Counting actin in contractile rings reveals novel contributions of cofilin and type II

myosins to fission yeast cytokinesis

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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 concentration 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 4,900/min to a

peak number of ~198,000 followed by a loss of actin at 5,400/min throughout ring constriction.

In *adf1-M3* mutant cells with cofilin that severs actin filaments poorly, contractile rings

accumulated polymerized actin at twice the normal rate and eventually had almost two-fold more

actin along with a proportional increase in type II myosins Myo2, Myp2 and formin Cdc12.

Although 30% of *adf1-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 alpha-actinin, capping protein, cofilin, 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 the myosins or actin-binding proteins owing to difficulty 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, 1990) 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 (Chen et al., 2012; Wu and Pollard, 2005).

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 molecules in the contractile ring. The filaments start at about 1.4 µm and shorten gradually during the ring constriction. Despite this progress, little is known about how actin filaments themselves turn over in contractile rings.

Cofilins promote actin turnover during endocytosis in yeast (Chen and Pollard, 2013; Okreglak and Drubin, 2007), at the leading edge of motile cells (Ghosh et al., 2004; Konzok et al., 1999) and in neurite growth cones (Zhang et al., 2012). Cofilin is required for the assembly of the cytokinetic contractile ring in fission yeast (Chen and Pollard, 2011; Nakano and Mabuchi, 2006). Cofilin mutations that reduce severing activity slowed or prevented the assembly of

contractile rings, because the precursors to the contractile ring, called cytokinetic nodes, are

pulled into large heterogeneous clusters around the equator rather than being organized into a

contractile ring. Rings that form slowly from these clusters constrict far more variably than those

in wild-type cells (Chen and Pollard, 2011).

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 (Fig. 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).

Specifically, we expressed from the endogenous locus Rlc1, the regulatory light chain for both

Myo2 and Myp2, tagged with 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 mins of maturation when the rate was constant at ~4,900 molecules/min (Fig. 1A-B and Table

1). At the end of the maturation phase, just before cleavage furrow ingression, fully assembled

contractile rings contained ~200,000 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 six minutes, which was not clear in our previous analysis (Courtemanche et

al., 2016), and then declined steadily at ~5,400 molecules/min for ~20 minutes (Fig. 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 two minutes,

together with the myosin regulatory light chain Rlc1 leaving behind actin patches along both

sides of the cleavage furrow (Fig. 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 the *adf1-*

M2 strain (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 (Fig. 2B) and much

more variable than in wild type cells (Fig. 2B-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 Fig. 2A, arrows). The peak number of molecules (100,000 to 600,000) was

much more variable in *adf1-M3* mutant cells than wild-type cells (Fig. 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 like wild-type cells, which have

~50 filaments in a bundle of ~125 nm wide (Courtemanche et al., 2016; Swulius et al., 2018).

We conclude that severing by cofilin limits and makes more reliable the 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 (Fig. 3C), while the

number of formin For3 was the same as wild-type cells (Fig. 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 ~7,000 myosin molecules (Fig. 3A-B and Table 2), consistent with previous

measurements (Goss et al., 2014; Wu and Pollard, 2005). 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 constrict had twice as many

myosin molecules as the wild-type cells, translating to one myosin motor domain for every 70

nm of filament (Fig. 3A-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 (Table 2).

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 revealed 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 final numbers of polymerized actin (Fig. 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 (Fig. S2), but those that constricted fully did so at an average rate similar to 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 ~5,800/min (Fig. 4A and B, Table 1), almost identical to 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 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 (Fig. 4C) and Myp2 (Fig. 4D) in the contractile ring through the first 20 minutes of constriction before they leave during the last 10 minutes of the constriction as observed earlier (Wu and Pollard, 2005). In contrast, these myosins persisted at nearly their highest levels for an hour and the time course of the process was much more variable in the *adf1-M3* mutant cells (Fig. 4C and D). In a few cofilin mutant cells, the myosins dwelled at the cell division for more than 10 minutes after the completion of the ring constriction (Fig. 5A). Myp2 oscillated as clusters along the contractile ring of the mutant cells, which was rarely observed in the wild-type cells (Fig. 5A).

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 (Fig. 5B and C). Similar structures were observed previously in 3D-reconstructions of

cofilin mutants *adf1-M2* and *-M3* (Cheffings et al., 2019; Chen and Pollard, 2011). Although the

structures containing myosin-II retracted back to the contractile ring, the bundles of actin

filaments did not (Fig. 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 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 *myp2Δ* 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 (Fig. 6A-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 *myp2Δ* (0.30 µm/min) cells than wild-

type cells (0.36 µm/min). These rates are slightly higher than 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 wild-type cells (Table 2).

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 (Fig. 6D). Treating wild-type cells with mature contractile rings with 10 µM

blebbistatin decreased the rate of actin disassembly by 60% (Fig. 6C). The ring constriction rate

was also lower by 30% (n = 16) (Fig. 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 (Chen et al., 2012; Wu and Pollard, 2005). Indirect probes can

produce artifacts, so we measured polymerized actin with a low concentration of GFP-Lifeact

that does not disturb endocytosis or cytokinesis (Courtemanche et al., 2016). Measurements on

beautiful EM-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 will be 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 prior to

the assembly of actin filaments (Wu and Pollard, 2005), so extra numbers of both proteins in the

contractile rings of *adf1-M3* cells implies proportionally more cytokinesis nodes. In fact, the

nodes were larger in *adf1-M3* mutant cells than 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 (Okada et al., 2019; Takaine et al.,

2015; Wu et al., 2003). Thus, the higher numbers of Myp2 molecules in 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 *ain1* mutants (Chen and Pollard, 2011).

Since 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 aggregation of

nodes that delays 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 myosin 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 fashion. 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 minutes. 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 discernable 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

hyper-sensitivity of this strain to Latrunculin A (Bohnert et al., 2013) as well as the genetic

interaction of the *cdc12-4A* mutation with many other cytokinetic mutants.

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 wild-type cells.

The role of type II myosins in constriction and disassembly of the contractile ring

Contractile rings with half the normal amount of actin filaments constrict 10% slower in the *myo2-E1* strain and 20% slower in the *myp2Δ* strain than wild-type cells (Laplante et al., 2015). Disassembly of actin filaments is slower in both myosin mutants than wild-type cells, although these rates are the same as wild-type when normalized for the starting actin content. Previously, the defects in ring constriction in the strains with myosin-II mutations was 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.

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

Yeast genetics

We followed the standard protocols for yeast cell culture and genetics. Tetrads were dissected using a SporePlay+ dissection microscope (Singer, UK). Table 3 lists all the strains used in this study.

Microscopy

For microscopy, 1 ml of exponentially growing yeast cells at 25°C with a density between 5.0 ×

10^6/ml and 1.0×10^7/ml in YE5s liquid media (unless specified), were harvested by

centrifugation at 4000 rpm for 1 min and resuspended in 50 µl YE5s. Due to the extremely slow

growth of the *adf1-M3* cells at 25°C, they were first inoculated at 30°C for a day from the plate,

before being inoculated at 25°C for 12 h. 6 µL of re-suspended cells were applied to a 25%

gelatin + YE5s pad and sealed under the coverslip with VALAP (a mix of an equal amounts of

Vaseline, Lanolin, and paraffin) (Wang et al., 2016). Live cell microscopy was carried out on an

Olympus IX71 microscope equipped with both a 100× (NA = 1.41) and a 60× (NA = 1.40)

objective lenses, a confocal spinning-disk unit (CSU-X1; Yokogawa, Japan), 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 nm (5% for 100ms except

for For3-3GFP: 7.5% for 150 ms) and 561 nm (5% for 50 ms) were used in the confocal

fluorescence microscope. In all experiments, the cells were imaged for 3 h (2-minutes interval

unless specified) by acquiring a Z-series of 15 slices at a step size of 0.5 µm using the 100×

objective (unless specified). Live cell microscopy was conducted in a room where the

temperature was maintained at around 22 ± 2°C. To minimize the variations in culture and

microscopy conditions, we imaged both control and experimental groups with randomized order

within a week. To measure the relative fluorescence of Cdc12-3GFP and For3-3GFP, a Z-series

of 8 slices with a spacing of 1 µm was used.

     To image the contractile ring head-on, cofilin mutant cells were observed in a petri dish

with a coverslip bottom. A sample of 20 µl of the cell culture was spotted onto a glass coverslip

(#1.5) at the bottom of a 10-mm petri dish (Cellvis, USA). The coverslip was precoated with 50

338   µl of 50 µg/ml lectin (Sigma, L2380) and allowed to dry overnight at 4°C. The cells were

339   allowed to attach to the lectin for 10 minutes at room temperature. YE5s medium (2 ml) was then

340   added to the dish just before microscopy. Images were acquired at 1-minute intervals.

Drug treatment

342   A sample of 20 µl of the cell culture was spotted onto a glass coverslip on the bottom of a petri

343   dish as described above. YE5s medium (2 ml) with either 10 µM Blebbistatin or 1% DMSO was

344   added to the dish before starting microscopy using the 60x objective. Image acquisition was

345   started exactly after 15 min of the drug treatment to minimize experimental variability.

Image processing and analysis

We used ImageJ (National Institutes of Health) to process all the images, with either freely

available or customized macros/plug-ins. For quantitative analysis, the fluorescence micrographs

were corrected for X-Y drifting using the StackReg plug-in (Thevenaz et al., 1998). Average

intensity projections of Z-slices were used for actin number measurements. Maximum intensity

projections of Z-slices were used for the contractile ring tracking. Kymographs of the rings

labeled with Rlc1-tdTomato were generated to determine both the start and end of ring closure.

The customized plug-in Ring Intensities Measurement-2015 was used to measure the GFP-LA

fluorescence during the constriction of rings that went on to complete constriction

(Courtemanche et al., 2016). The background fluorescence was calculated separately for each

cell used for GFP-LA measurement. The corrected GFP-LA fluorescence was then converted to

number of actin molecules based on the calibration as described (Morris et al., 2019).

The number of actin filaments in a cross-section of the contractile ring was calculated by

dividing total length of actin filaments with the circumference of a cell. Total length of actin

filaments (µm) is calculated as total number of actin molecules divided by 370. The

circumference of wild-type and *adf1-M3* cells was estimated at 10 and 13 µm respectively (Chen

and Pollard, 2011). The center-to-center spacing between actin filaments is assumed to be 15 nm

(Kanbe et al., 1989; Swulius et al., 2018) in estimating the width of a contractile ring.

Western Blots

For probing the expression level of GFP-Lifeact, 50 ml of exponentially growing cells were

collected by centrifuging at 3000g for 5 min. The pellet was resuspended in 300 µL of lysis

buffer containing Halt^{TM} protease inhibitor cocktail (#1862209, Thermo-Fisher Scientific) and

frozen at -20 ⁰C until lysis. The cells were lysed using BeadBug microtube homogenizer

(Benchmark Scientific). Proteins were resolved using Mini-PROTEAN TGX^{TM} Precast SDS-

PAGE gels (#4561084, BioRad). The gels were either stained with Coomassie blue or transferred

to PVDF membrane (Millipore) for immunoblots. Membranes were blotted with 1:1000 dilution

of primary anti-GFP (#11814460001, Sigma-Roche) over night, followed by 1:5000 dilution of

HRP-linked secondary antibody (#sc-516102, Santa Cruz) for 2 h. The blot was developed using

SuperSignal^{TM} West Pico PLUS chemiluminescent substrate (#34577, Thermo-Fisher Scientific).

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Table 1: Summary of the actin assembly and disassembly in the contractile ring

Genotype	Number of actin molecules in the mature ring $(x10^{-3})$*	Number of cells measured*	Net assembly rate (actin molecules $x10^{-3}$ min^{-1})[#]	Net disassembly rate (actin molecules $x10^{-3}$ min^{-1}) [#]	Normalized disassembly rate (min^{-1}) [&]
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%
myp2Δ	88 ± 21	33	N.M.	-2.3	-2.6%

382 *: The cells were pooled from at least two independent biological repeats.

383 [#]: Based on the best fits of linear regression. $R^2 > 0.90$.

384 [&]: Equals to the net disassembly rate divided by the total number of actin molecules in the ring.

385 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 (mins)*	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)
WT	43	530	~350	~7,000	1.5
adf1-M3	39	975	~520	~14,000	1.9

*: Based on the best fit of linear regression. Time = Number of actin molecules/net assembly rate.

392 **Table 3 Yeast strains**

Strains	Genotype	Source
QC-Y601	*h+ leu2::kanMX-Pcof1-mEGFP-Lifeact rlc1-tdTomato-Nat*	Lab stock
QC-Y620	*h+ leu2::kanMX-Pcof1-mEGFP-Lifeact rlc1-tdTomato-Nat adf1::KanMX6-Padf1-adf1-M3*	Lab stock
QC-Y1164	*h? leu2::kanMX-Pcof1-mEGFP-Lifeact rlc1-tdTomato-Nat Myo2-E1*	This study
QC-Y1185	*h? leu2::kanMX-Pcof1-mEGFP-Lifeact rlc1-tdTomato-Nat cdc12-4A:kanR ade6-M21X leu1-32 ura4-D18*	This study
QC-Y1196	*h? leu2::kanMX-Pcof1-mEGFP-Lifeact rlc1-tdTomato-Nat myp2::kanMX6*	This study
JW766	*h+ kanMX6-Pmyo2-GFP-myo2 ade6-M210 leu1-32 ura4-D18*	Lab stock (Jian-Qiu Wu)
QC-Y129	*h+ kanMX6-Pmyo2-GFP-myo2 leu1-32 ura4-D18 adf1M3A-KanMX6*	Lab stock
KV344	*h? cdc12-3XGFP::kanMX6 leu1-32 his3-D1 ura4-D18 ade6-M216*	Lab stock (David Kovar)
QC-Y142	*h+ cdc12-3XGFP::kanMX6 leu1-32 his3-D1 ura4-D18 ade6-M216 adf1M3-KanMX6 leu1-32 ura4-D18 ade6-M216*	Lab stock
QC-Y267	*h+ for3-3GFP-ura4+ ade6-M21 leu1-32 ura4-D18*	Lab stock
QC-Y1355	*h? adf1M3A-KanMX6 leu1-32 ura4-D18 his3-D1 ade6-M216 for3-3GFP-ura4+ ade6-M21 leu1-32 ura4-D18*	This study
QC-Y519	*h- myp2-GFP-kanMX6 ade6-M210 leu1-32 ura4-D18*	Lab stock
QC-Y1371	*h? adf1M3A-KanMX6 leu1-32 ura4-D18 his3-D1 ade6-M216 myp2-mEGFP-KanMX6 ade6-M210 leu1-32 ura4-D18*	This study

Figures and Figure legends

Figure 1

Figure 1. Counting the numbers of actin molecules in the contractile ring of fission yeast using GFP-Lifeact.

(A) Assembly and constriction of the contractile ring documented by time-lapse micrographs of a cell expressing both GFP-Lifeact (green) and Rlc1-tdTomato (red).

(B-C) Average time course of the number of actin molecules in the contractile rings during **(B)** their assembly and maturation and **(C)** constriction in wild-type cells. Clouds represent standard deviations. Time zero is defined as the start of the ring constriction. Red line represents the best linear fit. $R^2 > 0.9$.

Figure 2

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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* cells. Both expressed GFP-
Lifeact (fire colored) and Rlc1-tdTomato. Arrowheads: contractile rings at the stages of (1)
assembly, (2) maturation and (3) constriction.
(**B**) Time series of micrographs (pseudo-colored) of a cofilin mutant cell during cytokinesis.
(**C**) 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 fit; the cloud represents standard
deviations. The line for *WT* cells is from Fig. 1B.
(**D**) Average number of actin molecules in mature contractile rings. Measurements were taken
just before the start of the ring constriction.

(E) Time course of actin assembly in the contractile rings of formin *cdc12-4A* mutants. The line

for *WT* cells is from Fig. 1B. The slope of the *cdc12-4A* mutant cells is significantly smaller than

that of the wild-type ($P < 0.005$).

Figure 3

Figure 3. Architecture of the contractile ring in cofilin mutant cells.

(A-B) Numbers of type II myosins, **(A)** Myo2 and **(B)** Myp2 in contractile rings of wild-type and

adf1-M3 cells.

(C-D) The fluorescence intensities of two formins, **(C)** Cdc12-3GFP and **(D)** For3-3GFP in

contractile rings of wild-type and *adf1-M3* cells. ***: $P < 0.001$. N.S.: $P > 0.05$. Values were

calculated from two-tailed student t-tests.

Figure 4

Figure 4. Time courses of the numbers of actin and type II myosins in constricting contractile rings of WT and *adf1-M3* cells. Time zero is the onset of constriction.

(**A**) Time-series of micrographs of a constricting contractile ring in an *adf1-M3* mutant cell expressing GFP-Lifeact (top) and Rlc1-tdTomato (bottom, red). Interval = 2 min.

(**B**) Time course of the loss of actin molecules from contractile rings as they constricted. Red symbols and line are mean values; the cloud shows standard deviations.

(**C**) Time course of the numbers of GFP-Myo2 molecules in constricting contractile rings of WT and *adf1-M3* cells (n> 25). Red symbols and line are mean values; the cloud shows standard deviations, which are large in the mutant cells.

(**D**) Time course of the numbers of Myp2-GFP molecules in constricting contractile rings of WT and *adf1-M3* cells (n > 10). The thick lines are mean values.

Figure 5. Disassembly of myosins in the contractile ring of the cofilin mutant.

(**A**) Time series of micrographs of contractile rings in a wild-type and an *adf1-M3* cells

expressing Myp2-GFP. Dot: Start of ring constriction. Asterisk: Completion of ring constriction.

(**B**) Micrographs of attached *adf1-M3* cells in a petri dish. The spots around the contractile ring

are actin patches.

(**C**) Time series of micrographs of the contractile ring in a cofilin mutant cell. Arrowhead:

shedding actin filaments (upper panel) and myosin (lower panel).

Figure 6

Figure 6. Disassembly of contractile ring actin filaments in type II myosin mutants at the

permissive temperature of 22°C.

(A-B) Time courses of actin loss from constricting contractile rings of **(A)** *myo2-E1* and **(B)**

myp2Δ mutant cells. Dark red circles are mean values; red lines are the best linear fits; the cloud

shows standard deviations.

(C) Time courses of GFP-Lifeact fluorescence in contractile rings of the cells treated with either

10 μM blebbistatin or 1% DMSO (control). The slopes are -5489/min (DMSO) and

-2389/min (Blebbistatin) respectively. Only cells with a mature contractile ring before the

treatment were included. Circles are mean values; solid lines are the best linear fits; the cloud

shows standard deviations.

462   **(D)** Time series of micrographs of contractile rings marked by Rlc1-tdTomato in three wild-type

463   cells treated with either DMSO (top), or 10 (middle) or 20 µM (bottom) of blebbistatin. The

464   number in the time series of 20 µM blebbistatin-treated cell represents time in minutes.

Figure S1

Supplemental Figure S1: Expression of GFP-Lifeact. Top: Anti-GFP blot of the whole cell

lysate from wild-type, formin mutant and myosin mutants (left) and wild-type and cofilin mutant

cells (right). Bottom: Coomassie blue-stained SDS-PAGE gel of the whole cell lysate as loading

control.

Figure S2

Interval = 2'

Supplemental Figure S2: The contractile rings that failed to constrict in the cofilin mutant
cells. Time-series micrographs of two *adf1-M3* cells expressing Rlc1-tdTomato. Asterisk: start of
the contractile ring constriction. Arrowhead: fragmentation of the ring. Bracket: enlarging gap in
the contractile ring. Such fragmenting rings represent 30% of the contractile rings among the
cofilin mutant.

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