato) and used its fluorescence to segment the contractile ring and measure the total GFP-Lifeact fluorescence.

Actin accumulated in contractile rings, about half during assembly and about half during 18 min of maturation when the rate was constant at ~4900 molecules/min (Figure 1, A and B, and Table 1). At the end of the maturation phase, just before cleavage furrow ingression, fully assembled contractile rings contained ~198,000 polymerized actin molecules, consistent with previous measurements (Courtemanche et al., 2016). During contractile ring constriction the number of polymerized actin molecules was constant for the first 6 min, which was not clear in our previous analysis (Courtemanche et al., 2016), and then declined steadily at ~5400 molecules/min for ~20 min (Figure 1C). About 50,000 actin molecules remained in the contractile ring remnant at the end of constriction, which has not been reported previously. These filaments abruptly dispersed within 2 min, together with Rlc1-tdTomato, leaving behind actin patches along both sides of the cleavage furrow (Figure 1A).

**Contractile ring assembly and composition in the cofilin hypomorphic mutant *adf1-M3***

Cofilin is essential for viability, so we used the hypomorphic mutant *adf1-M3* to test the role of cofilin in the dynamics of contractile ring actin filaments. The *adf1-M3* mutation reduces the severing activity of cofilin by more than 50% and slows contractile ring assembly (Chen and Pollard, 2011). Actin patches and actin filament bundles stained brighter with Bodipy-phallacidin in fixed *adf1-M3* mutant cells than in wild-type cells (Chen and Pollard, 2011). However, at that time we lacked quantitative probes to measure polymerized...
actin in live cells (Chen et al., 2012). The cytokinesis defects were less severe in the adf1-M3 strain than in adf1-M2 (Chen and Pollard, 2011), allowing us to analyze the assembly and disassembly of larger numbers of mature rings.

Contractile ring assembly in adf1-M3 mutant cells was less orderly (Figure 2B) and much more variable than in wild-type cells (Figure 2, B–D). Contractile rings of adf1-M3 mutant cells accumulated actin twice as fast over a similar period of time as wild-type cells (Table 2). Therefore, mature rings of the mutant had on average about 1.9 times as much actin as wild-type cells (Table 1 and Figure 2A, arrows). The peak number of molecules (100,000–600,000) was much more variable in adf1-M3 mutant cells than in wild-type cells (Figure 2D). On average, the contractile rings of adf1-M3 mutant cells had enough actin molecules to assemble ∼950 μm of filaments (Table 2). A cross-section of such rings would contain ∼75 filaments in a bundle ∼160 nm wide, if the spacing between the actin filaments is 15 nm as in wild-type cells, which have ∼50 filaments in a bundle ∼125 nm wide (Courtemanche et al., 2016; Swulius et al., 2018). We conclude that severing by cofilin limits and ensures reliable assembly of actin filaments in the contractile ring.

To estimate the lengths of actin filaments in the contractile ring of adf1-M3 mutant cells, we measured the number of formins in fully assembled contractile rings just before they constricted. Assuming that barbed ends of all contractile ring actin filaments are associated with a formin (Coffman et al., 2013), the ratio of total actin to total formin molecules gives the average length of actin filaments, averaging ∼1.5 μm in wild-type cells (Table 2), similar to the previous estimate (Courtemanche et al., 2016).

The number of Cdc12-3GFP molecules in the contractile rings of adf1-M3 mutant cells was on average about twice that of wild-type cells and much more variable (Figure 3C), while the number of formin For3 was the same as in wild-type cells (Figure 3D). As a result, the combined number of the two formin molecules was ∼50% higher in the contractile rings of the mutant cells (Table 2). Consequently, the ratio of actin to formins was ∼25% higher, translating to an average length of 1.9 μm in the mutant cells (Table 2).

The numbers of type II myosins (Myo2 and Myp2) in mature contractile rings of wild-type cells peaked at ∼7000 myosin molecules (Figure 3, A and B, and Table 2), consistent with previous measurements (Wu and Pollard, 2005; Goss et al., 2014). This translates to one myosin motor domain for every 76 nm of actin filament and ∼20 motor domains for every actin filament in the contractile ring.

Contractile rings of adf1-M3 mutant cells that were able to contract had twice as many myosin molecules as the wild-type cells, translating to one myosin motor domain for every 70 nm of filament (Figure 3, A and B, and Table 2). We conclude that reduced severing by cofilin leads to contractile rings with twice the normal numbers of actin, Cdc12, and type II myosin molecules, so the ratios of these three proteins are about the same as in wild-type cells.

**Effects of a formin mutation on contractile ring assembly**

Measurements of the time course of actin accumulation in the contractile rings of the cdc12-4A formin mutant cells identified defects in actin that were not appreciated in previous work (Bohnert et al., 2013). The essential formin Cdc12 is required for assembly of actin filaments in contractile rings (Chang et al., 1996; Kovar et al., 2003). The hypomorphic cdc12-4A mutation prevents the phosphorylation of the formin by the essential SIN pathway kinase Sid2 (Bohnert et al., 2013). In prior work the mutant cells appeared to assemble normal contractile rings, but our quantitative measurements revealed that the cdc12-4A mutation reduced by about half both the rate of accumulation and the peak numbers of polymerized actin in the ring (Figure 2E and Table 1).

**Contractile ring disassembly during constriction in adf1-M3 mutant cells**

The contractile rings in 30% of adf1-M3 mutant cells (n = 65) either failed to constrict or halted constriction prematurely (Supplemental Figure S2), but those that constricted fully did so at an average rate similar to that of wild-type cells although with much more variability (Chen and Pollard, 2011). During ring constriction, the absolute number of actin molecules in the contractile rings of adf1-M3 cells declined linearly at ∼5800/min (Figure 4, A and B, and Table 1), almost identical to the rate in wild-type cells. However, the cofilin mutant cells had two defects. First, the large standard deviations showed that rate of actin disassembly varied much more in adf1-M3 cells than in wild-type cells. Second, the normalized disassembly rate, which took the number of actin molecules in the ring into consideration, was 40% lower in the mutant than in wild-type cells. Thus, normal severing by cofilin is not essential for the disassembly of actin filaments in constricting contractile rings but makes the process much more orderly.

Wild-type cells retain both Myo2 (Figure 4C) and Myp2 (Figure 4D) in the contractile ring through the first 20 min of constriction before they leave during the last 10 min of the constriction as observed earlier (Wu and Pollard, 2005). In contrast, these myosins persisted at nearly their highest levels for 1 h, and the time course of the process was much more variable in the adf1-M3 mutant cells (Figure 4, C and D). In a few cofilin mutant cells, the myosins dwelled at the cell division site for more than 10 min after the completion of the ring constriction (Figure 5A). Myp2 oscillated as clusters along the contractile ring of the mutant cells, which was rarely observed in the wild-type cells (Figure 5A).

**TABLE 1: Summary of the actin assembly and disassembly in the contractile ring.**

| Genotype | Number of actin molecules in the mature ring (x10³)a | Number of cells measureda | Net assembly rate (actin molecules x 10³ min⁻¹)b | Net disassembly rate (actin molecules x 10³ min⁻¹)b | Normalized disassembly rate (percent min⁻¹)c |
|----------|-----------------------------------------------------|---------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Wild type | 198 ± 42                                            | 80                        | +4.9                                          | −5.4                                          | −2.7                                          |
| adf1-M3  | 362 ± 176                                           | 21                        | +10.0                                         | −5.8                                          | −1.6                                          |
| cdc12-4A | 93 ± 23                                             | 38                        | +2.4                                          | N.M.                                         | N.M.                                          |
| myo2-E1  | 82 ± 28                                             | 38                        | N.M.                                          | −1.6                                          | −2.0                                          |
| myp2A    | 88 ± 21                                             | 33                        | N.M.                                          | −2.3                                          | −2.6                                          |

aThe cells were pooled from at least two independent biological repeats.

bBased on the best fits of linear regression. R² > 0.90.

N.M.: Not measured.
**TABLE 2:** Comparison of the architecture of the contractile ring between wild type and the cofilin mutant.

| Genotype  | Estimated assembly time of actin (min) | Total filament length in the mature ring (µm) | Number of formin dimers in the mature ring | Number of type II myosin motor domains in the mature ring | Average filament length in the mature ring (µm) |
|-----------|----------------------------------------|-----------------------------------------------|-------------------------------------------|-------------------------------------------------|-----------------------------------------------|
| Wild-type | 43                                     | 530                                           | −350                                      | −7000                                           | 1.5                                          |
| adf1-M3   | 39                                     | 975                                           | −520                                      | −14,000                                         | 1.9                                          |

*aBased on the best fit of linear regression. Time = Number of actin molecules/net assembly rate.
*bBased on the peak numbers of formins in the contractile ring (Figure 3, C and D).
*cBased on the peak numbers of type II myosins in the contractile ring (Figure 3, A and B).
*dDerived from division of the total filament length by the number of formin, For3 plus Cdc12, dimers.

**FIGURE 2:** Contractile ring assembly in cofilin mutant adf1-M3 and formin mutant cdc12-4A. (A) Fluorescence micrographs of the wild-type (left) and adf1-M3 (right) cells. Both expressed GFP-Lifeact (pseudo colored) and Rlc1-ttdTomato. Arrowheads: contractile rings at the stage of 1) assembly, 2) maturation, and 3) constriction. Bar represents the fluorescence intensity scale. (B) Time series of micrographs of a cofilin mutant cell expressing both GFP-Lifeact (top) and Rlc1-ttdTomato (middle). A merged time-lapse series is shown at the bottom. (C) Average time course of actin molecules accumulating in the contractile rings of adf1-M3 cells (n = 21). Red symbols are mean values; the line represents best linear fit ($R^2 > 0.9$); the cloud represents SDs. The line for WT is from Figure 1B. (D) Average number of actin molecules in mature contractile rings. Measurements were taken just before the start of the ring constriction. (E) Average time course of actin assembly in the contractile rings of cdc12-4A cells. The line is the best linear fit ($R^2 > 0.9$). The line for WT cells is from Figure 1B. The slope of cdc12-4A is significantly smaller than that of the wild type ($P < 0.005$).
Face views of constricting contractile rings of *adf1-M3* cells revealed linear structures containing both myosin-II isoforms and actin filaments that separated from 60% (*n* = 18) of the rings (Figure 5, B and C). Similar structures were observed previously in three-dimensional reconstructions of cofilin mutants *adf1-M2* and -M3 (Chen and Pollard, 2011; Cheffings et al., 2019). Although the structures containing myosin-II retracted back to the contractile ring, the bundles of actin filaments did not (Figure 5C, arrowheads). Such shedding of actin filaments was not observed in the wild-type cells. We conclude that contractile rings are far less stable in cofilin mutant cells than in wild-type cells.

Influence of type II myosins on assembly and disassembly of contractile ring actin filaments

Mutations of either type II myosin gene in the *myo2-E1* or *mp2Δ* strains reduced the numbers of actin molecules in contractile rings by more than half compared with wild-type cells at the end of the maturation period and the onset of constriction (Figure 6, A and B, and Table 1). This surprising finding was missed previously.

Starting with less than the normal amount of polymerized actin, contractile rings constricted slower in both *myo2-E1* (0.32 µm/min) and *mp2Δ* (0.30 µm/min) cells than in wild-type cells (0.36 µm/min).
These rates are slightly higher than in earlier studies (Laplante et al., 2015; Zambon et al., 2017). After normalization for the initial actin content, the rates that actin left constricting rings were only slightly less than normal in the myo2-E1 mutant and similar in the myp2Δ strain to that in wild-type cells (Table 1).

To rule out the possibility that the lower disassembly rates in the myosin mutants were indirectly tied to ring assembly defects, we measured the loss of actin from contractile rings of wild-type cells treated with blebbistatin to inhibit myosin-II (Straight et al., 2003). Contractile rings in wild-type cells either disintegrated or failed to assemble in blebbistatin concentrations of ≥20 µM (Figure 6D). Treating wild-type cells with mature contractile rings with 10 µM blebbistatin decreased the rate of actin disassembly by 60% (Figure 6C). The ring constriction rate was also lower by 30% (n = 16) (Figure 6D). We conclude that type II myosins contribute to both the assembly and disassembly of actin filaments in contractile rings.

DISCUSSION

Knowing the numbers and dynamics of polymerized actin molecules in the actomyosin contractile ring is essential for understanding the mechanism of cytokinesis, but such measurements have not been made due to the technical challenge of labeling actin directly without disrupting its activity (Wu and Pollard, 2005; Chen et al., 2012). Overexpression of indirect probes can produce artifacts, so we measured polymerized actin in a low concentration of GFP-Lifeact that does not perturb endocytosis or cytokinesis (Courtemanche et al., 2016). Measurements on beautiful electron microscopy-tomograms (Swulius et al., 2018) confirmed our earlier count of contractile ring actin with GFP-Lifeact (Courtemanche et al., 2016).

Our method provides valuable, new quantitative data on the accumulation and loss of polymerized actin in contractile rings but does not reveal the behavior, including the turnover, of individual filaments. Experiments with a probe directly on actin molecules are required to probe the underlying mechanisms.

Our measurements revealed several aspects of cytokinesis that were overlooked due to the lack of quantitative data on actin in contractile rings, including the phenotypes of yeast strains with mutations in cofilin, myosin, and formin genes. The protein products of each of these genes are essential for cytokinesis, and their roles are likely to have been conserved during evolution.

The role of cofilin in the assembly and composition of contractile rings

A mutation that reduces the severing activity of cofilin has a remarkable impact on the molecular composition of the contractile ring: about twice the wild-type numbers of polymerized actin, Myo2, Myp2, and formin Cdc12. First, we consider myosins and then formins and actin for discussion.

Myosins. Myo2 and formin Cdc12 are components of cytokinesis nodes that form before the assembly of actin filaments (Wu and Pollard, 2005), so extra numbers of myosins in the contractile rings of adf1-M3 cells implies proportionally more cytokinesis nodes. In fact, the nodes are larger in adf1-M3 mutant cells than in wild-type cells (Chen and Pollard, 2011) and likely represent clusters of larger numbers of small, unitary nodes as revealed in wild-type cells by super-resolution microscopy (Laplante et al., 2016). We do not know when these extra nodes form or how either reduced severing or more polymerized actin induce their formation.

On the other hand, Myp2 is recruited to fully formed contractile rings during the maturation period in a process that depends on actin filaments (Wu et al., 2003; Takaine et al., 2015; Okada et al., 2019). Thus, the higher numbers of Myp2 molecules in the contractile ring of adf1-M3 cells may follow directly from the high content of actin filaments.
Formins and actin. The essential formin Cdc12 nucleates and elongates actin filaments in the contractile ring (Chang et al., 1996; Kovar et al., 2003), so more formin Cdc12 in contractile rings of adf1-M3 cells likely contributes to the rapid accumulation of extra actin filaments, and the actin monomer concentration in the cytoplasm sets the rate of growth of individual filaments. On the other hand, no connection between slow severing and excess formin is known.

The longer actin filaments in the contractile rings of adf1-M3 cells are expected for cells with low actin filament severing activity (Chen and Pollard, 2011) and given evidence that cofilin stochastically severs actin filaments connecting the precursor nodes (Chen and Pollard, 2011). Longer filaments may also explain our observation of a negative genetic interaction between cofilin and α-actinin (ain1) mutants (Chen and Pollard, 2011).

Because actin filaments accumulate faster in the contractile rings of adf1-M3 mutant cells, some other mechanism must account for the slow assembly of full contractile rings (Chen and Pollard, 2011). The most likely mechanism is that slow severing results in the coalescence of the nodes into an organized contractile ring of actin oligomers (Chen and Pollard, 2011).

In contrast to the essential formin Cdc12, adf1-M3 cells do not accumulate excess formin For3, which is not a component of cytokinesis nodes and joins fully assembled contractile rings at a later stage and assembles peripheral actin bundles at the cell division plane (Coffman et al., 2013). Unlike Cdc12, its recruitment likely depends on the type V myosins and polarized membrane secretion (Coffman et al., 2013).

The role of cofilin in contractile ring constriction and disassembly

Multiple defects appear during constriction of contractile rings of adf1-M3 cells. First, constriction is much less uniform than in wild-type cells. About 30% of the rings fail to complete the constriction. Bundles of actin filaments containing myosins peel from the rings of mutant cells. Second, starting with contractile rings containing much more polymerized actin and both type II myosins, the cofilin mutant cells required more time for all three proteins to leave the rings in a highly variable manner. Third, type II myosin Myp2 oscillates along the contractile rings as clusters. Nevertheless, the adf1-M3 mutation does not significantly reduce the rate of net loss of actin filaments from contractile rings as they constrict.

The most likely explanation for the variability of contractile ring constriction in the adf1-M3 cells is that the loss of severing activity...
compromises the continuous, relatively rapid (tens of seconds) turnover of contractile ring components, which is required to maintain orderly force production in computer simulations of constriction (Stachowiak et al., 2014). Those simulations assume estimates of continuous protein turnover revealed by photobleaching experiments. Dialing down the turnover of polymerized actin, Cdc12 and Myo2 in these simulations, resulted in the loss of tension in less than 3 min. Cofilin contributes to this turnover by severing actin filaments, while other uncharacterized processes cause the exchange of Cdc12 and Myo2 with cytoplasmic pools.

Role of formins in the assembly of the contractile ring
Counting polymerized actin revealed unrecognized defects caused by the cdc12-4A mutation. This mutation prevents phosphorylation of Cdc12 by the SIN pathway kinase Sid2 but does not cause any discernible cytokinetic defects (Bohnert et al., 2013). Although cytokinesis appears normal, contractile rings in cdc12-4A mutant cells accumulate actin slower and in less than half the number of wild-type cells. This previously overlooked actin assembly defect may explain the hypersensitivity of this strain to latrunculin A as well as the genetic interaction of the cdc12-4A mutation with many other cytokinetic mutants (Bohnert et al., 2013).

Roles of type II myosins in the assembly of the contractile ring
Our experiments revealed to our surprise that mutations of type II myosins strongly reduce the actin content of contractile rings. This remarkable, unexpected finding had been missed in dozens of studies on these mutant strains due to the lack of methods to measure actin in live cells. During ring assembly, Myo2 in a given node pulls on actin filaments growing from nearby nodes (Vavylonis et al., 2008). In a reconstituted system, force on a filament growing from formin Cdc12 slows its elongation (Zimmermann et al., 2017). However, the loss of Myo2 activity in the myo2-E1 mutant has the opposite effect, resulting in more actin filaments, so the mechanism reducing the actin content is not clear. Myp2 is not present in contractile rings until after they form, so it must have its effect on the half of the actin that assembles during the maturation period, which is slower in the myp2Δ mutant than in wild-type cells.

In animal cells, the cortical flow of actin filaments toward the cleavage furrow contributes preformed actin filaments to the contractile ring (White and Borisy, 1983; Cao and Wang, 1990b; Khalilullin et al., 2018). Nonmuscle type II myosins drive this flow by compressing the equatorial cortex (Khalilullin et al., 2018). In comparison, fission yeast do not have an actin filament cortex, so formin Cdc12 associated with cytokinesis nodes assembles most of the contractile ring actin filaments de novo. Additional actin filaments in bundles formed by formin Cdc12 are also pulled into the ring from nonequatorial regions of dividing cells by Myo2 and Myp51 (Huang et al., 2012). Therefore, our finding that the accumulation of actin filaments in the contractile ring depends on myosins exhibits some parallels with the cortical flow process in animal cells.

The role of type II myosins in constriction and disassembly of the contractile ring
Contractile rings with half the normal number of actin filaments constrain 10% slower in the myo2-E1 strain and 20% slower in the mpy2Δ strain than in wild-type cells (Laplante et al., 2015). Disassembly of actin filaments is slower in both myosin mutants than in wild-type cells, although these rates are the same as in wild type when normalized for the starting actin content. Previously, the defects in ring constriction in the strains with myosin-II mutations were attributed entirely to loss of function of the myosins (Laplante et al., 2015; Zambon et al., 2017). However, these strains have a secondary defect, a loss of about half the normal polymerized actin. This insight emphasizes the importance of quantitative measurements of other contractile ring components in mutant strains.

Inhibition of myosins with 10 μM blebbistatin reduced both the disassembly rate of actin filaments and the ring constriction rate by ~60% and 30%, respectively. In addition to cofilin-mediated severing, forces produced by myosins may contribute to actin filament turnover during contractile ring constriction. The idea that such mechanical stress contributes to depolymerization/severing of actin filaments in contractile rings originated in experiments on sea urchin and Caenorhabditis elegans embryos (Schoeder, 1972; White and Borisy, 1983) and was supported by later work on animal cells in culture, where blebbistatin caused contractile ring actin filaments to turn over more slowly and persist longer than normal (Guha et al., 2005; Murthy and Wadsworth, 2005). Further studies of cofilin mutants are essential to determine whether severing of actin filaments contributes to the disassembly of the contractile ring in animal cell cytokinesis.

Collectively, our experiments demonstrate the value of measurements of polymerized actin in live cells. These measurements provided new, unanticipated features of contractile ring assembly and constriction, which will motivate future studies to characterize mechanisms.

MATERIALS AND METHODS
Request a protocol through Bio-protocol.

Yeast genetics
We followed the standard protocols for yeast cell culture and genetics. Tetradss were dissected using a SporePlay+ dissection microscope (Singer, UK). Supplemental Table S1 lists all the strains used in this study.

Microscopy
For microscopy, exponentially growing yeast cells (1 ml) at 25°C with a density between 5.0 × 10⁶/ml and 1.0 × 10⁷/ml in YEsS (yeast extract with 5 supplements) liquid media were harvested by centrifugation at 4000 rpm for 1 min and resuspended in 50 μl YEsS. Owing to slow growth of the adf1-M3 cells at 25°C, they were inoculated at 30°C for 1 d before being transferred to 25°C for inoculation of at least 12 h. Resuspended cells (6 μl) were applied to a 25% gelatin pad in YEs5 and sealed under coverslip with VALAP (a mix of an equal amount of Vaseline, lanolin, and paraffin) (Wang et al., 2016).

Live cell microscopy was carried out on a calibrated Olympus IX71 microscope equipped with two objective lenses (Olympus; Plan Apochromat, 100 × [NA = 1.41] and 60 × [NA = 1.40]), a confocal spinning-disk unit (CSU-X1; Yokogawa, Japan), and a motorized XY stage with a Piezo Z Top plate (ASI). The images were captured on an iXon-897 EMCCD camera controlled by iQ3.0 (Andor, Ireland). Solid-state lasers of 488 and 561 nm were used. In all experiments, the cells were imaged for 3 h with 2-min intervals. A Z-series of 15 slices at a step size of 0.5 μm was captured at each time point. The exceptions were measurement of the fluorescence of Cdc12-3GFP and For3-3GFP, for which Z-series of eight slices with a spacing of 1 μm were captured. Live cell microscopy was conducted in a room where the temperature was maintained at 22 ± 2°C. To minimize the experimental variations between the control and experimental groups, we imaged them with randomized order within 1 wk.

To obtain a face-on view of the contractile ring, yeast cells were imaged in a Petri dish with a coverslip at the bottom. Exponentially
ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award numbers R01GM026132 to T.D.P. and R15GM134496 to Q.C. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We thank Debatrayee Sinha and Abhishek Poddar for technical assistance.

REFERENCES

Bezanilla M, Wilson JM, Pollard TD (2000). Fission yeast myosin-II isoforms assemble into contractile rings at distinct times during mitosis. Curr Biol 10, 397–400.

Bolhert KA, Grzegorzekwa PA, Willet AH, Vander Kooi CW, Kovar DR, Gould KL (2013). SIN-dependent phosphoinhibition of formin multimerization controls fission yeast cytokinesis. Genes Dev 27, 2164–2177.

Cao LG, Wang YL (1990a). Mechanism of the formation of contractile ring in dividing cultured animal cells. I. Recruitment of preexisting actin filaments into the cleavage furrow. J Cell Biol 110, 1089–1095.

Cao LG, Wang YL (1990b). Mechanism of the formation of contractile ring in dividing cultured animal cells. II. Cortical movement of microinjected actin filaments. J Cell Biol 111, 1905–1911.

Chang F, Woolard A, Nurse P (1996). Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring. J Cell Sci 110 (Pt 1), 131–142.

Chellings TH, Burroughs NJ, Balasubramanian MK (2019). Actin turnover ensures uniform tension distribution during cytokinetic actomyosin ring contraction. Mol Biol Cell 30, 933–941.

Chen Q, Nag S, Pollard TD (2012). Formins filter modified actin subunits during prothetic elongation. J Struct Biol 177, 32–39.

Chen Q, Pollard TD (2011). Actin filament severing by coflin is more important for assembly than constriction of the cytokinetic contractile ring. J Cell Biol 195, 485–498.

Chen Q, Pollard TD (2013). Actin filament severing by coflin dismantles actin patches and promotes mother filaments for new patches. Curr Biol 23, 1154–1162.

Coffman VC, Sees JA, Kovar DR, Wu JQ (2013). The formins Cdc12 and For3 cooperate during contractile ring assembly in cytokinesis. J Cell Biol 203, 101–114.

Courtemanche N, Pollard TD, Chen Q (2016). Avoiding artefacts when counting polymerized actin in live cells with LifeAct fused to fluorescent proteins. Nat Cell Biol 18, 674–683.

Ghosh M, Song X, Mouneimne G, Sidani M, Lawrence DS, Condeelis JS (2004). Cofilin promotes actin polymerization and defines the direction of cell motility. Science 304, 743–746.

Goss JW, Kim S, Bledsoe H, Pollard TD (2014). Characterization of the roles of Bir1p in fission yeast cytokinesis. Mol Biol Cell 25, 1946–1957.

Guha M, Zhou M, Wang YL (2005). Cortical actin turnover during cytokinesis requires myosin II. Curr Biol 15, 732–736.

Huang J, Huang Y, Hu, Subramanian AD, Thadan R, Tao Y, Tang X, Wedlich-Soldner R, Balasubramanian MK (2012). Nonmedially assembled F-actin cables incorporate into the actomyosin ring in fission yeast. J Cell Biol 199, 831–847.

Kanbe T, Kobayashi I, Tanaka K (1989). Dynamics of cytoplasmic organelles in the cell cycle of the fission yeast Schizosaccharomyces pombe: three-dimensional reconstruction from serial sections. J Cell Sci 94 (Pt 4), 647–656.

Khaliullin RN, Green RA, Shi LZ, Gomez-Cavazos JS, Berns MW, Desai A, Oegema K (2018). A positive-feedback-based mechanism for constrict rate acceleration during cytokinesis in Caenorhabditis elegans. eLife 7, e3673.

Koroch A, Weber I, Simmeh E, Hacker U, Maniak M, Muller-Taubenberger A (1999). DAip1, a Dictostelium homologue of the yeast actin-interacting protein 1, is involved in endocytosis, cytokinesis, and motility. J Cell Biol 146, 453–464.

Kovar DR, Kuhn JR, Tichy AL, Pollard TD (2003). The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin. J Cell Biol 161, 875–887.

Laplante C, Berro J, Karatekin E, Hernandez-Leyva A, Lee R, Pollard TD (2015). Myosin contributions uniquely to the assembly and constriction of the fission yeast cytokinetic contractile ring. Curr Biol 25, 1955–1965.

Laplante C, Huang F, Tebbs IR, Bewersdorf J, Pollard TD (2016). Molecular organization of cytokinesis nodes and contractile rings by super-resolution fluorescence microscopy of live fission yeast. Proc Natl Acad Sci USA 113, E5876–E5885.

Luknavicius G, Reymond L, D’Este E, Masharina A, Gottfert F, Ta H, Guther A, Fournier M, Rizzo S, Waldmann H, et al. (2014). Fluorogenic

Volume 33 May 15, 2022 Polymerized actin in contractile rings | 9
probes for live-cell imaging of the cytoskeleton. Nat Methods 11, 731–733.
Morris Z, Sinha D, Poddar A, Morris B, Chen Q (2019). Fission yeast TRP channel Pkd2p localizes to the cleavage furrow and regulates cell separation during cytokinesis. Mol Biol Cell 30, 1791–1804.
Murthy K, Wadsworth P (2005). Myosin-II-dependent localization and dynamics of F-actin during cytokinesis. Curr Biol 15, 724–731.
Nakano K, Mabuchi I (2006). Actin-capping protein is involved in controlling organization of actin cytoskeleton together with ADF/cofilin, profilin and F-actin crosslinking proteins in fission yeast. Genes Cells 11, 893–905.
Okada H, Włoka C, Wu JQ, Bi E (2019). Distinct roles of myosin-II isoforms in cytokinesis under normal and stressed conditions. iScience 14, 69–87.
Okreglak V, Drubin DG (2007). Cofilin recruitment and function during actin-mediated endocytosis dictated by actin nucleotide state. J Cell Biol 178, 1251–1264.
Pollard TD, O'Shaughnessy B (2019). Molecular mechanism of cytokinesis. Annu Rev Biochem 88, 661–689.
Riedl J, Crevenna AH, Kessenbrock K, Yu JH, Neukirchen D, Bista M, Bradke F, Jenne D, Holak TA, Werb Z, et al. (2008). Lifeact: a versatile marker to visualize F-actin. Nat Methods 5, 605–607.
Schroeder TE (1972). The contractile ring. II. Determining its brief existence, volumetric changes, and vital role in cleaving Arbacia eggs. J Cell Biol 53, 419–434.
Stachowiak MR, Laplante C, Chin HF, Guirao B, Karatekin E, Pollard TD, O’Shaughnessy B (2014). Mechanism of cytokinetic contractile ring constriction in fission yeast. Dev Cell 29, 547–561.
Zambon P, Palani S, Kamnev A, Balasubramanian MK (2017). Myo2p is the major motor involved in actomyosin ring contraction in fission yeast. Mol Biol Cell 27, R99–R100.
Zhang XF, Hyland C, Van Goor D, Foscher P (2012). Calcineurin-dependent cofilin activation and increased retrograde actin flow drive S-HT-dependent neurite outgrowth in Aplysia bag cell neurons. Mol Biol Cell 23, 4833–4848.