Insight into Actin Organization and Function in Cytokinesis from Analysis of Fission Yeast Mutants

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ABSTRACT Actin is a key cytoskeletal protein with multiple roles in cellular processes such as polarized growth, cytokinesis, endocytosis, and cell migration. Actin is present in all eukaryotes as highly dynamic filamentous structures, such as linear cables and branched filaments. Detailed investigation of the molecular role of actin in various processes has been hampered due to the multifunctionality of the protein and the lack of alleles defective in specific processes. The actin cytoskeleton of the fission yeast, *Schizosaccharomyces pombe*, has been extensively characterized and contains structures analogous to those in other cell types. In this study, primarily with the view to uncover actin function in cytokinesis, we generated a large bank of fission yeast actin mutants that affect the organization of distinct actin structures and/or discrete physiological functions of actin. Our screen identified 17 mutants with specific defects in cytokinesis. Some of these cytokinesis mutants helped in dissecting the function of specific actin structures during ring assembly. Further genetic analysis of some of these actin mutants revealed multiple genetic interactions with mutants previously known to affect the actomyosin ring assembly. We also characterize a mutant allele of actin that is suppressed upon overexpression of Cdc8p-tropomyosin, underscoring the utility of this mutant bank. Another 22 mutant alleles, defective in polarized growth and/or other functions of actin obtained from this screen, are also described in this article. This mutant bank should be a valuable resource to study the physiological and biochemical functions of actin.

ACTIN is a small globular protein (~42 kDa) and is among the most conserved proteins in eukaryotes. In cells, actin transitions between the monomeric G-actin and filamentous F-actin forms that are regulated by the binding of ATP and the hydrolysis of ATP to ADP. The actin cytoskeleton in most eukaryotes comprises two major kinds of filamentous structures: Formin-nucleated unbranched linear filaments and the Arp2/3 complex-nucleated branched filaments (Pollard 2007). Over 100 different accessory proteins function in eukaryotic cells to regulate the assembly, turnover, and function of actin (Pollard and Borisy 2003). The

various F-actin structures play important roles in different cellular processes such as polarized growth, targeted secretion, endocytosis, and cytokinesis (Chhabra and Higgs 2007).

Although actin has been extensively studied over decades, the precise molecular basis of each distinct function of actin still remains unclear. While inhibitory drugs such as Latrunculin A (LatA), Cytochalasin A (CytA), and Cytochalasin D (CytD) that affect the dynamics of actin monomers and/or polymers have provided valuable insights, these inhibitors affect all aspects of actin function (Cooper 1987; Ayscough *et al.* 1997; Allingham *et al.* 2006; Uribe and Jay 2009). One approach to characterize distinct functions of actin involves the generation of mutant alleles specifically defective in individual actin-requiring processes. Such an allelic series has been isolated in the budding yeast *Saccharomyces cerevisiae* (Wertman *et al.* 1992). The actin mutants of budding yeast have been valuable in terms of understanding endocytosis, polarity, etc. (Kubler and Riezman 1993;

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Belmont and Drubin 1998; Kaksonen *et al.* 2005). However, studies of cytokinesis using the Wertman mutant bank have been relatively scarce.

The fission yeast, *Schizosaccharomyces pombe* (*S. pombe*) is an attractive model organism to study the actin cytoskeleton and its specific role in cytokinesis, since it divides using an actomyosin-based contractile ring. *S. pombe* cells are cylindrical and grow by tip extension through the use, in part, of actin cytoskeletal motor-based transport and actin-mediated endocytosis (Motegi *et al.* 2001; Castagnetti *et al.* 2005; Gachet and Hyams 2005; Ge *et al.* 2005; Lo Presti and Martin 2011). In fission yeast, actin is present in different cytoskeletal structures, such as patches containing branched actin filaments, cables, and the cytokinetic ring composed of linear and unbranched actin filaments (Marks *et al.* 1986; Machesky and Gould 1999; Feierbach and Chang 2001; Noguchi and Mabuchi 2001; Arai and Mabuchi 2002; Nakano *et al.* 2002; Kovar *et al.* 2003). These features combined with the ease of genetic manipulation and the availability of mutants affecting ~40 proteins involved in cytokinesis provide an opportunity to dissect actin function in all aspects of cell physiology, particularly in cytokinesis.

In this study, we utilized a highly efficient approach known as marker reconstitution mutagenesis (Tang *et al.* 2011) to generate an allelic series of actin mutants. We have identified 39 mutant alleles with amino acid changes distributed throughout the protein and, in most cases, defective in distinct cellular processes. Our mutant bank greatly expands the tools available to study the actin cytoskeleton (Ishiguro and Kobayashi 1996; Mccollum *et al.* 1999) and should facilitate the analysis of actin function, organization, and regulation.

Materials and Methods

Strains and medium

The *S. pombe* strains used in this study were grown on either yeast extract supplements (YES) medium or synthetic minimal medium (MM) as described elsewhere (Moreno *et al.* 1991). For the starvation experiment, minimal medium lacking nitrogen was used as described by Wang *et al.* (2002). A lithium acetate-based method was used for transformation (Okazaki *et al.* 1990). The *Escherichia coli* strain used for all cloning was XL1-blue.

Mutagenesis of *act1*⁺

Marker reconstitution mutagenesis was used to screen for mutants as described by Tang *et al.* (2011). First, a fusion PCR fragment was amplified using four primers (GGCGGA GATATC GTTTTC TTGCTC TGTTTT C, GGTACC ACCAGC TGAAGA TGATAC AACTCT AC, CATCTT CAGCTG GTGGTA CCACTA TGTATC, and GCTATA CGATAT CCAGAT CTACCC AAAGTT CCTCAT GAG, as p1, p2, p3, and p4, respectively), digested with *Bgl*II and *Eco*RV, and then subcloned into the *Nru*I-*Bgl*II site of *p208H5cdU4*⁺, generating *p208-act1*. This

plasmid was digested with *Pvu*II and then transformed into MBY6218 (*h*[−], *his5D leu1-32 ura4-D18 ade5D ade7Δ::ade5*⁺). Transformants were selected on minimal medium without uracil and insertion of *h5cd* at the 3'-UTR of *act1* was confirmed, generating a new strain MBY6501 (*h*[−], *his5D leu1-32 ura4-D18 ade5D ade7Δ::ade5*⁺ *act1*⁺::*his5cd::ura4*⁺). Another PCR fragment of *act1* whole gene was amplified using primers p5 (GTGCTAACGCTGTGTGTGG) and p4 and was subcloned into the *Nru*I-*Bgl*II site of *p205hc*⁺. The *act1-his5c*⁺ fragment was generated by mutagenic PCR, using primers p5 and p0. The PCR product then was transformed into yeast strain MBY6501. Transformants were selected on minimal medium without histidine at 24° and replicated onto YES with Phloxin B at 36°. Mutants were screened as colonies showing dark red on YES with Phloxin B at 36°. Mutants were backcrossed with MBY6217 (*h*⁺, *his5D leu1-32 ura4-D18 ade5D ade7Δ::ade5*⁺) to make sure mutations were tightly linked to *his5*⁺ and *ura4*⁺. Fragments of the actin gene were amplified from the genomic DNA of the mutants and sequenced.

Multicopy suppressor screen of *act1-329*

The *act1-329* mutant was transformed with *S. pombe* genomic libraries, pTN-L1 (Nakamura *et al.* 2001). The *Leu*⁺ transformants were selected at 24° and then replicated to grow on YES with Phloxin B at 36°. Two plasmids from two colonies were isolated and further sequenced. Both plasmids contained the *cdc8*⁺ gene. To confirm the suppression of *act1-329* by *cdc8*⁺, a plasmid was constructed by inserting the *cdc8*⁺ gene into the empty library vector. A pair of primers (1, GGC GGA CTCGAG GGAAAG TGGTGG GAATCG G; 2, GGGAGG ATCCAA CTAATTC CTAGTC TGTATG) was used to amplify a 1.4-kb fragment of the *cdc8*⁺ gene. The PCR product was digested with *Xho*I and *Bam*HI and then subcloned into the *Xho*I-*Bam*HI site of the empty library vector, generating pLEU2-*cdc8*. This plasmid and the vector as a control were separately transformed into two strains (*act1-329 leu1-32* and *act1-329 leu1-32, his1*⁺::*Pact1*::LAGFP) to confirm the suppression.

Microscopy and data analysis

For single time-point imaging, cells were directly put on a glass slide and imaged at the respective temperature. For time-lapse movies, midlog-phase cells were concentrated by centrifugation at 3000 rpm for 30 sec to 1 min. One microliter of cells was placed on a glass slide with either YES + 2% agarose pad or selection medium + 2% agarose pad and then sealed under a coverslip, using vaseline:lanolin:paraffin 1:1:1 (VALAP), and imaged at the respective temperature. For time-lapse movies at 36°, temperature was controlled and maintained by a full enclosure incubation chamber. Confocal images were acquired by an optimized spinning-disk microscope [Zeiss (Thornwood, NY) Axiovert 200M microscope with Plan-APOCHROMAT 100×/1.4 oil objective lens, a Yokogawa CSU-21 spinning-disk system, a Hamamatsu ORCA-ER camera, and MetaMorph software].

A Cobolt Calypso 491-nm solid-state laser and a Cobolt Jive 561-nm solid-state laser were used for excitation. Spinning-disk images were collected in three-dimensional (3D) mode (0.5 μm step size) with a time interval of 30 sec. Bright-field and epifluorescence images were captured using an Olympus IX71 microscope (PlanApo 100 $\times$ /1.45 oil objective lens) equipped with a Photometrics CoolSNAP HQ CCD camera and Metamorph software (Molecular Devices). Epifluorescence images were collected either in two-dimensional (2D) mode or in 3D mode (both with 0.5 μm step size). Images were analyzed using either ImageJ (<http://rsb.info.nih.gov/ij/>) or Metamorph. All 3D images were shown by 2D maximum-intensity projection. To visualize DNA, F-actin, and septum material, cells were fixed in 3.7% formaldehyde for 1 min and stained with 4',6-diamidino-2-phenylindole (DAPI), Alexa488 phalloidin, and calcofluor (Sigma, St. Louis), respectively, as described in Balasubramanian *et al.* (1997). The Spot function of Bitplane Imaris Software 7.6.1 was used to estimate the numbers of actin and coronin 1 patches in wild-type (WT) and *act1*-135 mutant cells.

Immunostaining

For all indirect immunofluorescence (Hagan and Hyams 1988), cells were fixed in formaldehyde and probed with anti-cdc4 antibodies (1:100 dilution; Molecular Probes, Eugene, OR), and anti-tubulin antibodies (1:200 dilution; a kind gift from Keith Gull, University of Oxford, Oxford, United Kingdom). Secondary antibodies of anti-rabbit and anti-mouse IgG conjugates (Molecular Probes) were used at a 1:200 dilution.

Synchronization by nitrogen starvation

Nonauxotrophic actin mutants were generated by crossing each mutant to the WT strain 972 (MBY99). Actin mutants and WT cells were grown in minimal medium overnight at 24 $^{\circ}$ to log phase ($\text{OD}_{595} < 0.4$). Cells were washed three times with minimal medium lacking nitrogen, resuspended in the same medium, and grown for 18 h at 24 $^{\circ}$ to arrest in the G₀ phase (Su *et al.* 1996). The culture was split into two parts. One part was shifted to 36 $^{\circ}$ for 1 hr and then transferred into YES (rich medium) to release cells from G₀ and allow the cells to recover from the arrest and regain polarized growth at 36 $^{\circ}$. The same was done for the second part of cells grown at 24 $^{\circ}$. Samples were taken just before the release (time-point 0) and every hour after release to assess the recovery of polarity and growth (Wang *et al.* 2002).

Results

Actin mutant screen using marker reconstitution mutagenesis

To isolate an allelic series of actin mutants in fission yeast, we performed a screen using marker reconstitution mutagenesis (Tang *et al.* 2011), a novel and highly efficient reverse genetics approach. As shown in Figure 1A, a mutagenic PCR fragment of *act1*-*his5c* (the whole *act1*⁺ gene and the

Figure 1 Screening for actin mutants, using marker reconstitution mutagenesis. (A) Schematic representation of marker reconstitution mutagenesis. Chromosomal integration of *his5cΔ* adjacent to *act1*⁺ was made using another selection marker, *ura4*⁺ (not shown). Mutation was represented as a four-angle star. (B) Spot assay comparing the viability of WT cells to that of the different actin mutants. Cultures of WT and mutants grown overnight at 24 $^{\circ}$ were serially diluted fivefold five times and the last two dilutions were spotted on YES agar plates and incubated at various growth temperatures. (C) Projection of all the point mutations on the predicted structure of actin, using the PyMOL Molecular Graphics System Version 1.2.8 (red denotes the sites mutated in mutants with a single amino acid change while yellow denotes sites mutated in mutants with more than one amino acid change and purple denotes the same site mutated in single as well as double mutants). (D) Schematic representation of the distribution of mutations over the open reading frame of the *act1* gene.

C-terminal part of the *his5*⁺ gene) was transformed into a strain with an integrated *his5cΔ* fragment (a fragment of *his5*⁺ with a 50-bp C-terminal part truncation) adjacent to the endogenous *act1*⁺ locus. Random mutations in the actin gene that were generated by mutagenic PCR were thus introduced into the chromosome through homologous recombination. This simultaneously reconstitutes *his5*⁺ by joining *his5cΔ* and *his5c* under the selection for *his5*⁺. Three independent rounds of screening were performed. From ~4000 *his5*⁺ transformants, we collected 67 stable temperature-sensitive mutants displaying phenotypes in cell division and/or cell morphology.

All the mutants were backcrossed with a WT strain to ensure that the mutations were tightly and stably linked to *his5*⁺. Most of the backcrossed strains were severely defective for growth and colony formation at 36 $^{\circ}$ (Figure 1B). Furthermore, microscopic examination of the strains that appeared to grow at 36 $^{\circ}$ (*act1*-115, *act1*-j34, and *act1*-d8), revealed that these strains were accumulating cell mass, but were still defective in processes such as cytokinesis and polarized growth (described in later sections). Genomic DNA was isolated from the mutants and the nucleotide sequence of the relevant region was determined. From these 67 mutants, we obtained 39 unique mutant alleles. All these alleles had at least one missense mutation in the coding sequence; some alleles had up to four mutations (Table 1). The mutations

Table 1 Classification of actin mutants

Allele <i>act1</i> -	Amino acid change	Temperature sensitivity (36°) ^a	Phenotype at 36°
111	S281P (Sd3)	L	Cytokinesis
112	F352L (Sd1)	L	Polarized growth
115	V96I (Sd1), I212T (Sd4), E276A (Sd3), Q318R (Sd3)	NL	Polarized growth
116	P27S (Sd1)	L	Polarized growth
117	Y362C (Sd1)	L	Multiple
121	I274T (Sd3), F352L (Sd1)	L	Multiple
123	D222G (Sd4), F255L (Sd4)	L	Multiple
125	V219G (Sd4)	L	Multiple
126	Y218N (Sd4)	L	Cytokinesis
128	T304P (Sd3)	L	Multiple
135	A22P (Sd1)	L	Multiple
138	I282N (Sd3)	L	Cytokinesis
145	H275R (Sd3), I309V (Sd3)	L	Cytokinesis
303	D81N (Sd1), V219A (Sd4), E237G (Sd4)	L	Polarized growth
313	F31S (Sd1)	L	Polarized growth
323	V96I (Sd1)	L	Polarized growth
327	K315E (Sd3)	L	Cytokinesis
328	R37G (Sd2), D80G (Sd1), L185S (Sd4)	L	Multiple
329	K291I (Sd3)	L	Cytokinesis
d1	D157G (Sd3)	L	Cytokinesis
d3	H88D (Sd1)	NL	Polarized growth
d5	S350F (Sd1)	NL	Polarized growth
d8	S52P (Sd2)	NL	Polarized growth
d13	E276G (Sd3), Y362H (Sd1)	NL	Cytokinesis
d14	I208V (Sd4)	NL	Cytokinesis
d16	S235P (Sd4)	NL	Polarized growth
j15	E259A (Sd4)	L	Multiple
j16	S348P (Sd1)	L	Cytokinesis
j21	N111S (Sd1)	NL	Cytokinesis
j23	M82T (Sd1), A135V (Sd1)	L	Polarized growth
j25	K373E (Sd1)	NL	Cytokinesis
j27	K68E (Sd2), I85T (Sd1), F132L (Sd1)	NL	Multiple
j28	F200S (Sd4), G308S (Sd3)	L	Cytokinesis
j30	F31L (Sd1), S60G (Sd2), M190I (Sd4)	L	Polarized growth
j32	F127L (Sd1), I309T (Sd3)	NL	Cytokinesis
j34	L67S (Sd2)	NL	Cytokinesis
j35	I250V(Sd4)	NL	Cytokinesis
j36	K50R (Sd2), I309T (Sd3)	L	Polarized growth
j39	L105S (Sd1), K215I (Sd4)	NL	Cytokinesis

^a L, lethal; NL, nonlethal.

were distributed evenly throughout the actin gene and the corresponding amino acid changes were scattered over the entire protein (Figure 1, C and D). Some of these mutations were on the surface of actin. Other mutations were situated in the interior of actin in positions where they could destabilize surface features or actin–actin subdomain interactions. Only a minor number of mutations lie in the core hydrophobic regions within a subdomain. Interestingly, one allele (*act1*-d1) with a point mutation next to the ATP binding pocket was also identified from this screen. Taken together, these mutations are likely to affect actin conformational plasticity and its interactions with itself and with other regulating partners. Hence, this screen was efficient in identifying many novel functionally compromised actin mutant alleles.

Classification of the actin mutants

Given the multifunctional nature of actin, we broadly classified mutants into three classes based on three major

phenotypes: mutants with defects in cytokinesis, mutants with defects in polarized growth/morphology, and those with multiple defects (Table 1 and [Supporting Information, Table S1](#)). To characterize these mutants, we fixed cells at both permissive and restrictive temperatures and stained them with aniline blue and DAPI, to visualize cell wall/septum and nuclei, respectively.

As shown in Figure 2 (top) and Table 1, the first group of mutants shows cytokinetic defects. In this class, polarized growth appeared to be normal but cytokinesis was defective (*act1*-126, *act1*-j28, and *act1*-j34). Most cells accumulated multiple nuclei after 4 hr at the restrictive temperature ([Figure S1, A and B](#)), were approximately twice as long as WT cells, and showed a phenotype similar to that of other known actomyosin ring assembly/stability mutants (Nurse 1976; Chang *et al.* 1996; Balasubramanian 1998).

The second group of mutants displayed abnormal cell shapes (including spherical/orb and rugby ball shapes) after

Figure 2 Classification of mutants. WT and actin mutant cells were grown at 24° and 36°, fixed with formaldehyde, and stained with DAPI and aniline blue to visualize the nucleus and septum. Class I, mutants with cytokinesis defects; class II, mutants with polarity/morphology defects; class III, mutants with multiple defects.

4 hr at 36°, owing to defects in polarized growth (Figure 2, *act1-303* and *act1-d8*). These mutants often showed minor defects in septum positioning and/or cell separation but were classified based on the most predominant phenotype observable.

The third group of mutants comprised those that showed multiple defects, including cytokinesis and polarized growth. Unlike WT cells that show aniline blue staining at cell ends during interphase, some mutants in this class showed aniline blue staining at the medial region in interphase cells (Figure 2, *act1-123*), suggesting a polarized growth defect. Furthermore, in binucleate cells, septation was found to be impaired, suggesting a defect in completing cytokinesis. Another mutant, *act1-j15* displayed a very thick septal deposition in the medial region (Figure 2, *act1-j15*). This might suggest either a defect in completion of septation during cytokinesis or a defect in reprogramming cell wall deposition to the cell ends upon entering the next cell cycle.

Actin mutants defective in polarized growth

The actin cytoskeleton is vital for fission yeast cells to localize growth to the cell tips and achieve a constant cylindrical shape (Miller and Johnson 1994; Feierbach *et al.* 2004). Thus, actin mutants defective in polarized growth could provide useful information specific to this function of actin. To analyze the ability of the actin mutants to establish polarized growth we performed the nitrogen starvation assay for many of the mutants. Upon nitrogen starvation, fission yeast cells arrest in the cell cycle before S phase, termed G₀. During this arrest cells remain dormant for growth, yet are metabolically active (Su *et al.* 1996). A

switch from a rod shape to a rounded morphology is characteristic of nitrogen-deprived G₀-arrested cells. Subsequently, cells were analyzed for their ability to reestablish polarized growth both at 24° and at 36° in rich medium. As shown in Figure 3A, while WT cells could reestablish a cylindrical shape at both 24° and 36° in rich medium, some actin mutants remained rounded and arrested even after 6 hr at 36° (*act1-135* and *act1-313*). At 24° most G₀-arrested mutants were able to reestablish a cylindrical shape when returned to rich medium. However, *act1-135* and *act1-313* cells reestablished polarized growth more slowly than WT cells even at 24°. To check whether the cytokinesis-defective mutants also have any defects in achieving polarized growth, we performed the nitrogen starvation experiment on selected mutants. Most cytokinesis-defective mutants (such as *act1-126*) were capable of reestablishing polarized growth and achieving a rod shape in rich medium after 4 hr at 36°. However, a few cytokinesis mutants such as *act1-128* also had difficulties in reestablishing polarized growth (Figure 3A).

To understand how polarized growth was affected in these actin mutants, phalloidin staining (Figure 3B) was used to examine the actin structure and organization at 36°. Some mutants showed actin staining evenly distributed around the cell cortex (*act1-135* and *act1-303*), while others displayed an abnormal concentration of actin at random locations in the cell (*act1-d8*, arrow). Thus, it is evident that localization of functional actin at the growing ends of the cell is very important for the rod shape of fission yeast cells.

Immunofluorescence staining for the myosin essential light chain, Cdc4p, in these morphology-defective mutants showed a fairly normal actomyosin ring formation in mitotic cells (Figure 3C). This indicates that these mutants have no defects in the assembly of the actomyosin ring. Although Cdc4p could be visualized as a ring during mitosis in many polarity-defective mutants, occasionally rings were displaced from the medial region as previously reported (Ge *et al.* 2005; Mishra *et al.* 2012). On the whole 13 novel actin mutants were thus isolated that displayed defects in achieving polarized growth. Among these, 8 mutants were inviable at 36° while others were able to grow at the restrictive temperature nevertheless with severe defects.

Actin is required for activated Cdc42p to localize to the growth tip

Polarized growth has been extensively studied in the budding yeast. Actin cables have been reported to be indispensable for polarized growth in budding yeast as well as in fission yeast (Pruyne and Bretscher 2000; Bendezu and Martin 2011). However, in fission yeast mutants that lack actin cables, growth has been shown to occur in a monopolar manner rather than a bipolar pattern of growth seen in WT cells (Bendezu and Martin 2011; Nakano *et al.* 2011). In our study, actin mutants with prominent actin patches showed no defects in polarized growth (Figure 4A, *act1-327*, *act1-329*, and *act1-j39*). The Rho family GTPase, Cdc42p is required for

Figure 3 Mutants defective in polarized growth. (A) Study of reestablishment of polarized growth by mutants upon nitrogen starvation and release. WT and actin mutant cells were first grown in minimal medium (MM) at 24°, subsequently starved in minimal medium without nitrogen source (MM-N2) for 18 hr at 24°, harvested and then regrown in rich medium (YES) at either 36° or 24°, and imaged every 1 hr up to 8 hr. (B) Visualization of actin structures in WT and actin mutant cells. Actin mutants were grown at 36° for 4 hr, fixed, and stained with DAPI (red) and phalloidin (green). (C) Immunofluorescence staining for anti-Cdc4p and anti- α -tubulin to analyze ring formation in mitotic cells. (D) Localization of CRIB-GFP (marker for active Cdc42p) in WT and mutant cells.

the establishment of membrane growth (Miller and Johnson 1994). We checked localization of Cdc42p in the actin mutants that were defective in polarized growth, using CRIB-GFP, a marker for the active form of Cdc42p (Tatebe *et al.* 2008). In WT cells, CRIB-GFP localized at the growth site and the division site (Figure 3D). The CRIB-GFP localization was as in WT in most morphology mutants at 24° (Figure 3D, *act1-303* and *act1-135*) except in the case of *act1-d8*, which showed random sites of growth even at 24°, wherever CRIB-GFP localization was observed (arrows). At 36° the polarity-defective mutants failed to localize CRIB-GFP to the cell ends whereas WT and cytokinesis-defective mutants (Figure 3D, *act1-j28*) could localize CRIB-GFP to the cell ends. Therefore, active Cdc42p localization at the cell ends depends on the proper organization of actin at the regions of growth; and it appears that an even or unpolarized distribution of the actin cytoskeleton may disrupt the localization of active Cdc42p.

Figure 4 Mutants defective in cytokinesis. (A) Visualization of actin structures in WT and actin mutant cells. Actin mutants were grown at 36° for 4 hr, fixed, and stained with DAPI (red) and phalloidin (green). (B) Microtubule and actin in live cells visualized using mCherry-Atb2p (red) and LAGFP (green). (C) Immunofluorescence staining for anti-Cdc4p and anti- α -tubulin to study defects in ring formation in mitotic cells.

Actin mutants defective in cytokinesis

Cytokinetic defects lead to the accumulation of nuclei without complete physical separation of the daughter cells. Seventeen novel actin mutants defective only in cytokinesis were isolated from this screen. There were also 9 mutants defective in both cytokinesis and polarized growth. To check the organization of the actin cytoskeleton in these mutants compared to WT, cells were fixed after growth at 36° for 4 hr and stained with Alexa 488 phalloidin and DAPI, to visualize F-actin and nuclei, respectively. As shown in Figure 4A, WT cells have actin patches at the cell end(s), actin cables in interphase, as well as the contractile actomyosin ring during cytokinesis. Among these mutants, some were found to have prominent actin patches when stained with phalloidin (Figure 4A, *act1-327*, *act1-329*, and *act1-j39*). However, actin could not be detected in linear cables or rings in these mutants. Another set of mutant alleles defective in cytokinesis appeared to assemble F-actin into linear cables during mitosis but lacked compact rings (*act1-111*, *act1-128*, and *act1-j34*). In a further subset of the cytokinesis-defective alleles, actin was difficult to visualize using phalloidin staining (*act1-126* and *act1-j28*).

Actin organization for cytokinetic ring assembly

We decided to take a closer look at how actin is organized for the assembly of the cytokinetic ring. Recruitment of actin to the medial cytokinetic actomyosin ring occurs differently in different organisms (Balasubramanian *et al.* 2004). The lack of good methods to observe the actin cytoskeleton in fission yeast cells has made it difficult to study actin during the assembly of the actomyosin ring. In a recent study from our laboratory, Lifeact-GFP (LAGFP) was used to study actin in fission yeast (Riedl *et al.* 2008; Huang *et al.* 2012). The analysis showed that nonmedially assembled actin cables appeared to be transported to the medial region and then incorporated into the ring during mitosis. We used LAGFP to visualize the actin cytoskeleton in these actin mutants. In WT, the localization pattern of LAGFP was similar to the pattern of actin distribution detected with phalloidin (Huang *et al.* 2012). As shown in Figure 4B, growing mutant cells expressing LAGFP at 36° allowed for the analysis of cytokinesis defects in many of the mutants. Similar to observations with phalloidin staining, *act1-j39* cells at 36° had more prominent patches with far fewer linear cables and an absence of rings during mitosis.

We also analyzed a few of the other sets of mutants that had actin cables but lacked compact actomyosin rings, using LAGFP. When LAGFP was expressed in the mutant, *act1-j34*, although cells showed no cytokinetic defects at 24°, defects in cytokinesis were present at 36° (Figures 2 and 4B). There were fewer actin patches while actin cables were more prominent in interphase cells and were much thicker than those in WT cells (data not shown). As the cells entered into mitosis the cables increased in number just as in WT. Although the actin cables at 36° appeared to be similar to those at 24°, these thick cables failed to compact into a ring even in anaphase by which time WT cells assemble medial compact rings. Instead, cables remained scattered in ~59% ($n = 49$) of postanaphase cells, too (Figure 4B). Since phalloidin staining was not informative for the mutant *act1-126*, we made use of LAGFP expression to analyze the actin cytoskeleton. Even though mitotic cables were found scattered around the whole cell in *act1-126*, cells failed in cytokinesis (Figure 4B). These data suggested that the cytokinetic actomyosin ring could be assembled from actin cables, but further processing factors might be required for recruiting and compacting these cables. Thus, in mutants such as *act1-j34* and *act1-126*, the failure of ring assembly might be due to the unstable interaction of actin and such processing factors at 36°.

Many essential components of the contractile actomyosin ring have been reported to form nodes early in mitosis, before actin is recruited to the medial region (Wu *et al.* 2003; Pollard and Wu 2010). Depletion of any of them has been shown to disrupt the assembly of the actomyosin ring. Previous studies have shown that treatment with Latrunculin A does not perturb the nodes (Wu *et al.* 2006; Huang *et al.* 2008; Vavylonis *et al.* 2008), suggesting that

the formation of these nodes has been shown to be independent of actin. To check whether the same was true in the cytokinesis-defective actin mutants and whether node formation in these mutants was also actin independent, the cytokinesis-specific actin mutants were fixed and stained with antibodies against tubulin and Cdc4p. In WT cells, Cdc4p forms nodes (Figure S2, A–D) that later compact into a ring during mitosis (Figure 3C). In many mutants with cytokinetic defects, although Cdc4p forms nodes (Figure S2D), they failed to assemble a ring at 36° and Cdc4p appeared as multiple short contiguous stretches of fluorescence scattered in the medial region during mitosis (Figure 4C and Figure S2, E and F). Thus, ring assembly in actin mutants phenocopies drug treatments and does not prevent node organization.

Organization and function of actin during cell separation

Cell separation is the last step in *S. pombe* cell division. Several actin mutants were found to have defects in cell separation. Although many molecules have been found to be required for cell separation (Gould and Simanis 1997), the direct role of the actin cytoskeleton in this process is not yet very clear. One mutant, *act1-135* with a single point mutation (A22P), was defective in cell separation at 24°, yet cells were able to survive. However, at 36° the mutant was found to be inviable with severe polarity defects (data not shown). The death at higher temperature was probably due to defective polarization as seen in the case of MOR mutants (Verde *et al.* 1998; Hirata *et al.* 2002; Ray *et al.* 2010). To investigate the cell separation defects observed at 24°, we stained the cells of this mutant and WT with aniline blue. The mutant cells displayed unsuccessful cell separation with a thick septum in the middle (Figure 5A). In an asynchronous population of WT, only 17% ($n = 103$) of cells showed a single septum that formed in the middle and none had two or more septa. Expression of LAGFP increased this proportion only slightly to 19% ($n = 104$). However, in *act1-135*, 41% ($n = 107$) of cells had a septum and 10% showed two or more septa (data not shown); most of the septa in *act1-135* were thicker than the ones in WT cells. Expression of LAGFP had little effect on the cell separation of *act1-135* (45% of the cells had a septum and 4% showed two or more septa, $n = 103$). Although the cytokinetic ring was assembled normally in *act1-135*, the cells had a predominance of actin cables over actin patches throughout the cell cycle (Figure 5, B and D). To check whether this difference in the actin cytoskeleton had any correlation with the cell separation defects seen in these cells, we made use of time-lapse movies to observe actin dynamics during cell separation. Since ring constriction was normal in this mutant, time-lapse movies were made to examine actin behavior in postanaphase cells after ring constriction. In WT cells, numerous actin patches could be observed at the division site as the actin ring constricted (Figure 5C, WT, 15 min after the spindle fully extended) until the two daughter cells

Figure 5 Functional analysis of actin during ring assembly and cell separation. (A) WT and actin mutant *act1-135* cells were grown and fixed at 24° and stained with DAPI and aniline blue to visualize the nucleus and septum. (B) Analysis of ring assembly in *act1-135* by observing actin by LAGFP (green) and microtubules by mCherry-Atb2 (red) in live cells grown at 24°. (C) Montage of a time-lapse movie to show actin (LAGFP) at the division site in WT and *act1-135* and cell separation defects observed at 24°. (D) Analysis of cortical endocytic actin patch accumulation at the division site in WT and *act1-135* cells by observing actin by LAGFP (green) and coronin1 by Crn1-tdTomato (red) in live cells grown at 24°.

separated completely (45 min). However, fewer actin patches were found at the division site in *act1-135* during ring constriction (Figure 5C). After the ring fully constricted, only a few actin patches appeared in this mutant. This was further confirmed when we performed dual-color imaging for LAGFP and Crn1-tdTomato (actin patch marker) (Pelham and Chang 2001) (Figure 5D). Analysis of cells with constricting actin rings revealed fewer actin patches in the mutant *act1-135* compared to WT cells visualized by both LAGFP and Crn1-tdTomato. While WT cells had an average of 49.4 ± 10.97 LAGFP patches and 34.05 ± 5.39 Crn1-tdTomato patches, *act1-135* cells had only 16.8 ± 5.47 LAGFP patches and 12.85 ± 6.56 Crn1-tdTomato patches (mean $\pm$ SD, $n = 20$). Hence, actin patches are likely to play an important role in cell separation.

Genetic interaction between actin alleles and other ring component genes

The mutations from the different actin alleles appear scattered throughout the structure of actin and those mutations on the surface or affecting surface features might affect the interaction of actin with other actin-binding proteins (ABPs). To investigate the molecular basis of the defects in the actin alleles we looked for synthetic lethal interactions in a pairwise combination between the cytokinesis-defective actin mutants and mutants of known ring components. As shown in Table 2, we tested for synthetic lethality between some of the actin mutants with defects in cytokinesis and other known cytokinetic mutants. Among all the alleles tested, *act1-117* showed synthetic lethality with four genes, which suggested that the mutation most severely affected the functional (direct) interaction between actin and its binding partners. Of the different ABPs, tropomyosin Cdc8p (a linear actin-stabilizing protein) is known to strengthen linear cables (Liu and Bretscher 1989). The tropomyosin mutant (*cdc8-110*) showed synthetic lethality with five actin alleles, which was surprising given that Cdc8p is a rather small protein. These five mutations were projected onto the 3D structure of actin (Figure 6A) and all except *act1-117* were found to cluster. This suggests that a high-specificity interaction between tropomyosin and actin is required for cytokinesis.

A multicopy suppressor screen was also performed to look for potential suppressors for some of these actin mutants. Two different plasmids were identified that partially rescued *act1-329* and were found to carry the chromosomal region of the *cdc8* gene. This rescue was further confirmed using a plasmid containing only the *cdc8* gene (Figure 6B). To further examine how the cytokinetic defect in *act1-329* was suppressed, cells were grown for 6 hr at 36° and stained with DAPI and aniline blue. As shown in Figure 6, C and D, 65.3% ($n = 153$) of *act1-329* cells carrying the empty vector appeared elongated with more than two nuclei and lacked a complete septum between the nuclei, while 23.5% of the cells were binucleate. In contrast, only 41.2% ($n = 201$) of *act1-329* cells with multicopy expression of *cdc8*⁺ appeared elongated with more than two nuclei (and usually with obvious septum formation), and 44.7% cells were binucleate. To further check actin organization, LAGFP was expressed in the mutant, as actin was difficult to visualize in *act1-329* using phalloidin (Figure 4A). As shown in Figure 6, E and F, after growth for 4 hr at 36°, 15.4% ($n = 130$) of *act1-329* cells carrying *pCdc8*⁺ showed actin rings compared with only 4.6% ($n = 130$) of *act1-329* cells carrying an empty vector. Therefore, the mutation in *act1-329* disrupted only the stability of actin cables and rings, which was consistent with the fact that this mutant predominantly formed actin patches. These data suggest the strong possibility that the defect in *act1-329* leads to poor interaction between actin and tropomyosin.

Discussion

In this study, we have isolated a large bank of mutants in the fission yeast actin gene, the majority of which are defective

Table 2 Genetic interaction network

	<i>act1-111</i>	<i>act1-117</i>	<i>act1-126</i>	<i>act1-128</i>	<i>act1-138</i>	<i>act1-145</i>	<i>act1-327</i>	<i>act1-329</i>	<i>act1-j28</i>
<i>cdc15-140</i>	No	No	No	No	No	No	No	No	No
<i>rng2-D5</i>	No	No	No	No	No	No	No	No	No
<i>rng3-65</i>	No	No	No	No	No	No	No	No	No
<i>cdc3-124</i>	Yes	Yes	No	^a	^a	Yes	No	No	Yes
<i>cdc8-110</i>	No	Yes	Yes	No	Yes	No	Yes	Yes	No
<i>myo2-E1</i>	No	Yes	No	No	No	No	No	^a	No
<i>cdc4-8</i>	No	No	No	No	^a	No	Yes	No	No
<i>cdc12-112</i>	No	Yes	No	No	Yes	No	No	No	No

^a Synthetic growth defect.

in specific cellular functions of actin and in many cases appear to affect specific actin-containing structures. Even though the mutagenesis procedure used does not give any indication of the extent of saturation of the screen, we have successfully recovered mutations spanning the entire gene sequence. Furthermore, mapping the amino acid alterations to the three-dimensional structure of the molecule revealed that such alterations were located both on the surface and internally. From this screen we recovered 39 distinct mutant alleles. Seventeen mutants were defective specifically in cytokinesis and 13 mutants affected polarized growth, while the rest were more complex.

We established 17 mutants to be defective in cytokinesis, since they were able to elongate and grow in a polarized manner with little change in cell morphology. Mapping the amino acids altered in the cytokinesis mutants to the three-dimensional structure did not reveal obvious molecular interactions that could be compromised in these mutants. However, our genetic analysis of some of these mutants revealed multiple and distinct synthetic lethal interactions between actin, Cdc8p-tropomyosin, Cdc3p-profilin, Cdc4p-EF hand protein, myosin II heavy chain (Myo2p), and Cdc12p-formin. In addition, we also isolated Cdc8p-tropomyosin as a high-dosage suppressor of *act1-329*. These observations establish a network of genetic interactions between actin and other actomyosin ring assembly factors, consistent with previous work on fission yeast cytokinesis.

Studies of the cytokinesis-defective alleles revealed that in some cases F-actin was present only in patches, but not in cables, whereas in others it was present in prominent cables that did not organize into a ring structure. Since the ring is composed of linear actin filaments 600–1000 nm in length, it is possible that the alleles with prominent patches (such as *act1-329* and *act1-j39*) do not assemble actin cables properly, leading to a defect in actomyosin ring assembly. The mutant *act1-j39* contains two mutations, L105S and K215I. While L105 is an internal residue, K215 is a surface residue that points directly toward tropomyosin with a distance of 5.5 Å to R167 of tropomyosin (Behrmann *et al.* 2012). The effect of *act1-329* arises from the single mutation K291I. K291 lies in actin subdomain 3 and forms a direct interaction with subdomain 4 of the next protomer in the filament. Furthermore, K291 interacts with M325, within the same protomer, to position K326 to point at tropomyosin residue

E187 [5.0 Å (Behrmann *et al.* 2012)]. Hence, there is a reasonable structural basis for both mutations interfering with tropomyosin binding. This is also consistent with the finding that the high-dosage expression of Cdc8p-tropomyosin partially rescues the lethality of *act1-329*. In our recent work, we showed that a significant number of actin filaments in the actomyosin ring are assembled *de novo* as well as through transport of nonmedially located actin cables through the activities of Myosin II and Myosin V (Huang *et al.* 2012). It is possible that cytokinesis-defective actin mutants with prominent actin cables (*act1-j34*) are not translocated by Myosin II and/or Myosin V. The *act1-j34* mutation at L67 in subdomain 2 will likely alter the stability of subdomain 2 and the G- to F-actin transition, since L67 interacts with A204 in subdomain 4 of F-actin [4.2 Å (Behrmann *et al.* 2012)]. Any destabilization of subdomain 2 has the potential to affect myosin binding, which interacts with subdomains 1 and 2. Purification and characterization of the product of *act1-j34* through Myosin II and Myosin V single-molecule motility studies should help further unravel the molecular basis of the actomyosin ring assembly defect in this mutant.

One mutant, *act1-135* (A22P), was able to assemble actomyosin rings but did not accumulate actin patches to the extent observed in WT cells. *act1-135* cells undergoing ring constriction had <40% of the number of actin patches in the medial region compared to WT. This mutation will change the geometry of the loop 22–25, a region that interacts with myosin and formin (Behrmann *et al.* 2012; Thompson *et al.* 2013). Coordinates are not currently available to assess whether this region also interacts with the Arp2/3 complex. Interestingly, although the ring constriction rate did not appear to be altered in this mutant, cell separation was significantly delayed. It is possible that the secretion of molecules involved in primary septum degradation or endocytic components involved in cell separation are affected in this mutant.

The 13 mutants that were defective in polarized growth, uncovered from our screen, were grouped mainly based on morphological observations as well as from studies of the ability/inability of these mutants to undergo polarized growth following G₀ arrest. These mutants were not defective in actomyosin ring assembly and constriction. In nearly all of these mutants, actin patches were clearly visualized but were delocalized, suggesting defective interaction of the

Figure 6 Genetic interaction between actin alleles and *cdc8*. (A) Tropomyosin mutant (*cdc8-110*) showed synthetic lethality with five actin alleles. The five mutations (m1–m5) are Y218N, K315G, I282N, K291I, and Y362C as shown projected onto the 3D structure of actin. (B–F) Overexpression of Cdc8p (plasmid pCdc8) rescues *act1-329*. (B) WT cells, *act1-329* with pCdc8, and *act1-329* with empty vector were grown on minimal medium at 24° and then replicated to grow on rich medium at 36°. (C) *act1-329* with empty vector and *act1-329* with pCdc8 were grown in minimal medium without leucine at 24° overnight and then shifted to 36° for 4 hr. Cells were fixed and then stained with DAPI and aniline blue to observe nuclear division and septum formation. (D) Quantification of nuclei numbers in *act1-329* with empty vector and *act1-329* with pCdc8 cells at 36°. (E) Visualization of actin (LAGFP) in live cells at 36° after growing cells as described in C. (F) Quantification of ring formation in *act1-329* with empty vector and *act1-329* with pCdc8 cells at 24° and 36°.

mutant protein with molecules that reinforce actin-based polarity. In actin mutants defective in cell polarity, CRIB-GFP was mislocalized, suggesting the interactions between Cdc42p GTPase localization and the actin cytoskeleton. This is indeed consistent with the earlier reports by multiple groups (Wedlich-Soldner *et al.* 2003; Irazoqui *et al.* 2005). Further genetic studies of polarity-defective actin mutants should provide insight into actin-based polarized growth in fission yeast.

In summary, we have generated a bank of actin mutants that will greatly assist in understanding actin function in various physiological processes. Although we have focused

on cytokinesis and polarity, the bank may equally well be useful to study the role of actin in other processes such as endocytosis, exocytosis, mating, and sporulation. The products of the mutant actins may also be useful in biochemical and cell biology studies to perform single-molecule analysis of interactions with motors, cross-linking proteins, and actin nucleators.

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Supporting Information

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Insight into Actin Organization and Function in Cytokinesis from Analysis of Fission Yeast Mutants

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A

B

Figure S1 Quantitative examination of nucleus numbers per cell in WT and actin mutant cells. (A) WT and mutant cells were grown at 24°C, fixed and stained using DAPI to count the number of nuclei per cell. (B) WT and mutant cells were grown overnight at 24°C and subsequently shifted to 36°C for 4hrs, fixed and stained using DAPI to count the number of nuclei per cell (N= approx. 50).

A

B

C

D

E

F

Figure S2 Quantitative examination of ring assembly in mitotic cells of WT and actin mutants by immunofluorescence staining for Cdc4p and α Tubulin. WT and mutant cells were grown at 24°C (A, B, C), or subsequently shifted up to 36°C for 4hours (D, E, F). Cells were fixed and subjected to immunofluorescence staining using antibodies against Cdc4p and α Tubulin. Ring assembly was assessed in cells with short spindles (A, D), mid-size spindles (B, E) and long spindles (C, F) [N= approx. 25 cells for each stage].

Table S1 Classification of Actin mutants

CLASS I - CYTOKINESIS DEFECTIVE (TOTAL= 17)				
<i>Allele act1-</i>	Amino acid Change	Temperature Sensitivity (36°C)*	Results of DAPI, Aniline Blue & Phalloidin Staining (Mutant Phenotype after 4-6hours at 36°C)	Results of Immunostaining for Cdc4p and Tubulin (Mutant Phenotype after 4-6hours at 36°C)
111	S281P(Sd3)	L	Many binucleates and multinucleate cells with septa. Improper rings or curly ribbons of actin seen in the medial region	Assembles nodes. Unable to form rings. Plenty of fragments observed in the medial region
126	Y218N (Sd4)	L	Many multinucleate cells with improper septum. Phalloidin staining faint. Long strands of actin cables with no rings	Assembles nodes. Unable to form rings. Plenty of fragments observed in the medial region
138	I282N (Sd3)	L	Many enlarged bi and multinucleate cells with improper septa. No rings. Only actin cable like structures seen.	Assembles nodes. Unable to form rings. Plenty of fragments observed in the medial region
145	H275R (Sd3), I309V (Sd3)	L	Large multinucleate multiseptate cells. Lack rings but have short cable like fragmented structures of actin in the medial region.	Assembles nodes. Unable to form rings. Plenty of fragments observed in the medial region
327	K315E (Sd3)	L	Elongated multinucleate, multiseptated cells. No actin rings. Plenty of actin patches seen.	Assembles nodes. Unable to form rings. Plenty of fragments observed in the medial region
329	K291I (Sd3)	L	Elongated multinucleate, with aberrant septa. Plenty of actin patches seen.	Assembles nodes but unable to form rings. Fragments seen in the medial region.
d1	D157G (Sd3)	L	Large elongated cells with severe cytokinetic defects. Cannot make out any actin structures either.	Assembles nodes. Cable like fragments seen but unable to form rings.
d13	E276G (Sd3), Y362H (Sd1)	NL	Long and multinucleate with aberrant septa. Occasional misplaced rings observed.	Nodes persisted even in late mitotic cells. No rings seen. Mostly fragments.

d14	I208V (Sd4)	NL	Many multinucleate cells seen with multiple aberrant septa. See some actin cables but no rings visible	Assembles nodes. Very few rings seen but mostly fragments in the medial region
j16	S348P (Sd1)	L	Many multinucleate cells with very sick and thick septum.	Cells very sick. Hard to stain and visualize structures.
j21	N111S (Sd1)	NL	Many long multinucleate multiseptate cells with thick aberrant septa. See actin cables and patches but no rings	Assembles nodes. Few weird rings seen but mostly fragments in the medial region
j25	K373E (Sd1)	NL	Many long multinucleate multiseptate cells. Predominant actin patches. Very few cables and few cells with rings observed	Cells assemble nodes and rings but very few and hard to find. Severe ring defects and plenty of long cables and fragments seen
j28	F200S (Sd4), G308S (Sd3)	L	Multinucleate cells with aberrant septa/septal material in the medial region. Hard to stain with phalloidin. Faint actin cables observed	Assembles nodes but unable to form rings. Fragments seen in the medial region.
j32	F127L (Sd1), I309T (Sd3)	NL	Long multinucleate cells with multiple septa seen. Actin patches and cables seen and occasional rings observed	Assembles nodes. Thick long cables seen but hardly any rings.
j34	L67S (Sd2)	NL	Long multinucleate cells with multiple septa seen. Actin cables seen but hardly observe any other structures.	Assembles nodes. Fragments seen in the medial region but very few rings seen late in mitosis.
j35	I250V(Sd4)	NL	Multinucleate cells with multiple septa. Actin patches and occasional rings can be seen	Assembles nodes. A few rings observed but mostly wriggly fragments
j39	L105S (Sd1), K215I (Sd4)	NL	Multinucleate cells with mostly single aberrant septa or septal material. Only see actin patches. No rings or cables seen.	Nodes look a bit larger. Mostly fragments with very few rings

CLASS II - POLARIZED GROWTH/MORPHOLOGY DEFECTIVE (TOTAL=13)				
<i>Allele act1-</i>	Amino acid Change	Temperature Sensitivity (36°C)*	Results of DAPI, Aniline Blue & Phalloidin Staining (Mutant Phenotype after 4-6hours at 36°C)	Results of Immunostaining for Cdc4p and Tubulin (Mutant Phenotype after 4-6hours at 36°C)
112	F352L (Sd1)	L	Plenty of large rounded cells with some occasional multinucleates. Actin patches present all over	Assembles nodes and rings. Some fragments observed and some misplaced rings seen
115	V96I (Sd1), I212T (Sd4), E276A (Sd3), Q318R (Sd3)	NL	Many different shapes of cells and some multinucleate. Rings appeared normal while patches were all over.	Assembles nodes and rings.
116	P27S (Sd1)	L	Bi- and uninucleate cells with heavy aniline blue staining in the middle. Poorly stained with phalloidin. See faint actin cables and patches.	Cells very sick upon protoplasting. Hard to stain and visualize structures.
303	D81N (Sd1), V219A (Sd4), E237G (Sd4)	L	Cells were short and fat with improper aniline blue staining. Either monopolar or all-around. Actin rings and cables seen	Very few mitotic cells seen. Inconclusive
313	F31S (Sd1)	L	Cells were rounded due to morphology defects and rounded.	Very sick with severe morphological defects and clumped. Inconclusive staining.
323	V96I (Sd1)	L	Severe morphology defects. Cells rounded with improper aniline blue deposition. Actin rings observed.	Very sick with severe morphological defects and clumped. Inconclusive staining.
d3	H88D (Sd1)	NL	Mostly fat and rounded cells with occasional multinucleate cells. Improper aniline blue deposition. Phalloidin staining inconclusive.	Assembles nodes and forms rings quite well although a small percentage of cells had incomplete and fragmented rings.
d5	S350F (Sd1)	NL	Cells appear to have severe morphological defects with some	Assembles nodes. Rings observed but some cells appeared to

			multinucleate multiseptate cells. Cells form actin rings and actin patches appear all over.	have fragments in the medial region.
d8	S52P (Sd2)	NL	Rounded cells with severe morphological defects. Cellwall deposition mislocalized. Actin rings form. Actin patches appear all over.	Assembles nodes, fragments and rings although rings were misplaced sometimes.
d16	S235P (Sd4)	NL	Severe morphology defects. Cells form actin rings and actin patches appear all over.	Very sick with severe morphological defects and clumped. Inconclusive staining.
j23	M82T (Sd1), A135V (Sd1)	L	Cells of all shapes. Some multinucleated and branched T-shapes. Forms actin rings but other structures hard to visualize.	Cells assemble nodes and rings but appear to be slow in formation.
j30	F31L (Sd1), S60G (Sd2), M190I (Sd4)	L	Severely rounded cells with severe morphological defects. Cellwall deposition mislocalized. Cells clump. Hard to image. Forms actin rings. Actin patches appear all over.	Very sick with severe morphological defects and clumped. Inconclusive staining.
j36	K50R (Sd2), I309T (Sd3)	L	Rounded cells with severe morphological defects. Cell wall deposition mislocalized. Multinucleate cells seen too. Actin rings form. Actin patches appear all over.	Assembles nodes and rings. However some improper rings and fragments were observed.

CLASS III – MULTIPLE DEFECTS (TOTAL=9)				
<i>Allele act1-</i>	Amino acid Change	Temperature Sensitivity (36°C)*	Results of DAPI, Aniline Blue & Phalloidin Staining (Mutant Phenotype after 4-6hours at 36°C)	Results of Immunostaining for Cdc4p and Tubulin (Mutant Phenotype after 4-6hours at 36°C)
117	Y362C (Sd1)	L	Plenty of multinucleate cells. Aniline blue staining brighter in the medial region. Phalloidin staining is inconclusive.	Cells very sick. Inconclusive staining.
121	I274T (Sd3), F352L (Sd1)	L	Larger fatter cells with aniline blue staining all around the cell. Thick septum also seen. Actin patches mislocalised and many misplaced rings seen.	Assembles nodes and rings but many misplaced rings were observed.
123	D222G (Sd4), F255L (Sd4)	L	Cells were smaller in size. Rings appeared normal while none of the other structures were hard to visualize.	Cells very sick. Inconclusive staining.
125	V219G (Sd4)	L	Bi and uninucleate cells with heavy aniline blue staining in the middle. Poorly stained with phalloidin.	Absence of mitotic cells. Hence inconclusive.
128	T304P (Sd3)	L	Cells looked larger. Cells appear to form distorted septa. Actin rings seen but mostly wriggly cables seen. Cells did not re-establish polarized growth post Nitrogen starvation.	Assembles nodes. Only few rings seen. Mostly observed fragments.
135	A22P (Sd1)	L	Rounded cells with severe morphological defects. Cell wall deposition mislocalized. Actin cables are more prominent than actin patches. Few cells with actin rings seen.	Cells look lysed and rounded. Inconclusive.
328	R37G (Sd2), D80G (Sd1), L185S (Sd4)	L	Cells appeared to deposit cell wall material to lateral regions and medial locations. Prominent actin cables seen but no other actin structures seen.	Hardly find mitotic cells. Hence inconclusive.

j15	E259A (Sd4)	L	Improper deposition of cell wall material with dense accumulation in the medial region. Faint Phalloidin staining revealing only actin cables. Unable to visualize other actin structures.	Very sick with hardly any mitotic cells. Inconclusive staining.
j27	K68E (Sd2), I85T (Sd1), F132L (Sd1)	NL	Long multinucleate and multiseptate cells often L and T shaped too. Unable to visualize actin structures by phalloidin staining.	Assembles nodes. However mostly fragments were seen. No rings seen.

Sd: Subdomain of Actin

L: Lethal NL: Non-Lethal

*Many of the mutants showed a strong phenotype even at the permissive temperature