Current Biology 20, 1415–1422, August 24, 2010 ©2010 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2010.06.020

Article

Fimbrin and Tropomyosin Competition Regulates Endocytosis and Cytokinesis Kinetics in Fission Yeast

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Summary

Background: Tropomyosin is an important actin filament-stabilizing protein that controls the access of other essential proteins to filaments, including myosin motors, Arp2/3 complex, formin, and cofilin. It is therefore critical to establish mechanisms for regulating the actin filament binding of tropomyosin. We examined how the actin filament crosslinking protein fimbrin Fim1p and tropomyosin Cdc8p affect each other's ability to bind filaments, localize to particular cellular structures, and regulate filament severing by cofilin Adf1p in fission yeast *Schizosaccharomyces pombe*.
Results: We discovered a novel mechanism for regulating actin filament dynamics in fission yeast. Fim1p inhibits Cdc8p binding to actin filaments in vitro, which permits Adf1p-mediated severing in the presence of Cdc8p. In cells, the balance between Fim1p and Cdc8p is important for both endocytic actin patch kinetics and contractile ring assembly during cytokinesis. High Fim1p concentrations prevent Cdc8p from associating with actin patches, allowing rapid patch turnover and motility. In the absence of Fim1p, ectopic localization of Cdc8p to actin patches increases patch lifetime while decreasing patch motility. Fim1p and Cdc8p also play antagonistic roles during cytokinesis, in which the deletion of Fim1p rescues the contractile ring assembly defects caused by mutation of Cdc8p.
Conclusion: Fimbrin Fim1p dissociates tropomyosin Cdc8p from actin filaments, permitting cofilin Adf1p-mediated severing. Therefore, we propose that in addition to actin filament crosslinking, Fim1p has a novel role as a positive actin-binding "selector" protein that promotes the access of other proteins to actin filaments by inhibiting Cdc8p.

Introduction

The fission yeast *Schizosaccharomyces pombe* assembles actin filaments with precise architecture and dynamics to drive different processes, including Arp2/3 complex-dependent endocytic patches, and to drive formin-dependent polarizing cables and the cytokinetic contractile ring [1]. Partially overlapping sets of actin-binding proteins are required for the establishment, maintenance, and disassembly of the actin filaments within these structures. A major challenge is to determine how specific subgroups of actin-binding proteins localize to a particular structure and collectively influence actin filament architecture, dynamics, and function.

For example, fission yeast contains a single copy of each of the actin filament crosslinking and stabilizing proteins fimbrin Fim1p and tropomyosin Cdc8p, as well as the severing protein cofilin Adf1p [2–5]. Although these proteins all bind to the side of actin filaments, they are differentially utilized for specific cellular processes. First, Fim1p is abundant in endocytic actin patches, whereas Cdc8p is not [2–4, 6]. Second, Cdc8p is absolutely required for contractile ring assembly [2], but it is unclear whether Fim1p plays a direct role in cytokinesis despite its localization to the division site [3, 4]. Third, Adf1p is important for both endocytic actin patches and contractile ring assembly [5], although the rate of actin filament turnover is faster in actin patches than the contractile ring.

Through a combination of in vitro and in vivo approaches, we discovered a novel paradigm for controlling actin filament dynamics in fission yeast. In addition to filament crosslinking, fimbrin Fim1p is a "selector" protein that controls the access of other proteins to actin filaments. Specifically, we found that Fim1p prevents tropomyosin Cdc8p from binding to actin filaments, permitting cofilin Adf1p-mediated severing. Therefore, the balance between Fim1p and Cdc8p is important for both endocytic actin patches and contractile ring assembly. By preventing Cdc8p from associating with actin patches, Fim1p allows rapid patch turnover and motility. Fim1p also antagonizes Cdc8p during contractile ring assembly, possibly promoting a limited amount of actin filament severing, which is necessary for cytokinesis.

Results

Fim1p Displaces Cdc8p from Actin Filaments In Vitro

Although fission yeast fimbrin Fim1p and tropomyosin Cdc8p both bind actin filaments with submicromolar affinity [3, 6–9], they preferentially localize to different actin-dependent structures in cells [2, 4, 6, 7]. To examine the basis for this phenomenon, we utilized in vitro cosedimentation assays to determine how purified Fim1p and Cdc8p influence each other's ability to bind actin filaments (Figure 1; see also Figures S1–S3 available online).

Cdc8p binds to and sediments with actin filaments following high-speed ultracentrifugation at 100,000 × g (Figure 1A). However, a range of Fim1p concentrations reduces the amount of Cdc8p in the actin pellet (Figures 1A and 1B). Maximal displacement of Cdc8p from filaments occurs at ~5 μM Fim1p (~1.5:1 Fim1p to actin), a concentration at which filaments are essentially saturated with Fim1p (Figure 1B). Fim1p prevents Cdc8p from binding actin filaments, whether Fim1p is added to filaments before Cdc8p, simultaneously with Cdc8p, or after Cdc8p (Figure 1B). Therefore, Cdc8p binds actin filaments with a K_d of ~40 nM in the absence of Fim1p, whereas no detectable binding of Cdc8p to actin filaments occurs in the presence of saturating amounts of Fim1p (Figure S1).

Because Fim1p is an actin filament crosslinking protein [3, 9], we performed low-speed centrifugation assays at 10,000 × g (Figures 1C and 1D). In the absence of Fim1p, unbundled actin filaments and Cdc8p remain in the supernatant. Fim1p has a biphasic effect on the appearance of Cdc8p in the low-speed pellet. Fim1p concentrations up to 250 nM progressively increase the amount of Cdc8p in the pellet, whereas

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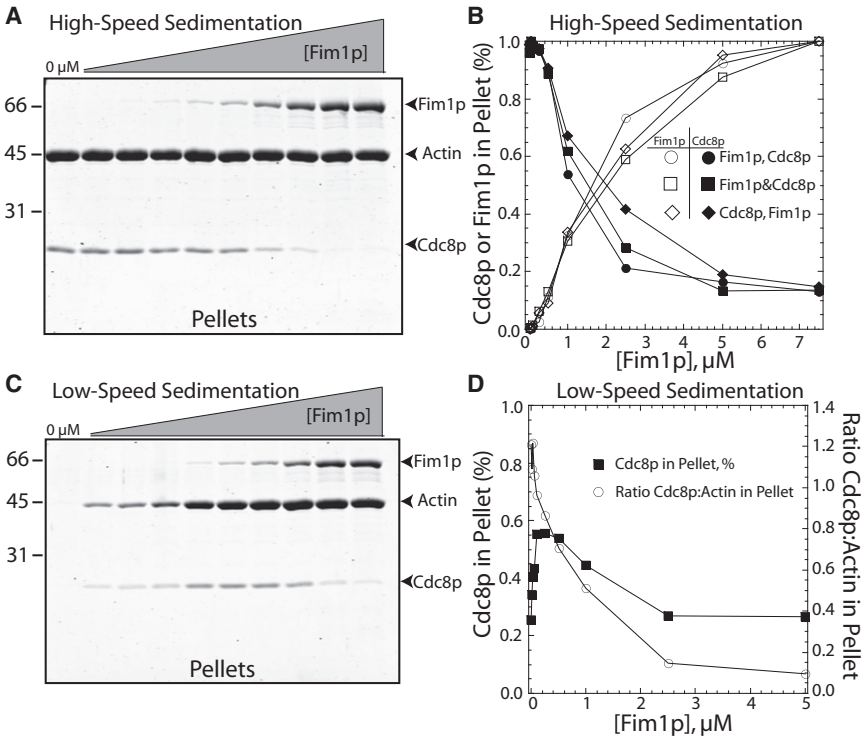

Figure 1. Fim1p Prevents Cdc8p from Binding Actin Filaments In Vitro

(A–D) Sedimentation of F-actin assembled from 3.0 μM Mg-ATP-actin monomers.

(A and C) Coomassie blue-stained gels of pellets after a range of Fim1p concentrations were added to filaments preincubated with 2.0 μM Cdc8p and then spun at high speed 100,000 × g (A) or low speed 10,000 × g (C). Fim1p, actin, and Cdc8p are marked on the right; molecular weights are indicated on the left.

(B) Plot of the amount of Cdc8p (closed symbols) or Fim1p (open symbols) in the high-speed pellet over a range of Fim1p concentrations. Results are shown for different orders of addition: Fim1p, Cdc8p, spin (●, ○); Fim1p and Cdc8p together, spin (■, □); or Cdc8p, Fim1p, spin (◆, ◇).

(D) Plot of the fraction of Cdc8p in the low-speed pellet (■) and the ratio of Cdc8p to actin in the low-speed pellet (○) over a range of Fim1p concentrations.

Fim1p concentrations over 250 nM progressively remove Cdc8p from the pellet (Figures 1C and 1D). Therefore, Cdc8p and Fim1p can simultaneously bind actin filaments at an optimal low Fim1p concentration that is capable of forming bundles but not inhibiting Cdc8p (~3:1:0.25, actin:Cdc8p:Fim1p). However, a plot of the ratio of Cdc8p to actin in the low-speed pellet over a range of Fim1p concentrations verifies that Fim1p displaces Cdc8p from actin filaments by similar amounts, whether spun at high or low speed (Figure 1D). Utilizing a truncated version of Fim1p (Fim1A2p) that contains only the second of two actin-binding domains, we verified that dissociation of Cdc8p from actin filaments is not dependent upon crosslinking by Fim1p (Figure S2). Conversely, Cdc8p does not affect the ability of Fim1p to bind or bundle actin filaments (Figure S3).

Fim1p Prevents Cdc8p from Localizing to Endocytic Actin Patches

Fim1p is highly enriched at endocytic actin patches, whereas Cdc8p is not [2–4, 6, 7, 10]. We examined the ability of Fim1p to inhibit the localization of Cdc8p to actin patches by observing integrated GFP-Cdc8p in live cells (Figure 2). GFP-Cdc8p associates primarily with the contractile ring in wild-type cells (Figures 2A and 2B) [6]. However, in the absence of Fim1p (*fim1Δ* cells), GFP-Cdc8p localizes to the contractile ring and to discrete puncta focused near the tips of interphase cells (Figures 2A and 2B).

To determine whether the GFP-Cdc8p puncta are endocytic actin patches, we visualized GFP-Cdc8p with the actin patch component ARPC5-mCherry, a subunit of the Arp2/3 complex (Figures 2C–2E). In wild-type cells, GFP-Cdc8p does not colocalize with ARPC5-mCherry (Figures 2C and 2D). However, in interphase *fim1Δ* cells, GFP-Cdc8p localizes to about 85% of ARPC5-mCherry actin patches (Figures 2C–2E). We confirmed that colocalization represents individual motile actin patches by time-lapse imaging (Figure 2D; Movie S1). Interestingly, GFP-Cdc8p colocalizes with ARPC5-mCherry at less than 20% of actin patches in dividing *fim1Δ* cells (Figure 2E). Therefore, Cdc8p is excluded from endocytic actin patches by Fim1p and is potentially recruited to actin filaments in the contractile ring.

Actin Patch Dynamics Are Disrupted in the Absence of Fim1p, Largely because of Ectopic Cdc8p Accumulation

We next examined actin patch behavior in the absence of Fim1p (Figure 3). Wild-type cells efficiently uptake the lipophilic dye FM4-64 within 20 min (Figures 3A and 3B), whereas endocytosis of FM4-64 is significantly reduced in *fim1Δ* cells (Figures 3A and 3B). Using the general actin marker GFP-CHD(rng2) [11], we tracked the lifetime and movement of individual interphase patches (Figures 3C–3E; Movie S2). GFP-CHD(rng2)-labeled wild-type patches exist for ~20 s, and 95% propel into the cell interior (Figures 3C and 3D) [12]. Actin patches in *fim1Δ* cells subsist twice as long (~40 s), and less than 20% propel into the cell interior (Figures 3C–3E).

Actin patch defects in *fim1Δ* cells could be due to the absence of Fim1p and/or the ectopic presence of Cdc8p. We tested whether reducing the amount of Cdc8p in patches, via the tropomyosin temperature-sensitive mutant *cdc8-27* [13], corrects patch dynamics in *fim1Δ* cells. We found that patch dynamics in *fim1Δ cdc8-27* double mutant cells at the semi-permissive temperature of 30°C are significantly restored (Figures 4A–4D; Figure S4).

First, whereas actin patches are abnormally retained at cell tips in *fim1Δ* cells, actin patch distribution is largely corrected in *fim1Δ cdc8-27* cells (Figure S4). Second, actin patch lifetime is significantly restored from ~25 s in *fim1Δ* cells to ~12 s in *fim1Δ cdc8-27* double mutant cells (Figures 4A and 4B; Movie S3). Third, patch motility from the cortex to the cell interior is substantially rescued in *fim1Δ cdc8-27* cells (Figure 4C). However, patches do not appear to regain full wild-type motility in *fim1Δ cdc8-27* cells, and endocytosis defects similar to *fim1Δ* cells persist (Figure 4D). Therefore, both the ectopic presence of Cdc8p and the loss of Fim1p-mediated actin filament bundling likely contribute to patch defects.

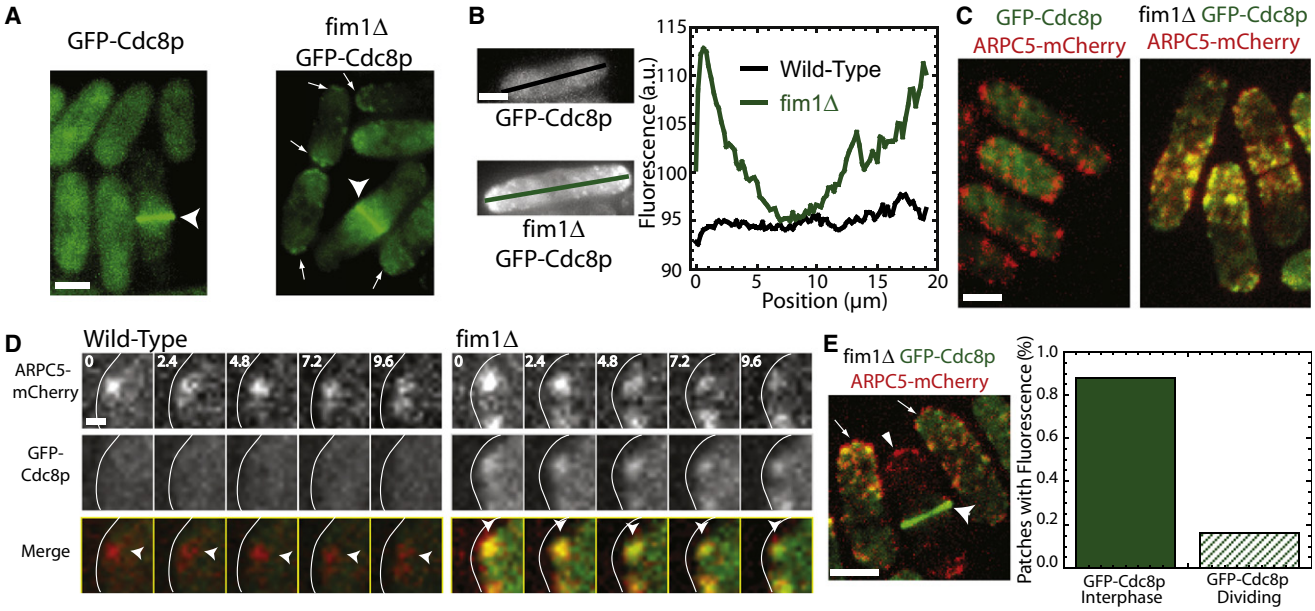

Figure 2. Fim1p Prevents Cdc8p from Localizing to Endocytic Actin Patches

(A and B) In the absence of Fim1p, GFP-Cdc8p accumulates in puncta at cell poles.

(A) Distribution of GFP-Cdc8p in wild-type (left) or *fim1*Δ (right) cells. Wedges and arrows indicate GFP-Cdc8p at the contractile ring and puncta. Scale bar represents 5 μm.

(B) Quantification of GFP-Cdc8p puncta fluorescence, determined from a line along the length of the cell (left) and plotted as a function of cell position (right; n = 25 for each strain). Scale bar represents 5 μm.

(C and D) GFP-Cdc8p colocalizes with ARPC5-mCherry at actin patches in *fim1*Δ cells.

(C) Z projections of interphase wild-type (left) and *fim1*Δ (right) cells. Scale bar represents 5 μm.

(D) Time-lapse micrographs of a single confocal section. Time is indicated (s). Top: ARPC5-mCherry; middle: GFP-Cdc8p; bottom: merge. Wedges indicate actin patches. Scale bar represents 1 μm. See Movie S1.

(E) GFP-Cdc8p is recruited to the contractile ring (wedge). Left: merge of interphase (arrow) and dividing (arrowhead) cells expressing ARPC5-mCherry and GFP-Cdc8p. Bar represents 5 μm. Right: percent of ARPC5-mCherry patches containing GFP-Cdc8p in interphase and dividing cells (n = 150 patches per condition).

The Balance between Fim1p and Cdc8p Plays a Role in Cytokinesis

Although Fim1p localizes primarily to endocytic patches, Fim1p is also suspected to play a role in cytokinesis [14]. Fim1p localizes to the division site [3, 4], possibly the contractile ring [3]. Deletion of Fim1p causes mild growth defects and is synthetic lethal with deletion of the major cytokinesis actin crosslinking protein α-actinin [3, 4]. We therefore examined whether Fim1p also antagonizes Cdc8p during cytokinesis (Figure 5).

The temperature-sensitive mutant *cdc8-27* has severe cytokinesis defects at the semipermissive temperature of 32°C (Figure 5A) [2, 13]. We found that the *fim1Δ cdc8-27* double mutant partially suppresses the severe cytokinesis defects of *cdc8-27* at 32°C, as quantified by several criteria (Figures 5A and 5B). First, the total area of *cdc8-27* cells is ~5-fold greater than *fim1Δ cdc8-27* double mutant cells. Second, more than 95% of *cdc8-27* cells contain more than two nuclei, compared with only 25% of *fim1Δ cdc8-27* cells. Third, at least 99% of septa are abnormal (misplaced, misoriented, or unusually broad) in *cdc8-27* cells, compared with ~70% abnormal septa in *fim1Δ cdc8-27* cells.

The Absence of Fim1p Rescues Contractile Ring Assembly Defects in a Cdc8p Mutant

We determined the stage of cytokinesis at which Cdc8p and Fim1p have antagonistic roles by imaging live cells over time (Figures 5C and 5D). Cytokinesis in fission yeast occurs through the coordinated progression of several major events that include prering cytokinesis node formation (broad band), completion of contractile ring assembly (sharp ring), constriction initiation, constriction completion (septum formation complete), disappearance of contractile ring components, and cell separation [14–16]. We measured the kinetics of cytokinesis in wild-type, *fim1Δ*, *cdc8-27*, and *fim1Δ cdc8-27* mutants at the semipermissive temperature of 30°C using differential interference contrast (DIC) and fluorescence microscopy of the general actin marker GFP-CHD(rng2) (Figures 5C and 5D).

Wild-type cells complete cytokinesis and undergo daughter cell separation ~75 min after actin fluorescence first appears at the division site (Figure 5D) [15, 16]. Contractile rings in *fim1Δ* cells assemble and constrict with similar timing as wild-type, but significant delays emerge at daughter cell separation (Figure 5D). This late delay suggests defects in actin patch-mediated delivery of septum components to the division site, consistent with Fim1p's major role in actin patch dynamics (Figure 3). In mutant *cdc8-27* cells, the early step of contractile ring assembly from the broad band of prering cytokinesis nodes is significantly delayed (Figure 5D) [15, 17], whereas later steps proceed normally. Removal of Fim1p rescues this early defect, because *fim1Δ cdc8-27* double mutant cells do not have contractile ring assembly delays (Figure 5D). Similarly, *fim1Δ cdc8-27* double mutant cells have a partial rescue of daughter cell separation defects seen in *fim1Δ* cells (Figure 5D). Therefore, Fim1p and Cdc8p have

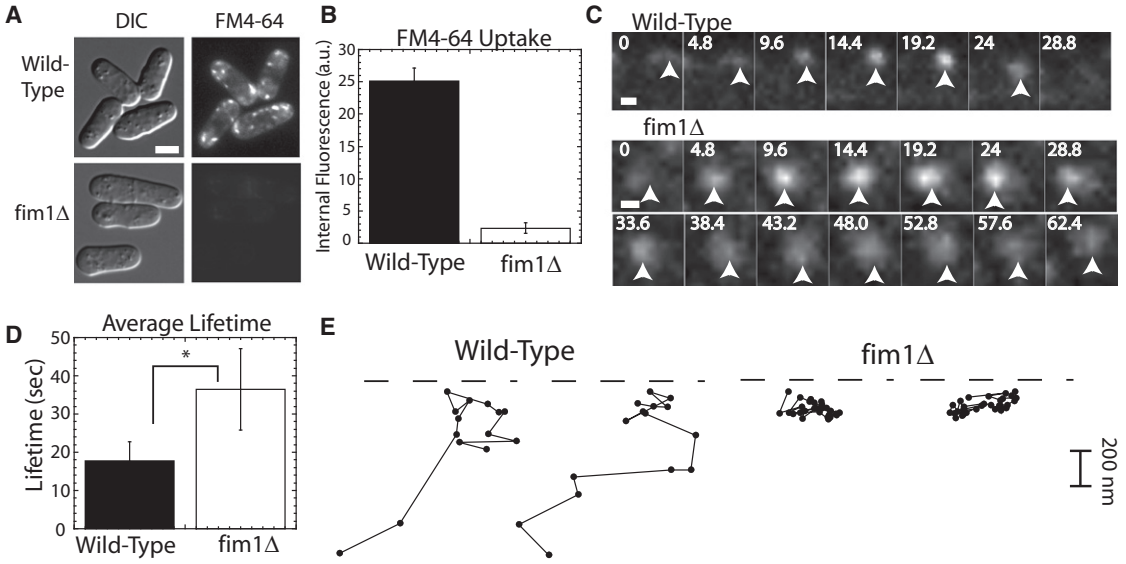

Figure 3. Deletion of Fim1p Causes Endocytic Actin Patch Defects

(A and B) Endocytosis is impaired in *fim1*Δ cells.

(A) Differential interference contrast (DIC) (left) and fluorescence (right) images of wild-type (top) and *fim1*Δ (bottom) cells following 20 min incubation with 2 μM lipophilic dye FM4-64. Scale bar represents 5 μm.

(B) Plot of FM4-64 fluorescence uptake. Values are mean ± standard deviation.

(C–E) Actin patch dynamics are impaired in *fim1*Δ cells.

(C) Time-lapse micrographs of single actin patches (wedges) in wild-type (top) and *fim1*Δ (bottom) cells expressing the general actin marker GFP-CHD(rng2). Time (s) is indicated at top. Scale bar represents 0.5 μm. See Movie S2.

(D) Plot of actin patch lifetime for wild-type and *fim1*Δ cells (n = 40 patches for each strain). Values are mean ± standard deviation. *p < 0.05.

(E) Position over time (1.2 s intervals) for two representative wild-type (left) and *fim1*Δ (right) actin patches. Dashed lines indicate cell cortex.

antagonistic roles during the contractile ring assembly and cell separation steps of cytokinesis.

Fim1p Allows Adf1p-Mediated Severing in the Presence of Cdc8p

We next investigated one possible mechanism by which competition between Fim1p and Cdc8p influences actin patch and contractile ring assembly kinetics. The actin filament-severing protein cofilin is the primary facilitator of rapid actin turnover in yeast actin patches [18]. Tropomyosins inhibit cofilin-mediated severing [19, 20], and overexpression of Cdc8p delocalizes cofilin Adf1p from contractile rings in fission yeast [5]. Therefore, we hypothesized that Fim1p might allow Adf1p-mediated severing by preventing Cdc8p from

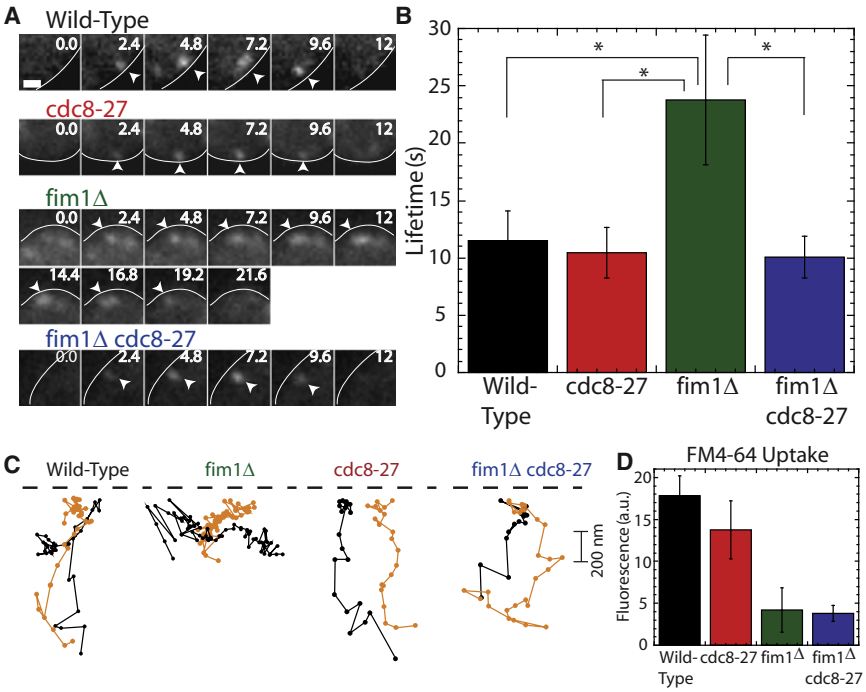

Figure 4. Actin Patch Dynamics in *fim1Δ* Cells Are Recovered by Depletion of Cdc8p

(A–C) Wild-type, *cdc8-27*, *fim1Δ*, and *fim1Δ cdc8-27* cells expressing GFP-CHD(rng2) at 30°C.

(A) Time-lapse micrographs (s) of single actin patches (wedges) in the indicated cells. Scale bar represents 1 μm. See Movie S3.

(B) Plot of average patch lifetime for the indicated cells (n = 25 for each strain). Values are mean ± standard deviation. *p < 0.05.

(C) Position over time (0.25 s intervals) for two representative actin patches (black and orange). Dashed lines indicate cell cortex.

(D) Plot of FM4-64 fluorescence uptake after 10 min at 30°C. Values are represented as mean ± standard deviation.

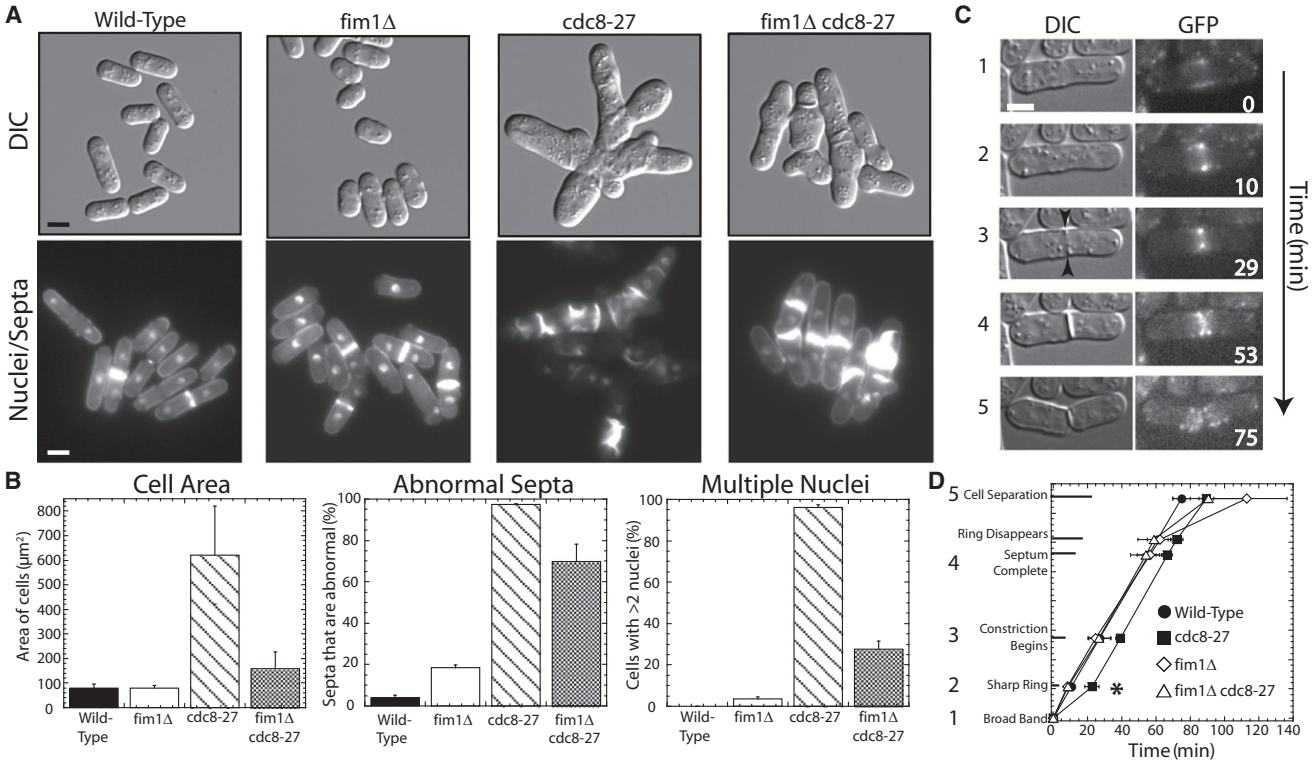

Figure 5. Fim1p and Cdc8p Have Antagonistic Roles during Cytokinesis

(A) Micrographs of the morphology (top) and nuclei and septa (bottom) of wild-type, *fim1*Δ, *cdc8-27*, and *fim1*Δ *cdc8-27* cells after 12 hr at 32°C. Scale bar represents 5 μm.

(B) Quantification of morphological phenotypes. Plots of cell area, abnormal septa (partial, misplaced, misaligned, and/or broad), and >2 nuclei (n = 250 for each strain). Values are mean ± standard deviation.

(C and D) Effect of *fim1*Δ and *cdc8-27* mutations on cytokinesis kinetics. Cells expressing GFP-CHD(rng2) at 30°C are shown.

(C) DIC and GFP time-lapse images of a representative wild-type cell undergoing cytokinesis: (1) broad band detected, (2) contractile ring assembly complete, (3) ring constriction and septum ingression (arrowheads) begins, (4) constriction ends/septum complete, and (5) cells separate. Scale bar represents 5 μm.

(D) Plot of major cytokinesis events over time for at least 10 wild-type (●), *cdc8-27* (■), *fim1Δ* (◇), and *fim1Δ cdc8-27* (△). *p < 0.05 for *cdc8-27* compared to other strains.

binding filaments. We first established that the balance of Fim1p and Cdc8p does regulate actin filament turnover. The absence of Fim1p significantly reduces the rate of latrunculin A (LatA)-mediated actin filament disassembly in actin patches, which is corrected by the partial loss of Cdc8p (Figures 6A and 6B).

We then examined the ability of Cdc8p to protect actin filaments from Adf1p in vitro in both the absence and presence of Fim1p. We began by visualizing the lengths of preassembled actin filaments following incubation with a range of concentrations of Adf1p for 30 min (Figure S5). We found that Adf1p efficiently reduces actin filament length in either the absence or presence of 250 nM Fim1p. On the other hand, 3.0 μM Cdc8p inhibits the reduction in filament length over a range of Adf1p concentrations up to 5.0 μM. In the presence of both Cdc8p and Fim1p, Adf1p reduces the average filament length by the same amount as in the presence of Fim1p only (Figure S5). We verified that Fim1p allows Adf1p-mediated severing in the presence of Cdc8p by directly observing actin filament severing with total internal reflection fluorescence (TIRF) microscopy (Figures 6C and 6D; Movie S4). We found that although 3.0 μM Cdc8p inhibits severing by 150 nM Adf1p, the severing rate is significantly increased in the presence of both Cdc8p and 250 nM Fim1p. These results are consistent with a mechanism whereby Fim1p allows cofilin-mediated severing by dissociating Cdc8p.

In fission yeast, Adf1p-mediated severing is thought to be important for both contractile ring assembly and endocytic patch dynamics [3, 5, 21]. As expected, we found that mutations in both Fim1p and Adf1p synergistically impair actin patches (Figures 6E and 6F). By visualizing the general actin marker GFP-CHD(rng2), we monitored patch dynamics in wild-type, *fim1Δ*, *adf1-1* [5], and *fim1Δ adf1-1* double mutant cells at 25°C (Figures 6E and 6F; Movie S5). Patch lifetime is significantly longer in *fim1Δ adf1-1* double mutant cells (>70 s) than *fim1Δ* (~20 to 40 s) and *adf1-1* (~20 to 40 s) single mutant cells (Movie S5). These data are consistent with the model whereby the ability of Fim1p to remove Cdc8p from actin filaments is critical for allowing severing by Adf1p in vivo.

Discussion

We have discovered that, in addition to actin filament cross-linking, fimbrin Fim1p plays an unexpected role in fission yeast by inhibiting the binding of tropomyosin Cdc8p to actin filaments; therefore, the balance between Fim1p and Cdc8p is critical in cells. We propose that Fim1p controls actin filament

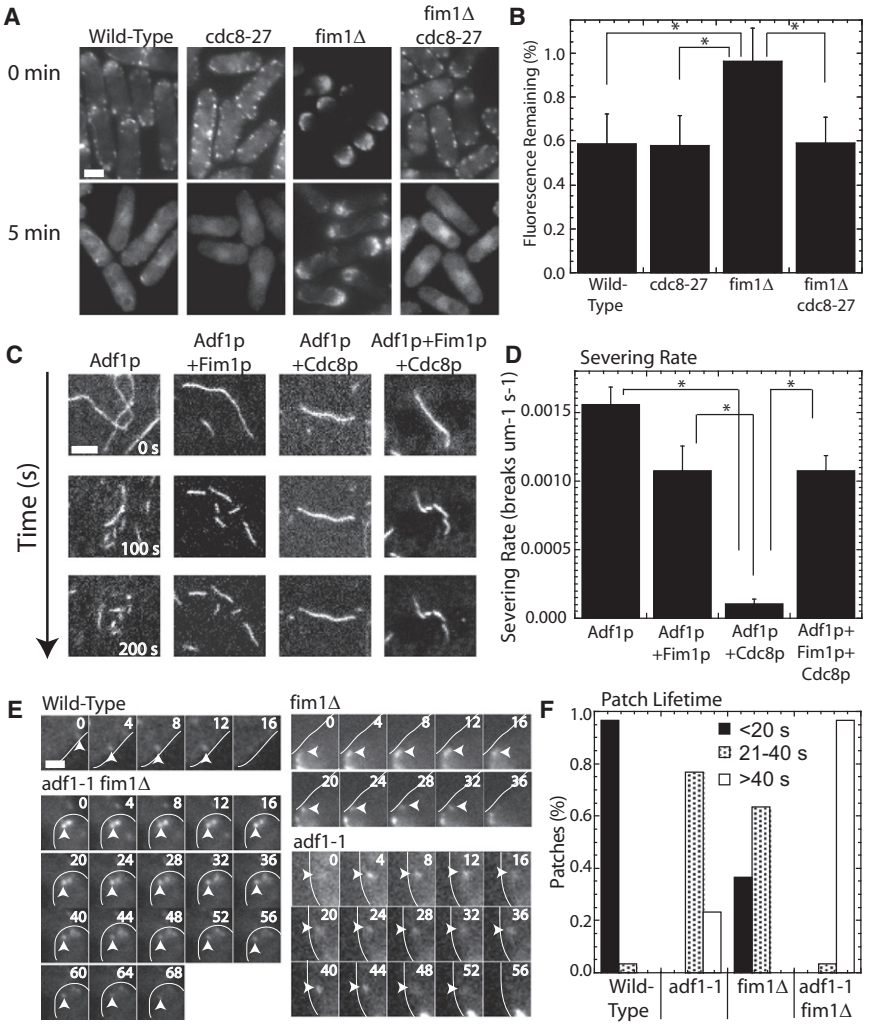

Figure 6. Fim1p Allows Adf1p-Mediated Severing in the Presence of Cdc8p

(A and B) Effect of 100 μM latrunculin A on F-actin in wild-type, *cdc8-27*, *fim1Δ*, and *fim1Δ cdc8-27* cells.

(A) Micrographs before and 5 min after addition of latrunculin A. Bar represents 5 μm.

(B) Average GFP-CHD(rng2) fluorescence at 20 cell poles. Values are mean ± standard deviation. *$p < 0.05$.

(C and D) Total internal reflection fluorescence microscopy observation of Adf1p-mediated severing. Scale bar represents 5 μm. Initially, 1.5 μM Mg-ATP-actin (33% labeled with Oregon green) was assembled in the presence of the indicated proteins. At time 0, 150 nM Adf1p was flowed into the chamber. See Movie S4.

(C) Time-lapse micrographs of the indicated reactions. Scale bar represents 5 μm.

(D) Plot of the severing rate (breaks μm^{-1} sec^{-1}) in the presence of Fim1p and/or Cdc8p (n = 15 filaments per condition). Values are mean ± standard deviation. *$p < 0.05$.

(E and F) Mutations in Fim1p and Adf1p have synergistic effects on actin patch dynamics. The indicated cells expressing GFP-CHD(rng2) at 25°C are shown.

(E) Time-lapse micrographs of representative actin patch (wedges) lifetimes (s). Scale bar represents 1.5 μm. See Movie S5.

(F) Plot of actin patch lifetime binned in 20 s intervals (n = 30 patches for each strain).

turnover at both endocytic actin patches and at assembling contractile rings by antagonizing Cdc8p, which in part "deprotects" actin filaments from cofilin Adf1p-mediated severing (Figure 7). Competition between Fim1p and Cdc8p represents a functionally novel paradigm for controlling actin dynamics and architecture in cells by selecting access of other proteins to filaments. In addition to regulating severing, the balance between Fim1p and Cdc8p might also delineate actin filament access for a range of other functionally important proteins, including nucleation factors (Arp2/3 complex and formin), myosin motors (Clayton et al. [22], this issue of *Current Biology*), and filament crosslinkers (α-actinin and formin).

Role of Fim1p in Endocytosis

Endocytic actin patches in yeast are thought to be structurally and functionally similar to the leading edge of a migrating cell, where rapid Arp2/3 complex-mediated assembly of short-branched actin filaments coupled to cofilin-mediated actin filament disassembly pushes clathrin-coated vesicles inward from the cortex [23–25]. Fimbrin is a major component of actin patches in both budding yeast and fission yeast [3, 4, 26, 27]. In fission yeast, the concentration of Fim1p at actin patches (100 μM) is almost 20 times the global cytoplasmic concentration [10]. Loss of fimbrin in both yeasts results in endocytosis failure because actin patches exhibit severe kinetic defects, including a doubled lifetime and failure to move away from the cortex (Figure 3; [27, 28]).

Given that fission yeast Fim1p and budding yeast fimbrin Sac6 belong to a well-established class of filament crosslinkers [4, 29], the simplest interpretation is that fimbrin's role for endocytic patches is to crosslink actin filaments. However, we found that the removal of Fim1p results in ectopic association of Cdc8p with actin patches (Figure 2). Importantly, reducing the concentration of Cdc8p with a temperature-sensitive mutant largely corrects actin patch dynamics in *fim1Δ cdc8-27* double mutant cells (Figure 4). Therefore, the presence of Cdc8p is a major contributor to patch defects in the absence of Fim1p. This is consistent with previous reports indicating that budding yeast actin patches are composed of short-branched unbundled filaments [30]. Because Arp2/3 nucleates branches at a 70° angle relative to the mother filaments and because the combined actions of actin capping protein and cofilin keep filament lengths at about 50 nm, fimbrin may not be able to form crosslinks in patches.

On the other hand, given that endocytosis of the lipophilic dye FM4-64 is not fully recovered in the *fim1Δ cdc8-27* double mutant (Figure 4D), the crosslinking activity of Fim1p is likely important. Recent evidence from budding yeast suggests that actin bundling is important for functional endocytic patches [31]. Alternatively, it is possible that residual amounts of Cdc8p remain on patches at the semipermissive temperature of 30°C or that Fim1p is also required to regulate association of additional actin filament-binding proteins such as α-actinin, transgelin, or IQGAP with patches [4, 9, 32].

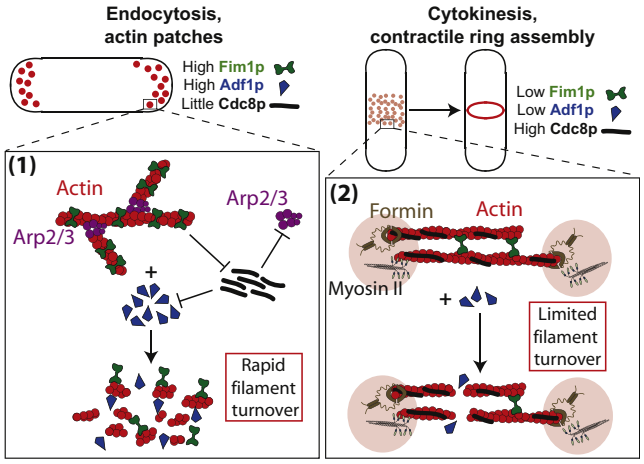

Figure 7. Cartoon Model for Regulation of Adf1p-Mediated Severing by Fim1p and Cdc8p in Fission Yeast

(1) At endocytic actin patches, high concentrations of Fim1p allow efficient Adf1p-mediated severing by preventing Cdc8p from binding to Arp2/3-nucleated actin filaments. In the absence of Fim1p, ectopic localization of Cdc8p to patches slows patch dynamics by inhibiting filament severing. Cdc8p may also inhibit actin filament nucleation by Arp2/3.

(2) During contractile ring assembly, lower concentrations of Fim1p allow limited actin filament severing by partially inhibiting Cdc8p. Previous studies have suggested that severing is necessary for contractile ring assembly despite the presence of high concentrations of Cdc8p [5, 21].

Roles of Cdc8p and Fim1p in Cytokinesis

Tropomyosin Cdc8p is essential for cytokinesis (Figure 2; Figure 5) [2, 6, 13], whereas it has been unclear whether Fim1p plays any role in the assembly or constriction of the contractile ring. Fim1p localizes to the division site, and deletion of fim1 is synthetically lethal with deletion of the contractile ring bundling protein α-actinin [3, 4]. Our results demonstrate that the balance between Fim1p and Cdc8p is important for contractile ring assembly, possibly to allow limited Adf1p-mediated actin filament severing (Figure 7).

One hypothesis is that contractile ring assembly in fission yeast occurs by the coalescence of ~65 prering cytokinesis nodes, which are interconnected through the activities of node components formin Cdc12p and myosin-II Myo2p [14, 16, 21]. Cdc12p stimulates actin filament assembly at nodes and remains processively associated with the elongating filament [33]. Myo2p on adjacent nodes captures the pointed end of Cdc12p-associated actin filaments, and nodes are pulled together through successive rounds of transient Myo2p-mediated pulling and breaking of the node connections [14]. The balance between periods of pulling and detachment is critical [21, 34].

Node connection could be broken by dissociation of either Myo2p or Cdc12p from the filament or by cofilin Adf1p-mediated filament severing. The importance of Adf1p-mediated severing in contractile ring assembly has been established [5, 21]. Our results indicate that filament severing could be regulated by limited removal of inhibitory Cdc8p by Fim1p (Figure 7). Supporting this, overexpression of Fim1p results in loss of the contractile ring and severe cytokinesis defects (unpublished data) [3, 4]. However, because *fim1*Δ cells do not have major cytokinesis defects, we predict that other contractile ring components such as α-actinin and IQGAP Rng2p may also regulate Adf1p-mediated severing.

In addition to inhibiting severing (Figure 6) [5], Cdc8p also regulates the cytokinesis myosin motor Myo2p and the cytokinesis formin Cdc12p [6, 8, 17]. Cdc8p theoretically lowers the number of Myo2p molecules required for processive coalescence of the contractile ring cytokinesis nodes by stabilizing the strong actin-bound state of Myo2p [17]. On the other hand, Cdc8p also allows Cdc12p-associated filaments to elongate twice as fast and to anneal end to end [8]. Therefore, Cdc8p appears to influence multiple aspects of contractile ring assembly in fission yeast.

Because the relative amounts of actin-binding proteins in particular cellular structures are critical, it is interesting to consider what regulates the amount of Fim1p and Cdc8p in the contractile ring. Endocytic actin patches may serve as sinks that accumulate Fim1p, and/or Fim1p may have an increased affinity for Arp2/3 complex-dependent branched actin filaments. Additionally, contractile ring proteins such as the actin filament crosslinking proteins α-actinin and IQGAP Rng2p may inhibit Fim1p. Conversely, Cdc8p prefers contractile ring filaments to actin patch filaments (Figure 2E), suggesting that Cdc8p is positively recruited to the contractile ring. Cdc8p binds more tightly to actin filaments nucleated by the cytokinesis formin Cdc12p [8], but other factors may also contribute to the recruitment of Cdc8p to the contractile ring.

Interaction among Actin-Binding Proteins Determines Actin Architecture

We revealed a novel paradigm for the regulation of cofilin-mediated actin severing through the competition between actin filament side-binding proteins fimbrin and tropomyosin. Additionally, these results demonstrate that interpretation of mutant phenotypes of actin-binding proteins can be extremely complicated. It may be necessary to determine how deletion of one actin-binding protein affects the localization of others, not only the proteins thought to play a role in the same process. In addition to Fim1p, Cdc8p, and Adf1p, fission yeast contains many other proteins that bind to the side of actin filaments and localize to specific cellular structures, including Arp2/3 complex, myosin, α-actinin, transgelin Stg1p, IQGAP Rng2p, coronin, and Aip1. Therefore, in addition to cofilin-mediated severing and myosin motor activity [22], numerous complex interactions remain to be sorted. Our results shed light on a mechanism by which interaction between actin-binding proteins may control global actin organization and dynamics.

Experimental Procedures

Microscopy of Actin Patches and Cytokinesis

For actin patch dynamics, cells grown in Edinburgh minimal media (EMM) liquid media were spotted onto EMM pads containing 25% gelatin on glass slides, sealed with VALAP, and imaged in the Z direction with 0.1 μm slices or in time-lapse every 165 ms in the GFP channel. Analysis of patch dynamics was quantified for 25 randomly chosen cells for each strain using ImageJ software (http://rsbweb.nih.gov/ij/). Only patches that originated and disappeared during the course of the movie were quantified. For confocal microscopy of actin patch dynamics, images were acquired with a 403/0.75 NA objective on a Zeiss Axiovert 200M equipped with a Yokogawa CSU-10 spinning-disk unit (McBain) and illuminated with a 50 mW 473 nm DPSS laser (Cobolt). Time-lapse acquisitions were captured at 1.2 s intervals on a Cascade 512B electron-multiplying charge-coupled device camera (Photometrics) with MetaMorph (Molecular Devices). Patch dynamics were quantified with ImageJ and previously described software [35]. Final numbers of patches analyzed per strain are indicated in the figure legends.

For cytokinesis, time-lapse imaging and analysis have been reported [15, 36]. Cells were spotted onto EMM pads containing 25% gelatin, sealed with VALAP, and imaged once per minute in both DIC and GFP channels for

at least 2 hr. Analysis of ring assembly was quantified for at least 10 randomly chosen cells for each strain using ImageJ software. Temperature was controlled using an air stream stage incubator (Nevtek) or an objective heater (BiOptechs).

Additional experimental procedures can be found in the Supplemental Experimental Procedures online.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, five figures, one table, and five movies and can be found with this article online at doi:10.1016/j.cub.2010.06.020.

Acknowledgments

The authors would like to thank D. Mulvihill (University of Kent, UK), I. Mabuchi (Gakushuin University, Japan), J.-Q. Wu (The Ohio State University), V. Sirotkin (The State University of New York, Upstate), M. Lord (Vermont), M. Glotzer (The University of Chicago), B. Galletta (Washington University, St. Louis), and members of the Kovar laboratory for strains, technical assistance, and insightful comments. This work was supported in part by National Institutes of Health (NIH) grant RO1GM079265 (to D.R.K.) and an NIH Molecular and Cellular Biology Training Grant T32 GM007183 (to C.T.S.).

Received: March 31, 2010
Revised: May 31, 2010
Accepted: June 10, 2010
Published online: August 12, 2010

References

1. La Carbona, S., Le Goff, C., and Le Goff, X. (2006). Fission yeast cytoskeletons and cell polarity factors: Connecting at the cortex. Biol. Cell *98*, 619–631.
2. Balasubramanian, M.K., Helfman, D.M., and Hemmingsen, S.M. (1992). A new tropomyosin essential for cytokinesis in the fission yeast S. pombe. Nature *360*, 84–87.
3. Nakano, K., Satoh, K., Morimatsu, A., Ohnuma, M., and Mabuchi, I. (2001). Interactions among a fimbrin, a capping protein, and an actin-depolymerizing factor in organization of the fission yeast actin cytoskeleton. Mol. Biol. Cell *12*, 3515–3526.
4. Wu, J.Q., Bähler, J., and Pringle, J.R. (2001). Roles of a fimbrin and an alpha-actinin-like protein in fission yeast cell polarization and cytokinesis. Mol. Biol. Cell *12*, 1061–1077.
5. Nakano, K., and Mabuchi, I. (2006). Actin-depolymerizing protein Adf1 is required for formation and maintenance of the contractile ring during cytokinesis in fission yeast. Mol. Biol. Cell *17*, 1933–1945.
6. Skoumpla, K., Coulton, A.T., Lehman, W., Geeves, M.A., and Mulvihill, D.P. (2007). Acetylation regulates tropomyosin function in the fission yeast Schizosaccharomyces pombe. J. Cell Sci. *120*, 1635–1645.
7. Arai, R., Nakano, K., and Mabuchi, I. (1998). Subcellular localization and possible function of actin, tropomyosin and actin-related protein 3 (Arp3) in the fission yeast Schizosaccharomyces pombe. Eur. J. Cell Biol. *76*, 288–295.
8. Skau, C.T., Neidt, E.M., and Kovar, D.R. (2009). Role of tropomyosin in formin-mediated contractile ring assembly in fission yeast. Mol. Biol. Cell *20*, 2160–2173.
9. Takaine, M., Numata, O., and Nakano, K. (2009). Fission yeast IQGAP arranges actin filaments into the cytokinetic contractile ring. EMBO J. *28*, 3117–3131.
10. Wu, J.Q., and Pollard, T.D. (2005). Counting cytokinesis proteins globally and locally in fission yeast. Science *310*, 310–314.
11. Martin, S.G., and Chang, F. (2006). Dynamics of the formin for3p in actin cable assembly. Curr. Biol. *16*, 1161–1170.
12. Sirotkin, V., Beltzner, C.C., Marchand, J.B., and Pollard, T.D. (2005). Interactions of WASp, myosin-I, and verprolin with Arp2/3 complex during actin patch assembly in fission yeast. J. Cell Biol. *170*, 637–648.
13. Nurse, P., Thuriaux, P., and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. Mol. Gen. Genet. *146*, 167–178.
14. Pollard, T.D., and Wu, J.Q. (2010). Understanding cytokinesis: Lessons from fission yeast. Nat. Rev. Mol. Cell Biol. *11*, 149–155.
15. Wu, J.Q., Kuhn, J.R., Kovar, D.R., and Pollard, T.D. (2003). Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. Dev. Cell *5*, 723–734.
16. Wu, J.Q., Sirotkin, V., Kovar, D.R., Lord, M., Beltzner, C.C., Kuhn, J.R., and Pollard, T.D. (2006). Assembly of the cytokinetic contractile ring from a broad band of nodes in fission yeast. J. Cell Biol. *174*, 391–402.
17. Stark, B.C., Sladewski, T.E., Pollard, L.W., and Lord, M. (2010). Tropomyosin and myosin-II cellular levels promote actomyosin ring assembly in fission yeast. Mol. Biol. Cell *21*, 989–1000.
18. Lin, M.C., Galletta, B.J., Sept, D., and Cooper, J.A. (2010). Overlapping and distinct functions for cofilin, coronin and Aip1 in actin dynamics in vivo. J. Cell Sci. *123*, 1329–1342.
19. Bernstein, B.W., and Bamburg, J.R. (1982). Tropomyosin binding to F-actin protects the F-actin from disassembly by brain actin-depolymerizing factor (ADF). Cell Motil. *2*, 1–8.
20. Ono, S., and Ono, K. (2002). Tropomyosin inhibits ADF/cofilin-dependent actin filament dynamics. J. Cell Biol. *156*, 1065–1076.
21. Vavylonis, D., Wu, J.Q., Hao, S., O'Shaughnessy, B., and Pollard, T.D. (2008). Assembly mechanism of the contractile ring for cytokinesis by fission yeast. Science *319*, 97–100.
22. Clayton, J.E., Sammons, M.R., Stark, B.C., Hodges, A.R., and Lord, M. (2010). Differential regulation of unconventional fission yeast myosins via the actin track. Curr. Biol. *20*, 1423–1431.
23. Engqvist-Goldstein, A.E., and Drubin, D.G. (2003). Actin assembly and endocytosis: From yeast to mammals. Annu. Rev. Cell Dev. Biol. *19*, 287–332.
24. Galletta, B.J., and Cooper, J.A. (2009). Actin and endocytosis: Mechanisms and phylogeny. Curr. Opin. Cell Biol. *21*, 20–27.
25. Kaksonen, M., Toret, C.P., and Drubin, D.G. (2006). Harnessing actin dynamics for clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. *7*, 404–414.
26. Adams, A.E.M., Botstein, D., and Drubin, D.G. (1991). Requirement of yeast fimbrin for actin organization and morphogenesis in vivo. Nature *354*, 404–408.
27. Kübler, E., and Riezman, H. (1993). Actin and fimbrin are required for the internalization step of endocytosis in yeast. EMBO J. *12*, 2855–2862.
28. Kaksonen, M., Toret, C.P., and Drubin, D.G. (2005). A modular design for the clathrin- and actin-mediated endocytosis machinery. Cell *123*, 305–320.
29. Matsudaira, P. (1991). Modular organization of actin crosslinking proteins. Trends Biochem. Sci. *16*, 87–92.
30. Young, M.E., Cooper, J.A., and Bridgman, P.C. (2004). Yeast actin patches are networks of branched actin filaments. J. Cell Biol. *166*, 629–635.
31. Gheorghe, D.M., Aghamohammadzadeh, S., Smaczynska-de Rooij, I.I., Allwood, E.G., Winder, S.J., and Ayscough, K.R. (2008). Interactions between the yeast SM22 homologue Scp1 and actin demonstrate the importance of actin bundling in endocytosis. J. Biol. Chem. *283*, 15037–15046.
32. Nakano, K., Bunai, F., and Numata, O. (2005). Stg 1 is a novel SM22/transgelin-like actin-modulating protein in fission yeast. FEBS Lett. *579*, 6311–6316.
33. Kovar, D.R., and Pollard, T.D. (2004). Insertional assembly of actin filament barbed ends in association with formins produces piconewton forces. Proc. Natl. Acad. Sci. USA *101*, 14725–14730.
34. Coffman, V.C., Nile, A.H., Lee, I.J., Liu, H., and Wu, J.Q. (2009). Roles of formin nodes and myosin motor activity in Mid1p-dependent contractile-ring assembly during fission yeast cytokinesis. Mol. Biol. Cell *20*, 5195–5210.
35. Carlsson, A.E., Shah, A.D., Elking, D., Karpova, T.S., and Cooper, J.A. (2002). Quantitative analysis of actin patch movement in yeast. Biophys. J. *82*, 2333–2343.
36. Kovar, D.R., Wu, J.Q., and Pollard, T.D. (2005). Profilin-mediated competition between capping protein and formin Cdc12p during cytokinesis in fission yeast. Mol. Biol. Cell *16*, 2313–2324.