Myosin concentration underlies cell size–dependent scalability of actomyosin ring constriction

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In eukaryotes, cytokinesis is accomplished by an actomyosin-based contractile ring. Although in Caenorhabditis elegans embryos larger cells divide at a faster rate than smaller cells, it remains unknown whether a similar mode of scalability operates in other cells. We investigated cytokinesis in the filamentous fungus Neurospora crassa, which exhibits a wide range of hyphal circumferences. We found that N. crassa cells divide using an actomyosin ring and larger rings constricted faster than smaller rings. However, unlike in C. elegans, the total amount of myosin remained constant throughout constriction, and there was a size-dependent increase in the starting concentration of myosin in the ring. We predict that the increased number of ring-associated myosin motors in larger rings leads to the increased constriction rate. Accordingly, reduction or inhibition of ring-associated myosin slows down the rate of constriction. Because the mechanical characteristics of contractile rings are conserved, we predict that these findings will be relevant to actomyosin ring constriction in other cell types.

Introduction

Throughout biology there are examples of cellular mechanisms regulated by cell size. Size dependence governs cell cycle signaling, as in the initiation of mitosis in the fission yeast Schizosaccharomyces pombe (Nurse, 1975) and also structural processes of cell division, such as the length and rate of spindle elongation in Caenorhabditis elegans (Hara and Kimura, 2009). The rate of cytokinesis was recently determined to depend on cell size in C. elegans embryos (Carvalho et al., 2009). In these cells, the duration of ring closure was constant and independent of starting size, implying that the contractile rings of larger cells must constrict at a faster rate than those of smaller cells. This size-dependent variation in the rate of constriction was scalable, such that a cell with twice the perimeter constricted at twice the rate. This resulted in the total duration of cytokinesis remaining uniform between subsequent embryonic divisions (Carvalho et al., 2009).

In the majority of eukaryotes cytokinesis is accomplished by the constriction of a cortical actomyosin-based ring (Balasubramanian et al., 2004; Barr and Gruneberg, 2007; Stark et al., 2010). It is established that F-actin and a number of actin-binding proteins form the structural framework of the ring (Maupin and Pollard, 1986; Pelham and Chang, 2002; Field et al., 2005). Myosin II motors associate with the actin filaments at the ring and are thought to provide the predominant contractile force by regulating actin turnover and disassembly (Mabuchi and Okuno, 1977; Guha et al., 2005; Murthy and Wadsworth, 2005). Though well conserved, the precise architecture of these interactions and the mechanism by which myosin II promotes actin turnover and contractility during cytokinesis has yet to be fully elucidated.

To determine if scalability of the rate of cytokinesis is also a property of contractile actomyosin rings in fungi, we investigated septum formation in the filamentous fungus, N. crassa. In this organism a wide range of hyphal diameters are observed within a single colony, and septum formation is preceded by the assembly and constriction of an actin ring (Rasmussen and Glass, 2005). We performed live-cell imaging of F-actin and myosin II during septation and found that both the rate and the total...
components. Homologues of the majority of ring components were found to be present. Thus, the structural features of the contractile actomyosin ring are likely to be well conserved within this organism (Table I).

Septation in N. crassa is regulated by type II myosin

Type II myosin motors drive actomyosin ring constriction during cytokinesis in most eukaryotic organisms (Mabuchi and Okuno, 1977; Maupin and Pollard, 1986; De Lozanne and Spudich, 1987; Knecht and Loomis, 1988; Manstein et al., 1989; May et al., 1997; Shelton et al., 1999). By in silico genomic analysis we determined that only one type II myosin gene is encoded in the genome of N. crassa, referred to hereafter as myo2. Upon further characterization, the N. crassa myo2 gene was found to span approximately 7.3 kb, and be comprised of three coding exons interrupted by two short introns and followed by a 301 bp 3′UTR (Fig. 2 A). Determination of the complete coding sequence of myo2 revealed that it encodes a protein of 2,418 amino acids consisting of an N-terminal SH3 domain and myosin head (motor domain) and a C-terminal myosin tail (Fig. 2 A). Sequence comparison showed the region of highest homology among eukaryotes is within the motor domain (56% identity and 39% identity with myosin heavy chain from S. pombe and H. sapiens, respectively). To determine the gene function, we examined septation in a myo2-deleted strain. In wild-type cells septa were observed within all hyphae with an approximate frequency of two septa within 50 µm from branch sites (see arrows in Fig.1 and Fig. 2 B). By contrast, a sublethal myo2-deleted heterokaryon strain was completely lacking in observable septa (Fig. 2 C), suggesting that septation is defective when myosin II function is compromised. To observe the localization and dynamics of myosin II within the division apparatus during septation in N. crassa, we constructed a strain expressing myosin II as a GFP fusion protein under control of its native promoter at its genomic location. Myo2-GFP appeared as a diffuse cortical band of 1–2 µm before septation (Fig. 2 D; 0 min) and then compacted into a dense ring structure at the onset of ring constriction. As constriction proceeded, Myo2-GFP remained tightly associated with the contractile apparatus until the completion duration of cytokinesis increased with increasing cell size. We examined the dynamics of the ring components both during ring assembly and throughout constriction and determined that the amount of myosin incorporated into the assembling ring also increases with ring size. Here, we demonstrate that the rate of ring constriction is mediated by both the amount and the activity of ring-associated myosin, and that myosin is therefore a likely determinant of the size-dependent scalability of ring constriction.

Results

N. crassa is an attractive model for studies of scalability

To investigate the potentially size-dependent scalability of the rate of cytokinesis within nonembryonic cells, we sought a model system in which cellular diameter varied significantly among wild-type cells. Primary and secondary hyphae in N. crassa exhibit large differences in diameter, and within a single colony we observed up to a fourfold range in hyphal diameter (Fig. 1). Given this intrinsic variation in size, its genetic tractability, and the ease of live-cell confocal imaging, we concluded that N. crassa is an attractive model for studying the scalability of the rate of cytokinesis.

Identification of conserved actomyosin ring components in N. crassa

In N. crassa, septation occurs at frequent intervals along the length of the hyphae, and actin has been shown to localize to the sites of septation (Rasmussen and Glass, 2005; Berepiki et al., 2010). In another filamentous fungus, Aspergillus nidulans, homologues of a number of well-known regulators of cytokinesis have been characterized, including α-actinin (Bruno et al., 2001; Wang et al., 2009) and the formin, SepA (Harris et al., 1997). Though actin rings are known to be involved in septation in N. crassa, other components of the contractile ring have not been well characterized in this organism. To determine whether proteins important for actomyosin ring function and cytokinesis in higher eukaryotes are conserved in N. crassa, a BLAST search was performed within the genome (Galagan et al., 2003) to identify conserved actomyosin ring components. Homologues of the majority of ring components were found to be present. Thus, the structural features of the contractile actomyosin ring are likely to be well conserved within this organism (Table I).

Figure 1. Range of hyphal diameters observed in N. crassa. White arrows indicate positions and diameters of septa. All images are to the same scale. Bar, 10 µm.
throughout constriction (Fig. 2 E). Observation of labeled membrane using the vital dye FM4-64 (Hickey et al., 2005) and the localization of Myo2-GFP revealed that the ring of myosin II stays at the leading edge of nascent membrane formation during septation (Fig. 2 D and Video 1). Observation of labeled membrane using the vital dye FM4-64 (Hickey et al., 2005) and the localization of Myo2-GFP revealed that the ring of myosin II stays at the leading edge of nascent membrane formation throughout constriction (Fig. 2 E).

F-actin assembles into a constricting ring at the septation site in N. crassa

We then observed the dynamics of F-actin during septation in N. crassa using genomically integrated Lifeact-GFP, a probe shown to bind to actin cables and contractile rings in N. crassa, budding yeast, and mammalian fibroblasts (Riedl et al., 2008; Berepiki et al., 2010). Before the initiation of ring constriction, Lifeact-GFP accumulated along a 9–28-µm region of the cortex (Fig. 3 A; 1 min) and then rapidly assembled into a tight ring. After ring assembly, the ring constricted steadily until septation was complete. Lifeact-GFP remained associated with the ring throughout constriction (Fig. 3 A and Video 2). The behavior of both F-actin and myosin II during septation and the lack of septation in cells defective in myosin II establish that N. crassa cytokinesis relies upon the function of an actomyosin-based contractile ring.

Ring constriction is followed by invagination of the plasma membrane and cell wall deposition and occurs at a constant rate

Using cell wall and membrane labels (calcofluor and FM4-64, respectively) in conjunction with the Lifeact-GFP actin marker, we observed that ring constriction was tightly coupled with plasma membrane invagination and nascent cell wall deposition at the cleavage site (Fig. 3 B and Video 3). As was observed with myosin II, actin occupied the leading edge of the contractile ring, and new membrane deposition followed centripetally behind the closing ring. Analysis of kymographs and graphical plots of the rate of ring constriction over time in individual cells revealed that, once initiated, the constriction occurred at a constant rate (Figs. 2 E, 3 C, and 4 A).

The rate of ring constriction in N. crassa increases with increasing cell size

In early C. elegans embryos, the scalability of the rate of ring constriction causes the duration of cytokinesis to be constant and independent of the starting cell size (Carvalho et al., 2009). Given that the hyphae in N. crassa are cylindrical in shape with a circular cross-section perpendicular to the plane of growth (as seen in Fig. 3 B), we calculated ring circumference as 2πrc where rc is the ring radius, and determined that the starting circumference of the contractile ring varied between 12 and 48 µm in our sample population. We measured the decrease in actomyosin ring circumference during constriction over time from hyphae with a range of circumferences (Fig. 4 A). Interestingly, the rate of ring constriction was higher in hyphae with a larger circumference. Smaller hyphae (circumference of ~15 µm) constricted at 0.07 µm/s on average, whereas larger hyphae with threefold the circumference (~45 µm) constricted at less than threefold the rate of smaller hyphae, at an average rate of 0.17 µm/s (Fig. 4 B). We therefore refer to this increased rate.

Table I. Conservation of actomyosin ring components among divergent eukaryotes

<table>
<thead>
<tr>
<th>Protein</th>
<th>S. pombe</th>
<th>C. elegans</th>
<th>N. crassa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Profilin</td>
<td>Cdc3 (Balasubramanian et al., 1994)</td>
<td>Phn-1 (Severson et al., 2002)</td>
<td>S.p., C.e. NCU06397*</td>
</tr>
<tr>
<td>Myosin essential light chain</td>
<td>Cdc4 (McCollum et al., 1995)</td>
<td>ND</td>
<td>S.p. CU06617*</td>
</tr>
<tr>
<td>Traponomyosin</td>
<td>Cdc8 (Balasubramanian et al., 1992)</td>
<td>Tmy-1 (Ono and Ono, 2004)</td>
<td>S.p. NCU01204*, C.e. NCU01878</td>
</tr>
<tr>
<td>F-BAR domain–containing protein</td>
<td>Cdc15 (Farkhauser et al., 1995)</td>
<td>ND</td>
<td>S.p. NCU10905</td>
</tr>
<tr>
<td>Formin</td>
<td>Cdc12 (Chang et al., 1997)</td>
<td>Cyk-1 (Severson et al., 2002)</td>
<td>S.p., C.e. NCU01431* (SepA)</td>
</tr>
<tr>
<td>Myosin type II heavy chain</td>
<td>Myo2 (Kitayama et al., 1997; May et al., 1997)</td>
<td>Nmy2 (Shelton et al., 1999)</td>
<td>S.p., C.e. NCU00551</td>
</tr>
<tr>
<td>ADF/cofilin</td>
<td>Adf1 (Nakano and Mabuchi, 2006)</td>
<td>Unc-60 (McKim et al., 1994)</td>
<td>S.p., C.e. NCU01587</td>
</tr>
<tr>
<td>IQGAP</td>
<td>Rng2 (Eng et al., 1998)</td>
<td>Pes-7 (Skop et al., 2004)</td>
<td>S.p., C.e. NCU03116* (ras-GAP)</td>
</tr>
<tr>
<td>UCS domain–containing protein</td>
<td>Rng3 (Balasubramanian et al., 1998)</td>
<td>Unc-45 (Kachur et al., 2004)</td>
<td>S.p., C.e. NCU06821* (Cro1)</td>
</tr>
<tr>
<td>Myosin regulatory light chain</td>
<td>Rlc1 (Le Goff et al., 2000; Naqvi et al., 2000)</td>
<td>Mic-4 (Shelton et al., 1999)</td>
<td>S.p., C.e. NCU04120* (Calmodulin)</td>
</tr>
<tr>
<td>Septin</td>
<td>Spn1, Spn2, Spn3, Spn4 (Wu et al., 2003; An et al., 2004)</td>
<td>Unc-59 (Nguyen et al., 2000)</td>
<td>S.p., C.e. NCU08297* (Cdc3), NCU03515* (Cdc10), NCU02464* (septin-1), NCU03795* (Cdc12)</td>
</tr>
<tr>
<td>F-BAR domain–containing protein</td>
<td>Imp2 (Demeter and Sazer, 1998)</td>
<td>ND</td>
<td>S.p. NCU10905</td>
</tr>
<tr>
<td>Alpha actinin</td>
<td>Ain1 (Wu et al., 2001)</td>
<td>Atn-1 (Barstead et al., 1991)</td>
<td>S.p., C.e. NCU06429</td>
</tr>
<tr>
<td>Paxilin</td>
<td>Pxl1 (Ge and Balasubramanian, 2008; Pinar et al., 2008)</td>
<td>ND</td>
<td>S.p. NCU09812.3</td>
</tr>
</tbody>
</table>

Genes encoding proteins homologous to highly conserved actomyosin ring components in both S. pombe and C. elegans are represented within the N. crassa genome. S. pombe has been included here because many of the proteins involved in cytokinesis are best characterized in this model system. Asterisk indicates annotated homologue; C.e., C. elegans homologue.

of septation (Fig. 2 D and Video 1). Observation of labeled membrane using the vital dye FM4-64 (Hickey et al., 2005) and the localization of Myo2-GFP revealed that the ring of myosin II stays at the leading edge of nascent membrane formation throughout constriction (Fig. 2 E).
Figure 2. Characterization of Myosin II in N. crassa. (A) Schematic representation of N. crassa myo2 gene structure and the corresponding encoded protein domains (drawn to scale). The three coding exons (I, II, and III), two small introns, and 3’ UTR are indicated. SH3, SH3 domain; MH, myosin head (motor domain); MT, myosin tail. Numbering indicates the corresponding amino acid residues within the protein sequence. (B) Septa in a wild-type N. crassa strain. The wild-type FGSC#9719 strain was labeled with FM4-64 to visualize membranes and septa. White arrows indicate positions of septa. The image was created from 11 overlapping individual confocal images by merging and cropping in Adobe Photoshop. Bar, 50 µm. (C) Septation is defective in a heterokaryon knockout strain of the myosin II homologue, NCU00551. NCU00551 deleted heterokaryons were labeled with FM4-64 to visualize membranes and septa. The image was created from 12 overlapping individual confocal images by merging and cropping in Adobe Photoshop. Bar, 50 µm. (D) Myo2-GFP localized to a discreet region on the hyphal cortex, assembled into a contractile ring, and persisted at the ring throughout septation. The time is indicated in minutes; arrowhead indicates the region of preassembly accumulation. Bar, 5 µm. See also Video 1. (E) A kymograph showing the decrease in ring diameter over time within a representative hypha expressing Myo2-GFP (green) and membranes labeled with FM4-64 (red) and a merge of the two.
Larger rings initiate constriction with a higher concentration of myosin II

In order for myosin or actin to be providing scalability to our system, we expected to see an increased total amount of the potential rate-determinant protein with increasing hyphal circumference. We measured the fluorescence intensity of Myo2-GFP and Lifeact-GFP at the initiation of constriction and quantified the total amount of both markers present in the ring. The total amount of both of Myo2-GFP and Lifeact-GFP assembled into the ring at the start of ring constriction is higher in larger hyphae (Fig. 5 A). To explore this further, we then measured the amount of each marker per unit length and found that the concentration of Myo2-GFP also increased significantly in larger hyphae whereas the concentration of Lifeact-GFP remained constant and independent of starting circumference (Fig. 5 B). We hypothesize that the increased concentration of myosin II motors of ring constriction in larger hyphae as “partial scalability.”

The duration of septation, though not constant across hyphae of different sizes, did not show as much of a size-dependent increase as would be predicted if the constriction rate was constant among all cells (Fig. 4 C). During the vegetative growth phase, colonies of *N. crassa* consist of wider, primary hyphae that branch subapically into the narrower secondary hyphae. To rule out the possibility that the detected differences in rate are not size-dependent but in fact due to biological differences between primary and secondary hyphae, we compared the constriction rates of a subset of equally sized primary and secondary hyphae. There was no difference in the mean rate of constriction due to hyphal rank alone (Fig. S1). Together, these experiments led to the conclusion that, as in *C. elegans* embryonic cells, the rate of actomyosin ring constriction in *N. crassa* increases substantially with increasing cell size.
in larger hyphae could provide additional contractile force, enabling larger rings to complete constriction at a faster rate than smaller rings.

**The concentration of ring-associated myosin II increases during constriction**

We then performed further quantitative live-cell confocal microscopy in order to understand the molecular behavior of the ring components during ring constriction. We observed that the intensity of Myo2-GFP within the ring appeared to increase during constriction, whereas the intensity of Lifeact-GFP remained constant. To verify this, we quantified the concentration of Myo2-GFP and Lifeact-GFP associated with the ring by measuring the intensity of both markers during the constant phase of ring constriction. In the 3 min immediately after the initiation of constriction, before completion of septation, the concentration of Myo2-GFP increased by more than 80%, whereas the concentration of Lifeact-GFP remained unchanged (Fig. 6 A). To determine if the amount of myosin II in a given ring is proportional to the ring circumference, we then compared the amount of myosin II within an initially large ring after 3 min of constriction, and that of a smaller ring that initiated constriction at the same size. Larger rings consistently contained more myosin II at 3 min into constriction than smaller rings of the equivalent size, and in all rings we observed that the total amount of myosin II assembled into a ring at the start is retained throughout constriction (Fig. 6, B and C). Examination of the redistribution of myosin during constriction also confirmed that the concentration of myosin II increased during constriction and was always greater in larger rings (Fig. 6 C). Taken together, these data demonstrated first that the total number and concentration of myosin motors associated with the ring is dependent upon the starting hyphal size, and second that the number of myosin motors incorporated into the ring at assembly remains constant throughout ring constriction.

**Myosin undergoes turnover during ring constriction**

Though previous studies in fission yeast and Dictyostelium discoideum have shown that ring-associated myosin II turns over continuously (Yumura, 2001; Pelham and Chang, 2002), more recent work in C. elegans and Drosophila melanogaster cells found myosin becomes stably associated with the ring after the initiation of constriction (Carvalho et al., 2009; Uehara et al., 2010). To determine whether the individual myosin subunits are stably associated with the ring in N. crassa, we performed fluorescence recovery after photobleaching (FRAP) experiments on the Myo2-GFP strain. We photobleached entire rings after the initiation of constriction, and monitored the recovery of the signal across the entire ring in three dimensions (Fig. 7 A and Video 4). Within 2 min after bleaching, Myo2-GFP was again detectable in the constricting ring, and the levels continued to recover until reaching 80% of pre-bleach levels by the time ring constriction was complete, with a half-time of recovery of 56 s (Fig. 7 B). These data suggest that a significant portion of the individual ring-associated myosin is stable and does not undergo turnover during constriction.
Decreased ring-associated myosin II activity causes a decreased rate of ring constriction

If the size-dependent increase in the concentration of myosin licenses larger rings to constrict at an increased rate, a simple prediction is that decreasing the amount of ring-associated myosin or myosin activity should slow ring constriction. To test this, we treated live cells with a variety of concentrations of the drug blebbistatin, which has been shown to inhibit myosin II activity by blocking myosin II heads in an actin-detached state (Straight, et al., 2003; Kovács et al., 2004). At a high concentration (1 mM), blebbistatin caused total dissociation of Myo2-GFP from contractile rings and stalled septation completely (Fig. 8 A). At lower concentrations (100 µM), treatment with blebbistatin caused a 15% decrease in the amount of Myo2-GFP at the ring (Fig. 8 B), resulting in constriction rates around half that of control cells of the same size (Fig. 8 C and Video 5). The amount of actin associated with the ring remained unchanged after blebbistatin treatment (Fig. S2). A second approach to perturbing myosin II activity is to genetically alter the protein itself. We observed that the introduction of the C-terminal GFP tag to the endogenous myosin II caused a slight reduction in ring constriction rate relative to the Lifeact-GFP strain (Fig. 8 D), though growth rate, septal frequency, and hyphal morphology were unaffected. To explore this hypomorphic phenotype, we developed a heterokaryon strain in which the amount of Myo2-GFP was reduced to around one third of the myosin II subunits turn over throughout ring constriction, though the total amount remains constant.

Ring constriction requires continual assembly and turnover of actin filaments

In C. elegans embryos, ring-associated actin filaments were determined to be stably associated with the contractile ring. Inhibition of new actin polymerization with the drug latrunculin A had no effect on the ability of the ring to constrict, suggesting that constriction is coupled with disassembly, but not turnover, of actin filaments (Carvalho et al., 2009). Though in N. crassa the actin appears to be lost progressively from the ring during constriction, whether the actin filaments are stably associated with the ring is not clear from our quantitative fluorescence microscopy alone. Because the recovery dynamics of Lifeact-GFP after FRAP may not accurately reflect that of molecular actin itself, we decided to also use latrunculin A treatment to examine if actin turnover was required for ring constriction. The drug was solubilized in DMSO containing FM4-64 membrane stain in order to monitor uptake. Although the control cells, treated with DMSO only, were able to assemble rings and complete ring constriction normally, latrunculin A caused immediate delocalization of Lifeact-GFP from the ring and stalled septation, as is evident from the incomplete membrane ingestion (Fig. 7 C). No ring assembly, ring-associated actin, or ring constriction was observed after latrunculin A treatment. This suggests that continual turnover of actin filaments is required for septation in N. crassa.
and yeasts. A particularly unique feature of N. crassa is that within a single multinucleate coenocytic colony, hyphal circumference can vary over a fourfold range. This intrinsic variability in cell size allows us to look at the size dependence of cellular processes without the need for mutants, drugs, or other perturbations to normal growth and metabolism. This work establishes the use of N. crassa as a model for both cytokinesis and for the size-dependent scalability of cellular mechanisms in general.

We have shown that ring constriction in N. crassa occurs at a constant rate within a given hyphae and that there is an increase in the rate of ring constriction with increasing initial ring circumference. Previous studies in dividing cells in developing embryos of C. elegans have shown that the rate of cytokinesis increases with increasing cell perimeter (Carvalho et al., 2009). That we find a similar cell size-dependent scalability of actomyosin ring constriction rate in N. crassa suggests that scalability is likely to be a general property of the contractile mechanism during cytokinesis and not specific to embryonic cell types.

Larger rings assemble with a higher concentration of myosin II, which increases further during constriction. What mechanisms might account for the observed cell size-dependent scalability of ring constriction rates? To investigate the

Discussion

N. crassa is a well-suited model organism for studies of cytokinesis and scalability. In this study, we have determined that hyphal septation in N. crassa relies upon a contractile actomyosin ring. Importantly, we identified genes encoding the majority of essential ring components within the N. crassa genome, leading us to conclude that this organism shares a basic cytokinetic mechanism with animals and yeasts. A particularly unique feature of N. crassa is that within a single multinucleate coenocytic colony, hyphal circumference can vary over a fourfold range. This intrinsic variability in cell size allows us to look at the size dependence of cellular processes without the need for mutants, drugs, or other perturbations to normal growth and metabolism. This work establishes the use of N. crassa as a model for both cytokinesis and for the size-dependent scalability of cellular mechanisms in general.

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contributions of the ring components involved in the scalability of ring constriction, we performed the first characterization of myosin II in \textit{N. crassa}. We examined the distribution of myosin II and detected a size-dependent increase in the total amount and concentration of myosin II in the contractile ring upon assembly. The total myosin II levels within the ring remained constant as the ring constricted and thus the concentration increased. The retention of myosin II during ring constriction is a property conserved in many eukaryotes and our findings are in good agreement with this paradigm (Robinson et al., 2002; Wu et al., 2003).

**Changes in ring-associated myosin II amount or activity directly affects the rate of ring constriction**

One key finding from our study is that an initially larger ring (of circumference \(a\)) that has constricted to a given size (circumference \(b\), where \(a > b\)) will contain more myosin than a smaller ring initiating constriction at this same size (circumference \(b\)). Increased recruitment of myosin II is therefore a pre-constriction property of larger rings that is retained throughout constriction. If this is the property that allows larger rings to constrict at a faster rate, then increasing the recruitment of myosin II to the ring should increase constriction rates further. Though experimental overexpression of myosin II could theoretically address this prediction, a recent study in \textit{S. pombe} (Stark et al., 2010) found that high levels of ectopic expression of myosin II in fact caused a decrease in constriction rates, potentially due to defects in ring assembly. Additionally, studies in \textit{S. pombe} and \textit{D. discoideum} have shown that only 10–27% of the total cytoplasmic pool of myosin proteins accumulate locally at the division site, suggesting that the cytoplasmic pool of expressed protein is already in excess of the fraction incorporated into the ring (Robinson et al., 2002; Wu and Pollard, 2005). Though to date we have been unable to ectopically increase the amount of ring-associated myosin II, a converse prediction is that decreasing the amount of ring-associated myosin II should decrease constriction rates. Indeed, when we reduced both the total amount and the activity of ring-associated myosin II through blebbistatin treatment or the use of a hypomorphic strain carrying only the Myo2-GFP fusion protein, we observed a resultant decrease in the rate of ring constriction. Based on our findings, we propose that it is not an increased availability of free myosin II but a size-dependent recruitment of myosin II motors to the ring that regulates cell size-dependent scalability of actomyosin ring constriction in \textit{N. crassa}.

How might the accumulation of myosin II in the assembling ring be regulated in a size-dependent way? Recent work examining the force–velocity relationship of myosin II and actin filaments in intact cells determined that reductions in force were primarily due to decreased numbers of myosin motors bound to each filament. This force–velocity relationship was load dependent, such that the number of attached motors was proportional to the filament load (Piazzesi et al., 2007). Given the sensitivity of velocity to an opposing force, increased numbers of myosin motors could speed up ring constriction by increasing contractile force in response to increased load. One size-dependent source of filament load within our system is internal hyphal pressure due to turgor and the force of cytoplasmic mass flow (Lew, 2005). However, because the growing \textit{N. crassa} colony is a coenocytic interconnected system,
the turgor pressure should be equivalent across all hyphae and thus size independent. If filament load is equivalent in rings of all sizes, the cortical actin filaments themselves may be reorganized in a size-dependent way as to provide additional myosin-binding sites in larger rings. Studies of both filament distribution and load may shed more light on the mechanism of cytokinesis by uncovering size-dependent differences in actomyosin ring ultrastructure.

Figure 8. Inhibition of myosin II after treatment with blebbistatin. (A) Observation of Myo2-GFP (green) and membranes labeled with FM4-64 (red) after inhibition of myosin II with blebbistatin. MeOH alone as a control (top) and 1 mM blebbistatin (bottom). Bar, 10 µm. (B) Concentration of Myo2-GFP in constricting rings in medium sized cells (mean initial circumference = 25 µm) treated with MeOH alone (red, n = 27, top panel insert) or 100 nM blebbistatin (blue, n = 20, bottom panel insert) expressed in relative units of fluorescence intensity per confocal volume; error bars represent the standard deviation from the mean. Bar, 10 µm. Asterisk indicates statistical significance (unpaired t test; P < 0.0001). (C) Average rate of constriction in medium-sized cells (mean initial circumference = 25 µm) in cells treated with MeOH alone (red, n = 26) or 100 µM blebbistatin (blue, n = 22); error bars represent the standard deviation from the mean. Asterisk indicates statistical significance (unpaired t test, P < 0.0001). See also Video 5. (D) Average rate of constriction in medium-sized cells (mean initial circumference = 25 µm) of the Myo2-GFP homokaryon (red, n = 10), Myo2-GFP heterokaryon (blue, n = 11), and Lifeact-GFP (green, n = 38) strains; error bars represent the standard deviation from the mean. Asterisk indicates statistical significance (unpaired t test, P < 0.0001). (E) Concentration of Myo2-GFP in the Myo2-GFP homokaryon (red triangles, n = 25) and Myo2-GFP heterokaryon (blue circles, n = 22) expressed in relative units of fluorescence intensity per confocal volume, as a function of hyphal circumference.
The dynamics of actomyosin ring components

The common finding of cell size–dependent scalability of actomyosin ring constriction in *N. crassa* and *C. elegans* suggests that scalability may represent an evolutionarily conserved property of actomyosin ring constriction. However, we have uncovered important mechanistic differences with respect to the dynamics of actin and myosin between these two systems. Though FRAP studies reveal that ring-associated myosin II turns over during constriction as demonstrated here in *N. crassa* and in other systems (Yumura, 2001; Pelham and Chang, 2002), the *C. elegans* study found that myosin in the contractile ring did not undergo rapid exchange with cytoplasmic pools after photobleaching (Carvalho et al., 2009). Unlike myosin II, the total amount of actin in the contractile rings in *N. crassa* decreased throughout constriction. Additionally, inhibition of actin polymerization with latrunculin A revealed that in this system, as in other cell types, the actin filaments in the ring undergo rapid turnover throughout ring constriction (Pelham and Chang, 2002; Guha et al., 2005; Murthy and Wadsworth, 2005). In contrast, treatment of *C. elegans* cells undergoing cleavage furrow constriction with latrunculin A did not impair the progression of cytokinesis (Carvalho et al., 2009). To explore these mechanistic differences, we developed a physical model to explain the size dependence of the contractile mechanism of the actomyosin ring that accounts for the divergent behaviors of the ring components seen in the two systems.

We initially considered an actomyosin ring with myosin motors distributed along its length and actin filaments stably associated throughout constriction. The contractile ring stress is opposed by cytoplasmic viscous stress, so using a radial stress balance the ring constriction rate can be expressed as

\[
(-\dot{r}) = \rho R_0
\]

where \( \dot{r} \) is the radial velocity and \( R_0 \) is the initial ring radius (Zhang and Robinson 2005). \( \phi = 4\pi^2 f \eta \rho / \mu \) is the contractile rate constant, where \( f \) is the motor force per myosin head, \( \eta \) is the duty ratio, \( \rho \) is the concentration of myosin motors per unit length of filament and \( \mu \) is the viscous dissipation constant. If the concentration of myosin motors \( \rho \) is constant, the ring constriction rate scales linearly with the initial ring size and the consequence is full scalability as shown in Fig. 9 A, such that the duration of cytokinesis is independent of initial ring size, a result clearly observed in *C. elegans* embryos (Carvalho et al., 2009).

As shown in Fig. 5 B, the initial myosin concentration in *N. crassa* is not constant, but in fact scales with ring size \( R_0 \). Because the myosin concentration varies linearly with ring size, the constriction rate becomes

\[
(-\dot{r}) = \phi_0 (\zeta R_0 + 1) R_0
\]

where \( \zeta \) is a fitting constant for the size dependent increase in myosin concentration. Larger rings have higher initial myosin concentration \( (\zeta > 0) \), and therefore cytokinesis is predicted to be completed in a shorter period of time compared with smaller rings, a phenomena we refer to here as super scalability, as shown in Fig. 9 B (using \( \phi_0 = 0.5 \) and \( \zeta = 1 \)). Because the ring constriction rate in *N. crassa* is in fact partially scalable and not super scalable, the actual actomyosin ring dynamics cannot be explained by myosin motor activity alone. In vitro studies demonstrate that additional contractile stress can be generated in the absence of motor proteins by the dynamics of actin filaments and their cross-linking proteins alone (Mogilner and Oster, 2003; Footer et al., 2007; Zumdieck et al., 2007). Though actin turnover is distinctly absent in the scalable system, as observed in *C. elegans* (Carvalho et al., 2009), it is possible that actin turnover in *N. crassa* could provide an additional force contribution resulting in partial scalability. Indeed, our Latrunculin A experiment shows that actin is continually turning over in *N. crassa*, as discussed in the preceding sections.

To explore this possibility, we consider an additional myosin-independent contractile force contribution (\( \lambda \)) due to actin turnover:

\[
(-\dot{r}) = \phi_0 (\zeta R_0 + 1) R_0 + \lambda.
\]

If the effect of actin turnover were to scale linearly with size, i.e., \( \lambda \propto (R_0)^n \) and \( n = 1 \), then the result would either be full scalability (if \( \zeta = 0 \)) or super scalability (if \( \zeta > 0 \)), as shown in Fig. 9 C. However, if the contribution of actin turnover were to be either independent of initial ring size \( (n = 0) \) or minimally scalable \( (n < 1) \), then partial scalability could be possible. Accordingly, we fit the model with our experimental data using parameters \( \zeta = 0.3 \pm 0.05 \) (slope of Fig. 5 B, top) and \( \phi_0 = 0.00085 \pm 0.00015 \) s\(^{-1}\) (including uncertainty estimates). The best fit of the model to the data are achieved if we input an actin turnover rate of 52 ± 12 nm/s, which is very close to 3 μm/min or 50 nm/s, the typical rate of actin turnover measured in other systems in vivo (Pantalonei et al., 2001). As shown in Fig. 9 D, using these experimentally derived parameters we reach a good agreement between the fitted model and experimental results. Thus, we hypothesize that a size-independent force contribution due to actin turnover, along with the size-dependent variation in myosin motor force, may account for the observed partial scalability in *N. crassa*.

Studies in fission yeast and HeLa cells have revealed that while individual actin filaments progressively disassemble and shorten during ring constriction, the absolute number of filaments within the assembled ring remains constant (Maupin and Pollard, 1986; Kamasaki et al., 2007). Because we find that the amount of myosin II remains constant, the ratio between the absolute number of actin filaments and the number of myosin II motors per filament should also remain constant throughout constriction. We therefore hypothesize that it may be the ratio between the number of motors and the number of actin filaments in a given ring that determines the rate of actomyosin ring constriction during cytokinesis. This would account for why the rate of ring constriction remains constant and does not accelerate, despite the increasing concentration of myosin motors that occurs during constriction.
What mechanism might account for the size-independent contribution of actin filament turnover? If the actin turnover rate is dependent upon the number of filament ends and the number of filaments scales linearly with ring size, then we would expect to see either full or super scalability, as shown in Fig. 9 C. However, our model predicts that this is not the case. Because the total amount of actin in the newly assembled ring is size dependent (see Fig. 5 A), there are two possibilities that could account for this: either that the individual filaments in larger rings are longer, or that scalable differences in myosin motor density may have a nonlinear effect on the rate of actin turnover. Again, more detailed ultrastructural studies are necessary to directly observe size-dependent differences in actomyosin ring composition.

Summary

In summary, we have demonstrated that the scalability of actomyosin ring constriction is a conserved mechanism among different cell types, and not unique to embryonic cells. Additionally, we find that the assembly of myosin motors into the ring appears to be modulated in a size-dependent way. This may provide additional structural integrity as well as increased velocity to the process of ring constriction. Our findings expand upon both the understanding of myosin II dynamics and the regulation of actomyosin ring constriction in general. Importantly, our work establishes 

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<th>N. crassa</th>
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as an ideal model system for studying the size dependence of cytokinetic mechanisms in a wild-type and nonembryonic organism.

Materials and methods

Strains and growth conditions

The wild type 

| N. crassa |

strain used was FGSC#9719 (Δmus52::bar+, mat a); the Lifeact-GFP construct was inserted into a histidine auxotrophic strain, FGSC#9720 (his-3, Δmus52::bar+, mat A). The myo2-deleted heterokaryon was obtained from the Neurospora Functional Genomics knockout collection (FGSC#11485). All strains were grown at 30°C on solid Vogel’s normal synthetic medium as described previously (Davis and de Serres, 1970).

Construction of Lifeact-GFP expressing strain

The Lifeact-GFP probe was constructed by Klenow extension of the oligonucleotide pair encoding the Lifeact peptide (LAF and LAR), followed by cloning into the GFP expression plasmid pMW272 (Freitag et al., 2004). Conidia from the 

| N. crassa |

strain FGSC#9720 were transformed with this plasmid by electroporation (Yann, 1995; see Table II for oligonucleotide sequences).

Construction of Myo2-GFP expressing strain

To produce Myo2-GFP, we integrated GFP into the downstream region of the chromosomal locus of myo2 (NCU00551) into wild-type strain FGSC#9719 using Marker Fusion Tagging (MFT) as described previously.
Live-cell Imaging
Sample preparation was via the inverted agar block method described previously (Ng et al., 2009). In brief, the fungal mycelium was grown on agar in a Petri dish, then a block of the agar from the periphery of the colony, measuring 1–4 cm² (including the growing hyphal tip) was cut out and gently inverted into a droplet of liquid Vogel’s medium (and any required dye or treatment) on a coverslip. Cells were labeled with 1 µM FM4-64 (Invitrogen) and 20 µg/ml calcofluor where noted. For treatment with latrunculin A (Sigma-Aldrich), a 10 mM stock solution was prepared in DMSO and this was diluted into Vogel’s medium containing 1 µM FM4-64. The DMSO control was prepared the same way. For treatment with S(+)-blebbistatin (Sigma-Aldrich), a 5 mM stock solution was prepared in MeOH, and this was diluted into Vogel’s medium containing 1 µM FM4-64. The MeOH control was prepared the same way. Live-cell imaging was performed at room temperature using a confocal laser scanning microscope (model TCS SP5, Leica; operated with the LAS AF acquisition software) equipped with a Plan Apochromat 63×/1.2 NA water immersion objective lens (270×1.45 NA oil immersion objective lens at room temperature. For photobleaching, this was not possible; both the coverslip surface and the agar surface caused artifacts in the imaging such that it was impossible to accurately segment the 3D images.

Image processing
Image processing and figure preparations were performed using a combination of Leica LAS AF Lite,Bio-formats (for 3D maximum intensity projection (MIP) volume rendering in Figs. 3 B, 7 A, and Video 3), AutoQuant (for Blind deconvolution for Fig. 3 B), ImageJ (http://rsbweb.nih.gov/ij) and Fiji (http://fiji.sc/wiki/index.php/Fiji) using the LOCI Bioformats (http://www.loni.wisc.edu/bio-formats/about), Stackreg (Thévenaz, et al., 1998, for the kymographs in Figs. 2 E and 3 C), and Adobe Photoshop and SigmaPlot for figure preparation.

Image analysis methods to determine duration and rates of contractility
The decrease in ring diameter over time was measured for each constriction event. For quantitation of the rate of constriction the raw Leica .lif files were opened in ImageJ using the LOCI Bioformats plugins. To ensure uniformity of illumination across the field of view we performed the field illumination confocal performance test as described by Zucker (2006). There was less than 5% variation over the field of view and no position-dependent correlation of variance was detected. The images were acquired at the medial focal plane, its thickness being determined through the combination of wavelength, objective lens, immersion medium, and confocal pinhole size. The Leica LAS AF software gives a value for confocal slice thickness based on these parameters, which for these experiments was 1.281 µm. Initially the diameter of the ring was measured. Pre-constriction rings that were still undergoing assembly were excluded from the analysis as described above. Two circular regions of interest (9 pixels in diameter) were then drawn over the two intense spots of the ring and their intensities (integrated density) were measured (Fig. S4 B). The data were then taken over to Microsoft Excel where the mean of the two measurements was calculated and used as the protein concentration for that ring. To calculate the total protein in the ring, this average was divided by the confocal volume (a cylindrical volume as the product of the area of the 2D ROI and the confocal slice thickness) and multiplied by the circumference of the ring (Fig. S4 C). Before settling on this methodology, attempts were made to use 3D segmentation to measure the total protein in the ring directly. However, the nature of the sample preparation meant that this was not possible; both the coverslip surface and the agar surface caused artifacts in the imaging such that it was impossible to accurately segment the 3D images.

Fluorescence recovery after photobleaching (FRAP)
FRAP experiments were performed on an inverted confocal laser scanning microscope (model FV1000, Olympus; operated with the Olympus FV-ASW acquisition software) equipped with a Plan Apochromat 60×/1.45 NA oil immersion objective lens at room temperature. For photobleaching, the multi-line Argon laser was set to 30% power on the 488-nm line. An oval ROI encompassing the maximum diameter of the ring was defined and continually bleached (tornado mode) for 8 s, during which time the z-position was adjusted to cover the full extent of the ring. For image acquisition, 8% of the 488-nm laser was used for the excitation of GFP (Em. 500–550 nm) and 405 nm diode laser (3% transmission at the AOTF) was used for the excitation of the GFP (Em. 500–550 nm) and a 405-nm diode laser (3% transmission at the AOTF) for the calcofluor (Em. 415–478 nm). The standard Leica photo-multiplier tubes (PMTs) were used as detectors.

Online supplemental material
Fig. S1 shows mean rates of constriction in primary and secondary hyphae. Fig. S2 shows quantitation of the concentration of ring-associated Lifeact-GFP after treatment with low-dose blebbistatin. Fig. S3 shows quantitation of the concentration of ring-associated Myo2-GFP in homokaryon and heterokaryon strains. Fig. S4 shows image analysis methodology.
Video 1 shows Myo2p-GFP localization during cortical ring formation and constriction. Video 2 shows Lifeact-GFP localization during cortical ring formation and constriction. Video 3 shows membrane formation, cell wall deposition, and actomyosin ring constriction in N. crassa. Video 4 shows turnover of myosin II during ring constriction. Video 5 shows that decreased ring-associated myosin II causes a decreased rate of ring constriction. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201101055/DC1.

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Figure S1. **Mean rates of constriction in primary and secondary hyphae.** The rate of constriction was calculated in cells with a mean circumference of 25 µm from both primary (red, \( n = 10 \)) and secondary (blue, \( n = 10 \)) hyphae. Error bars indicate the standard deviation from the mean.

Figure S2. **Quantitation of the concentration of ring-associated Lifeact-GFP after treatment with low-dose blebbistatin.** Concentration of Lifeact-GFP in constraining rings in medium-sized cells (mean initial circumference = 24 µm) treated with MeOH alone (red, \( n = 16 \)) or 100 µM blebbistatin (blue, \( n = 15 \)), expressed in relative units of fluorescence intensity per confocal volume. Error bars indicate the standard deviation from the mean.
Figure S3. **Quantitation of the concentration of ring-associated Myo2-GFP in homokaryon and heterokaryon strains.** The Myo2-GFP intensity in medium-sized cells (mean circumference = 25 μm) of the Myo2-GFP homokaryon (red, n = 10) and heterokaryon (blue, n = 14) strains, expressed in arbitrary units of fluorescence intensity. Error bars indicate the standard deviation from the mean.

Figure S4. **Image analysis methodology.** (A) The duration and rate of contractility were determined by measuring the decrease in ring diameter during constriction for time-series acquired at a medial focal plane. The raw data were initially smoothed, then segmented by an intensity threshold, before using the Object Tracker plug-in in ImageJ. (B) The fluorescence intensity was measured in single images, acquired at a medial focal plane with a known confocal slice thickness, just after ring constriction had initiated. The diameter of the ring and intensity (integrated density) within two circular regions of interest (ROIs), drawn over the two intense spots of the ring, were measured using ImageJ. (C) The ring-associated protein concentration was determined as the mean of the two measurements described in B divided by the confocal volume (a cylinder equaling the product of the circular ROIs and the confocal slice thickness). Total protein in the ring was calculated by multiplying the concentration by the circumference of the ring.
Video 1. **Myo2p-GFP localization during cortical ring formation and constriction.** Endogenously labeled Myo2-GFP was observed in wild-type *N. crassa* during vegetative growth by time-lapse (images acquired every 15 s for the duration of ring constriction) microscopy using a confocal laser scanning microscope (model TCS SP5; Leica). The time is indicated in minutes. Bar, 10 µm.

Video 2. **Lifeact-GFP localization during cortical ring formation and constriction.** The actin-binding probe Lifeact-GFP was transformed into wild-type *N. crassa* and observed during vegetative growth by time-lapse (images acquired every 15 s for the duration of ring constriction) microscopy using a confocal laser scanning microscope (model TCS SP5; Leica). The time is indicated in minutes. Bar, 10 µm.

Video 3. **Membrane formation, cell wall deposition, and actomyosin ring constriction in *N. crassa*.** The process of septation was observed in wild-type *N. crassa* during vegetative growth using Lifeact-GFP (green), FM4-64 (red), and calcofluor (blue) to label actin, cell membrane, and cell wall, respectively. A 3D time-lapse was acquired (z-slices every 1 µm) every 38 s for 5 min using a confocal laser scanning microscope (model TCS SP5; Leica) then rendered into the video shown using Imaris software (Bitplane). The time is indicated in minutes. Bar, 5 µm.

Video 4. **Turnover of myosin II during ring constriction.** Fluorescence recovery after photobleaching (FRAP) of Myo2-GFP (green) in a hypha with two rings in close proximity, rendered into a 3D image. The ring in the foreground was selectively photobleached after the first image while the second ring in the background remained unbleached. A 3D time-lapse was acquired (z-slices every 0.8 µm) every 20 s for 10 min using a confocal laser scanning microscope (model FV1000; Olympus) then rendered into the video shown using Imaris software (Bitplane). The time is indicated in minutes. Bar, 10 µm.

Video 5. **Decreased ring-associated myosin II causes a decreased rate of ring constriction.** Endogenously labeled Myo2-GFP (green) and FM4-64 membrane dye (red) were observed in wild-type *N. crassa* after treatment with MeOH alone (top) or 100 µM blebbistatin (bottom). The images were acquired every 30 s for up to 24 min by time-lapse microscopy using a confocal laser scanning microscope (model TCS SP5; Leica). The time is indicated in minutes. Bar, 10 µm.