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Schizosaccharomyces pombe Arc3 is a conserved subunit of the Arp2/3 complex required for polarity, actin organization, and endocytosis

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Abstract

We characterized the *Schizosaccharomyces pombe arc3* gene, whose product shares sequence homology with that of the budding yeast *ARC18* and human *ARPC3*/p21 subunits of the Arp2/3 complex. Our data showed that Arc3p co-localizes with F-actin patches at the cell ends, but not with F-actin cables or the equatorial actin ring, and binds other subunits of the Arp2/3 complex. Gene deletion analysis showed that *arc3* is essential for viability. When *arc3* expression was repressed, F-actin patches became dispersed throughout the cell with greatly reduced mobility. Furthermore in *arc3*-repressed cells, endocytosis was also inhibited. Human ARPC3 rescued the viability of the *S. pombe arc3* null mutant; in addition, ARPC3 also localizes to F-actin patches in human cells. These data suggest that Arc3p is an evolutionarily conserved subunit of the Arp2/3 complex required for proper F-actin organization and efficient endocytosis.

INTRODUCTION

The Arp2/3 complex is a well-established nucleator of actin polymerization and responsible for the formation and dynamics of several kinds of actin structures in a number of model organisms (Goley and Welch, 2006). The Arp2/3 complex is composed of at least seven highly conserved subunits, two of which, Arp2 and Arp3 are structurally related to actin and proposed to act as nuclei to promote actin polymerization (Machesky and Gould, 1999). Since the Arp2/3 complex frequently associates with the sides of preexisting actin filaments and initiates polymerization at an angle, its activity often leads to the formation of highly branched F-actin sturctures (Wear, et al., 2000). The remaining subunits have been hypothesized to play regulatory roles (Welch, et al., 1997) as well as maintain the structural integrity of the complex (Zhao, et al., 2001). Reconstitution experiments suggest that the p41, p21 and p16 subunits are located in the periphery of the complex and seem to influence actin polymerization efficiency and activation by WASP (Gournier, et al., 2001). p41 can be phosphorylated by PAK1 to influence cell migration (Vadlamudi, et al., 2004). The p20 and p34 subunits seem to be confined to the complex's core and to be required for the structural integrity of the complex and its ability to bind existing actin filaments (Gournier, et al., 2001). Arc18, the predicted *S.cerevisiae* homolog of the *arc3*/p21 subunit is recruited to the mitochondria and the *arc18* deletion mutant shows impaired mitochondrial transport (Fehrenbacher, et al., 2005).

The Arp2/3 complex is known to associate with and participate in actin polymerization in both *Schizosaccharomyces pombe* (Sirotkin, et al., 2005) and *Saccharomyces cerevisiae* (Winter, et al., 1997). In *S. pombe*, three F-actin structures are readily identifiable. During

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interphase, F-actin patches concentrate at the growing ends of the cell (Marks, et al., 1986; Verde, et al., 1995), and during early mitosis, these patches relocate to the cell equator (Wu, et al., 2006). F-actin also foms cable-like structures that extend along the long axis of the cell throughout the cell cycle. Just before anaphase-B , F-actin filaments also form a ring encircling the cell equator, which plays a key role in providing the constrictive force needed for cytokinesis (Noguchi, et al., 2001; Wu, et al., 2006). The Arp2/3 complex associates with F-actin patches, but not with F-actin cables or the equatorial ring, and is required for the proper organization and mobility of these patches (McCollum, et al., 1996; Pelham and Chang, 2001). They are spatially associated with endocytic vesicles (Gachet and Hyams, 2005) and have been proposed to play a role in their formation and internalization (Girao, et al., 2008). This is supported by the observations that the *S. cerevisiae* WASP (Wiskott-Aldrich Syndrome Protein) ortholog, *LAS17*, and *S. pombe* Cdc42, activators of the Arp2/3 complex, are requried for clathrin-mediated endocytosis (Murray and Johnson, 2001; Naqvi, et al., 1998). However, whether all Arp2/3 subunits are required for efficient endocytosis has not been determined. Furthermore, although Arp2/3 complex subunit orthologs from different species are highly conserved at the protein sequence level, it is not know whether the function of different subunits is also conserved across evolution.

In a recent study of a mutant (carrying deletion in the *yin6* gene) with defective proteasomes, we isolated the *S. pombe arc3* gene, which is highly homologus to human *ARPC3* and *S. cerevisiae ARC18* (Welch, et al., 1997), and showed that it is needed for proteasomes to maintain high mobility (Cabrera, et al., 2010). In this study, we further characterize the role of *arc3* in regulating F-actin organization and endocytosis. Our results showed that unlike its *S. cerevisiae* ortholog *ARC18*, *arc3* is essential for viability. We further showed that Arc3 is required for proper organization and high mobility of F-actin patches and efficient endocytosis. The essential function of the *arc3* gene can be fully rescued by the human ARPC3, which also localizes to F-actin patches in human cells, suggesting that their functions are evolutionarily conserved.

MATERIALS AND METHODS

Growth conditions and reagents

Cells were grown in either yeast extract (rich) medium (YEAU) or synthetic minimal medium (MM) with appropriate supplements (Chen, et al., 1999). To depolymerize F-actin, Lat A stock solution (1 mM, Sigma) was prepared in DMSO. To repress the *nmt* promoter, thiamine was added from a 20 μM stock (Sigma) after autoclaving. We carried out all the experiments with cells pregrown to early logarithmic phase ($2–5\times10^6$ cells/ml). For growth experiments on plates, cells were serially diluted 1:5. HeLa cells were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine (Gibco). Subconfluent cells were transfected with 200 ng of plasmid using Lipofectamine 2000 (Invitrogen).

Plasmid constructions

The pREP41ARC3 expression vector was described elsewhere (Cabrera, et al., 2010). Full length *arc3* was amplified from an *S. pombe* cDNA library (Norbury and Moreno, 1997) and cloned into the BamHI site of pVJL11 to generate pVJLARC3. Full length *arc4* was amplified from the *S. pombe* cDNA library described above and cloned into the BamHI site of pGADgh to generate pGADARC4. *ARPC3* was amplified from cDNA was obtained from Open Biosystems and cloned into the SalI sites of pEGFP-N1 or pREP1 to generate pARPC3EGFP and pREP1ARPC3.

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Strain constructions

The parental wild-type *S. pombe* strain used in this study was SP870 (*h*^{90}, *ade6-M210, leu1-32, ura4-D18*) unless indicated otherwise. Cells expressing Arc3-MYC and GFP were generated by tagging Arc3 with 13× MYC and GFP respectively at the C-terminus using homologous recombination via a PCR-based method (Bähler, et al., 1998). The creation of an *arc3::ura4*/+ strain was described elsewhere (Cabrera, et al., 2010). Crn1-GFP was kindly provided by Dr. Fred Chang. To determine if *arc3* is an essential gene, *arc3::ura4*/+ cells were induced to sporulate, and the viability of individual spores was assayed by tetrad analysis. To study the function of *arc3*, its expression was repressed in the ARC3NMT strain (Cabrera, et al., 2010), which carries an *arc3* deletion (*arc3::ura4*) and the pREP41ARC3 plasmid to express *arc3* from a thiamine-represible *nmt* promoter. Repression of *arc3* for microscopy experiments was achieved by incubating cells in MM with 200 nM thiamine for 16 to 20 hours. Repression of *arc3* to assay growth was achieved by growing ARC3NMT cells on MM plates containing 1 nM thiamine.

Immunoprecipitation

Approximately 50 OD units of cells expressing Arc3-Myc were lysed using glass beads in PBS (pH 7.4) containg 0.1% Triton X-100. Lysate was centrifuged for 5 minutes at 16000×g and the supernatant was added to 40 µl of Protein A beads (Roche) and incubated in the presence of either anti-Myc antibody (9E10), anti-Arp2 (Morrell, et al., 1999) or mouse IgG (Roche) as a control. The beads were washed three times with PBS plus 0.1% Triton X-100 and solubilized in 1× SDS loading buffer. The resulting samples were analyzed by western blot with anti-Myc (9E10, 1:100), anti-Arp2 ((Morrell, et al., 1999), 1:1000) and anti-tubulin (TAT1, 1:1000) antibodies.

Fluorescence Microscopy

The general procedures for staining F-actin in fixed yeast cells were as described (Sawin and Nurse, 1998) except staining was done with both Rhodamine-Phalloidin and Alexa Fluor 488-Phalloidin as required. To stain F-actin in HeLa cells, the cells were washed with PBS, fixed in PBS plus 4% paraformaldehyde, washed and permeabilized with PBS plus 0.5% Triton X-100. These cells were then incubated with PBS containing 0.2 units/ml Rhodamine-Phalloidin, washed, and mounted for observation. Representative images were collected using Olympus IX70 and BX61 fluorescent microscopes. To depolymerize actin, yeast cells were grown in YEAU, treated with 10 µM Latrunculin A for 30 mins and then washed in YEAU and grown for 1 hour. Images were collected at 30 min after Latrunculin A and 1 hour after washing. Identical treatment was given to HeLa cells. To determine actin patch localization, projection images of deconvolved stacks of Z-sections of cells stained with Alexa-488 Phalloidin were used. To measure Crn1-GFP velocity, time lapse microscopy was performed. A given Crn1-GFP dot was tracked frame by frame (0.5 sec/frame) for a total of 20 sec by the MetaMorph software (Molecular Devices), and the total distance it traveled was measured. To assay endocytosis, *arc3*^{nmt} cells were pregrown to early log phase at 30 °C in minimal medium, concentrated 10-fold before FM 4–64 (8.15 µM) was added, and observed by time lapse microscopy (Gachet and Hyams, 2005).

RESULTS AND DISCUSSION

Arc3 is a subunit of the Arp2/3 complex and associates with F-actin patches

The predicted Arc3 protein is 51 and 54 % identical to the human ARPC3 and *S. cerevisiae* Arc18p protein, respectively. Because both ARPC3 and Arc18p represent the p21 subunits of the Arp2/3 complex, we named this molecue Arc3 with the assumption that it is an Arp2/3 complex subunit. To test whether Arc3 associates with the Arp2/3 complex, we

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constructed a strain in which the endogenous Arc3 protein was C-terminally tagged with 13 copies of the c-Myc epitope by homologous recombination. We performed immunoprecipitation experiments and found that Arc3 immunoprecipitated Arp3 and *vice versa* (Figure 1A). We also found that Arc3 can bind the predicted Arp2/3 subunit Arc4 in a yeast two-hybrid assay (Figure 1B), an interaction also observed between the *S. Cervisiae* Arc18p and Arc4p orthologs (Zhao, et al., 2001). These results confirm that Arc3 binds components of the Arp2/3 complex.

We then tested whether Arc3 would localize to F-actin patches. We generated a strain that expresses the Arc3 fused at its C-terminus to GFP from its endogenous promoter by homologous recombination. Expression of this fusion protein as the sole source of Arc3 does not result in growth abnormalities, suggesting that it is functional. As shown in Figure 1C, Arc3-GFP localizes to cortical dots at the end of the cell that overlap with F-actin patches, as visualized by rhodamine-phalloidin. In dividing cells, both Arc3 and the F-actin patches are visible at the division plane (Figure 1D and data not shown). We note that Arc3-GFP dots do not associate with F-actin cables or F-actin ring at the cell equator. To determine whether this association with F-actin patches is dependent on the integrity of F-actin, we treated cells with the F-actin polymerization inhibitor Latrunculin A, which readily caused the majority of Arc3-GFP to be dispersed throughout the cell, a defect that was efficiently rescued when Latrunculin A was washed out (Figure 1D). We conclude from these results that Arc3 associates with Arp2/3 complex and F-actin patches in a F-actin-dependent manner.

Creation of an *arc3* mutant reveals that *arc3* is essential for viability

In order to study the function of the *arc3* gene, we generated a diploid strain in which one copy of the *arc3* was deleted and replaced by the *ura4* selectable marker (*arc3*Δ or *arc3::ura4*) by homologous recombination. Tetrad analysis revealed that *arc3* is essential for viability, since only 2 spores of every tetrad were able to form colonies, both of which are $arc3^{+}$ (Figure 2A and data not shown).

To facilitate the study of Arc3 functions, we generated a conditional *arc3* mutant by transforming $arc3^{+}$/*arc3*Δ cells with a plasmid expressing Arc3 from a thiamine-repressible promoter. Upon meiosis and sporulation, we selected the thiamine repressible haploid *arc3* mutant cells ($arc3^{nmt}$). Upon addition of high concentrations of thiamine (200 μM), the expression of Arc3 was repressed with a concurrent increase of lethality which eventually affected nearly 100% of cells (Figure 2B and data not shown). We also observed that under semi-permissive conditions (low thiamine, 1 nM), these cells show impaired growth in the cold, similar to that observed in many mutants with defective actin cytoskeleton (e.g., (Balasubramanian, et al., 1996; McCollum, et al., 1999; McCollum, et al., 1996)) (Figure 2C).

Arc3 is required for actin organization and endocytosis

The actin cytoskeleton has been implicated in the regulation of cell polarity (Marks, et al., 1986). In fission yeast, the cell maintains an elongated morphology by growing from two ends of the cell, which are marked by F-actin patches. We examined the effect of reducing *arc3* expression on cell shape and F-actin organization. Our data show that while normal cells maintain the typical rod-like shape, *arc3*-repressed cells became round or ellipsoid in shape (Figure 3A). Staining cells to visualize F-actin structures with Alexa Fluor 488 Phalloidin revealed a defect in actin organization. Whereas F-actin patches are mostly restricted to the cell ends in interphase cells expressing *arc3* ($arc3^{+}$), in *arc3*-repressed cells ($arc3^{-}$), F-actin patches were more scattered and could be readily seen in the middle of the cell (Figure 3B). However, *arc3* deficiency does not seem to affect other F-actin structures

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such as F-actin cables, which still extend along the cell body with no significant reduction in length or number, and rings that encircle the cell equator (data not shown). These observations agree with those of the study of Arp2 (Morrell, et al., 1999). These results suggest that Arc3 is required for bi-polar distribution of F-actin patches in fission yeast cells.

F-actin patches are highly mobile and their mobility requires F-actin polymerization and functional Arp2 (Pelham and Chang, 2001). F-actin patches can be tracked by following the localization of GFP-tagged coronin (Crn1), which interacts with Arp2/3 (Pelham and Chang, 2001). We therefore tested whether Arc3 is also required for F-actin patch mobility by following the trayectory of Crn1-GFP containing patches through time. We determined that the average velocity of Crn1-GFP containing patches is reduced nearly three-fold in *arc3*$^{-}$ cells, and the distance travelled by individual patches is also much shorter (Figure 3C). The formation of endocytic vesicles is also believed to be driven by the Arp2/3 complex, and *S. cerevisiae* mutants defective in the Arp2/3 complex are deficient in endocytosis (Daugherty and Goode, 2008). We thus tested whether Arc3 is required for endocytosis in *S. pombe* by measuring the rate of internalization of the membrane dye FM 4–64. Our data showed that while FM 4–64 internalized readily in *arc3*$^{+}$ cells, in *arc3*$^{-}$ cells, FM 4–64 mainly associated with the plasma membrane and did not internalize efficiently (Figure 3D). These results suggest that Arc3 is involved in the movement of F-actin patch function and endocytosis. Collectively, since Arc3 associates with Arp2/3 and controls the same set of functions known to be controlled by Arp2, we conclude that Arc3 is an authentic Arp2/3 subunit in *S. pombe*.

Yeast Arc3 and human ARPC3 have an evolutionarily conserved function

Based on sequence analysis, *S. pombe* Arc3 is most likely the ortholog of human *ARPC3*, whose functions have not been reported. We first examined whether human *APRC3*, like *S. pombe* Arc3, can associate with F-actin patches in human cells. We took HeLa cells ectopically expressing GFP-tagged *ARPC3* and found that it colocalized with F-actin-rich patches visualized by phalloidin staining (Figure 4A). Treatment with Latrunculin A seemed to cause disappearance of Arc3-GFP structures in the cell with a concurrent increase of diffuse signal in cytoplasm (Figure 4B). To determine if the function of the two orthologs is conserved, we transformed *arc3*$^{+}$*/arc3::ura4 (arc3*$^{+}$*/arc3Δ)* cells with plasmids expressing either human *ARPC3* or *S. pombe arc3* or with the vector control. After sporulation, haploid spores were plated on media without uracil to select *arc3*Δ cells. Our data show that we could only recover viable *arc3*Δ cells when they carried either human *ARPC3* or *S. pombe arc3*, indicating that human *ARPC3* can efficiently rescue colony formation of *S. pombe arc3*Δ cells (Figure 4C). These cells were further examined by microscopy, and the data show that while most of the cells are elongated in cell-shape, they appeared to be more round, and wider and shorther than normal, and the septum in some mitotic cells was not properly positioned (Figure 4D). While human *ARPC3* can replace *S. pombe arc3*Δ when the cell is grown in solid medium, when *arc3*Δ cells expressing *ARPC3* were transferred to liquid medium, the resulting cells could barely grow. We conclude that *S. pombe arc3* is an ortholog of human *ARPC3* and that they control a similar set of functions, although human *ARPC3* does not fully rescue the phenotype of *S. pombe arc3*Δ cells.

CONCLUDING REMARKS

In this study, we identified the *S. pombe* Arc3 subunit of the Arp2/3 complex. As expected, Arc3 binds other subunits of the Arp2/3 complex, and localizes to F-actin patches. Cells deficient in Arc3, as with the *arp2* mutant, contain an F-actin network that is disorganized. In particular, in these *arc3*-repressed cells, F-actin patches are dispersed throughout the cells with greatly reduced mobility. *S. pombe arc3* mutant is also deficient in endocytosis, which is consistent with the observation that proper formation of F-actin patches correlates with

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efficient endocytosis. Our data also strongly suggest that Arc3 is the ortholog of human ARPC3 and that its function is conserved because (1) ARPC3 expression in *S. pombe* efficiently rescues lethality of *arc3*Δ cells in solid medium and (2) ARPC3 also localizes to patches and in HeLa cells the Arp2/3 complex has been shown to be recruited to sites of clathrin-mediated endocytosis (Carreno, et al., 2004). While in *S. cerevisiae,* deleting many genes encoding Arp2/3 subunits severely impairs cell growth (Winter, et al., 1999), *arc18*Δ cells are viable. By contrast, in *S. pombe,* deleting *arp2,* as well as *arc3,* induced lethality. Thus while the Arp2/3 complexes in both yeasts control F-actin patch organization and endocytosis, the essential function of Arc3 in *S. pombe* may be absent or performed by another protein in *S. cerevisiae*.

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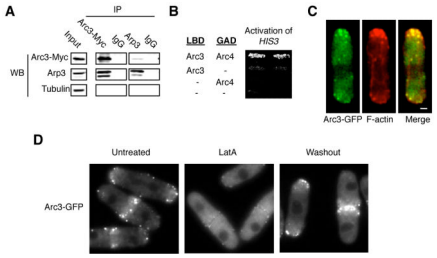

Figure 1.

Arc3 associates with the Arp2/3 complex in a F-actin dependent manner. (A) Lysates from cells expressing Arc3-MYC were prepared for immunoprecipitation (IP) — antibodies used are as indicated (top) and purified mouse IgG was used as a control. Immunoprecipitated proteins were analyzed by Western blots using antibodies for the indicated proteins. (B) The binding between Arc3 and Arc4 was determined by the yeast two-hybrid system by measuring the activation of the HIS3 reporter gene as described (Chang, et al., 1994). pVJLARC3 and pGADARC4 express Arc3 and Arc4 fused to the LexA DNA binding domain (LBD) and the Gal4 activation domain (GAD), respectively. (C) Cells expressing Arc3-GFP were fixed and stained with rhodamine-conjugated phalloidin to visualize F-actin. We found that nearly every GFP dot overlaps with an F-actin dot/patch, but not with F-actin cables, in all the cells that we examined. A representative cell is shown. (D) Arc3-GFP expressing cells were treated with 10 μM Latrunculin A (LatA) for 30 mins to depolymerize F-actin followed by washing and incubation in regular media (Wash out). Images of Arc3-GFP distribution were collected from untreated cells, from cells treated with LatA for 30 mins and from cells 1 hour after washing out. Bar, 1 μm.

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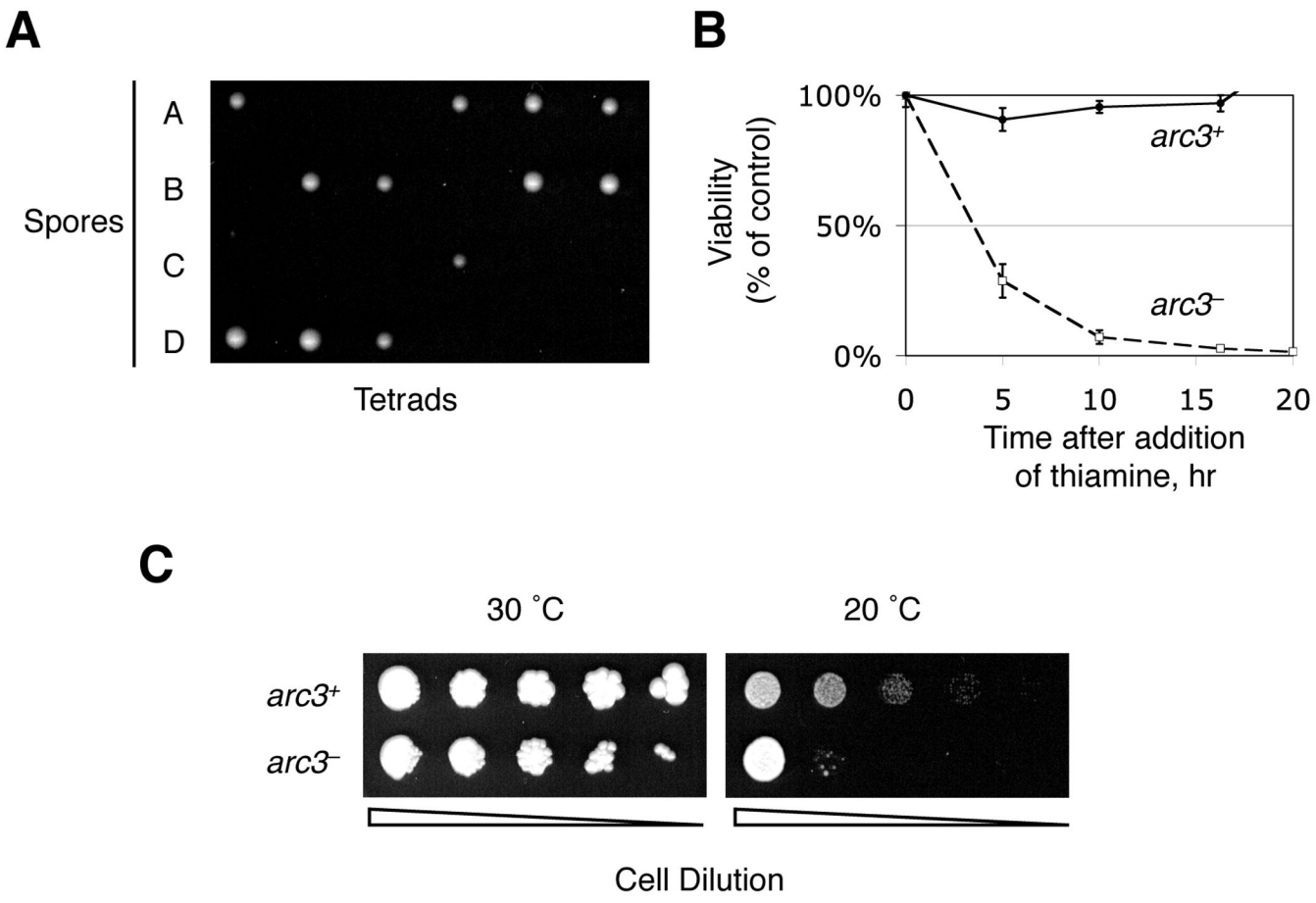

Figure 2.

arc3 is essential for viability. (A) *arc3* null heterozygous diploid cells, *arc3*Δ/+ (*arc3::ura4*/$^{+}$), were induced to sporulate. After tetrad dissection, the spores were allowed to grow at 30°C on YEAU plates. Emerged colonies were further examined for autotrophy markers and none were Ura+, indicating that these were *arc3*$^{+}$ cells. (B) The *arc3*^{nmt} mutant cells (strain ARC3NMT), whose *arc3* is expressed under the control of the thiamine-repressible *nmt* promoter, were first treated with (*arc3*$^{-}$) or without (*arc3*$^{+}$) 200 nM thiamine to regulate *arc3* expression. Time points were taken and identical numbers of cells were spread on MM plates lacking thiamine to measure viability by colony formation (left). (C) WT or *arc3*^{nmt} mutant cells were serially diluted and spotted on MM plates contatining 1 nM thiamine and incubated at the indicated temperatures.

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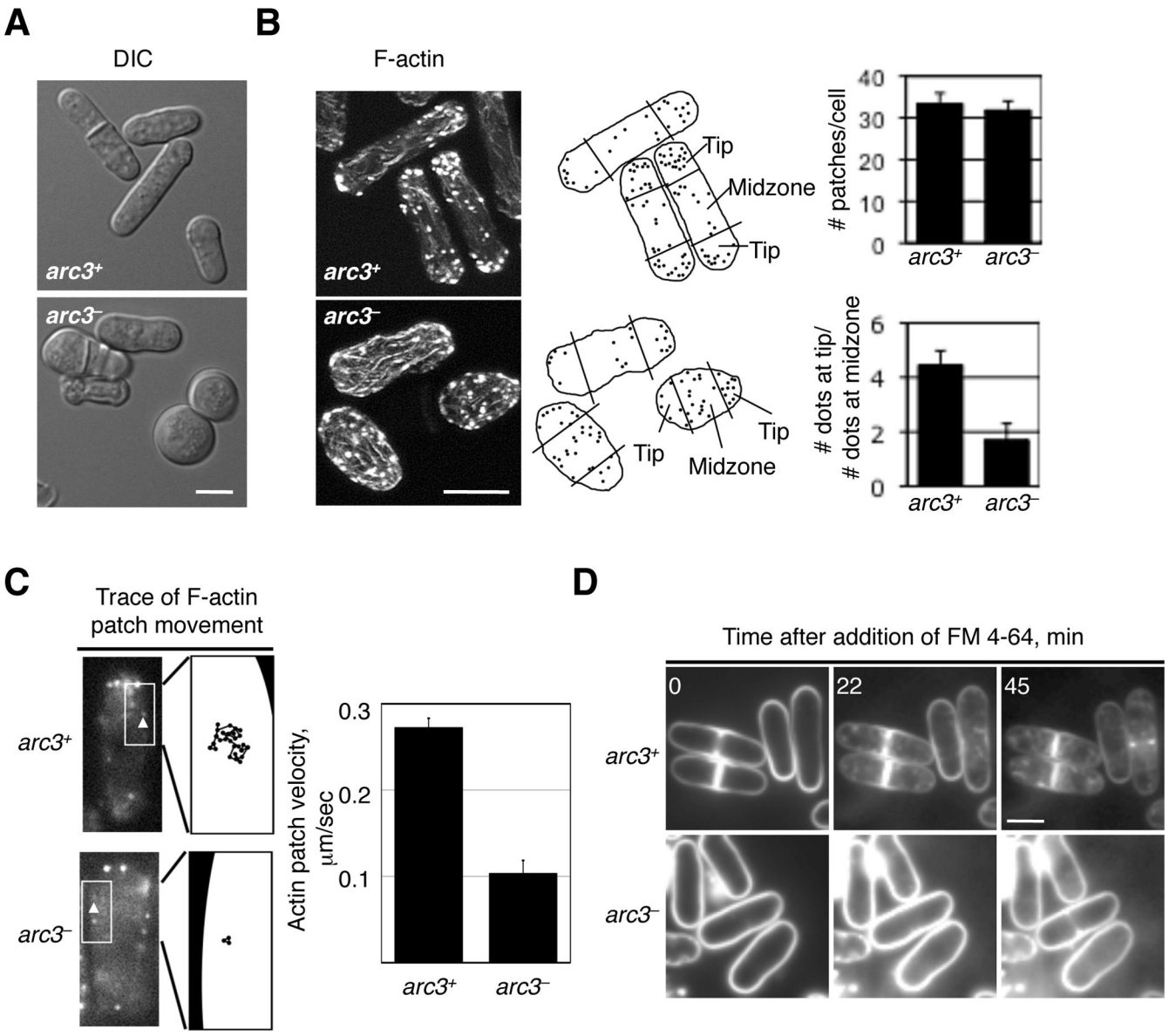

Figure 3.

arc3 is required for organization and mobility of F-actin patches and endocytosis. (A) *arc3* expression was repressed as in Figure 2B for 20 hours, and both *arc3*$^{-}$ and *arc3*$^{+}$cells were visualized by DIC microscopy. (B) *arc3* expression was repressed as in A, the resulting cells were fixed and stained with the F-actin dye Alexa 488-Phalloidin. Shown here are projection images after deconvolution. The length of the cell was measured and dots located in the 25% of the length nearest to the end were counted as localized to that cell end. While F-actin patches are concentrated at the cell ends in *arc3*$^{+}$ interphase cells, in *arc3*-repressed (*arc3*$^{-}$) cells, these patches are more diffused whether the cell is round or not (right). The number and the size of F-actin patches are similar in these two types of cells. (C) *arc3*^{nmt} cells expressing Crn1-GFP were treated with (*arc3*$^{-}$) or without 200 nM thiamine (*arc3*$^{+}$) for 16 hours and observed by time lapse microscopy. Crn1-GFP dots (left, arrowheads) were individually tracked and the velocity calculated (right). A total of 15 dots in wild type cells and 30 dots in the *arc3* mutants were analyzed. We note that the measured Crn1-GFP

velocity in wild type cells matches that reported previously (Pelham and Chang, 2001). (D) The *arc3* expression was repressed as in (C) before the fluorescent dye FM 4–64 (8.15 mM) was added, and the cells were then observed by time lapse microscopy. This dye first bound the plasma membrane and then readily entered *arc3*$^{+}$ cells and ultimately accumulated in the vacuoles. In contrast, in *arc3*$^{-}$ cells, this dye could only be detected at the plasma membrane. Bars, 5 μm.

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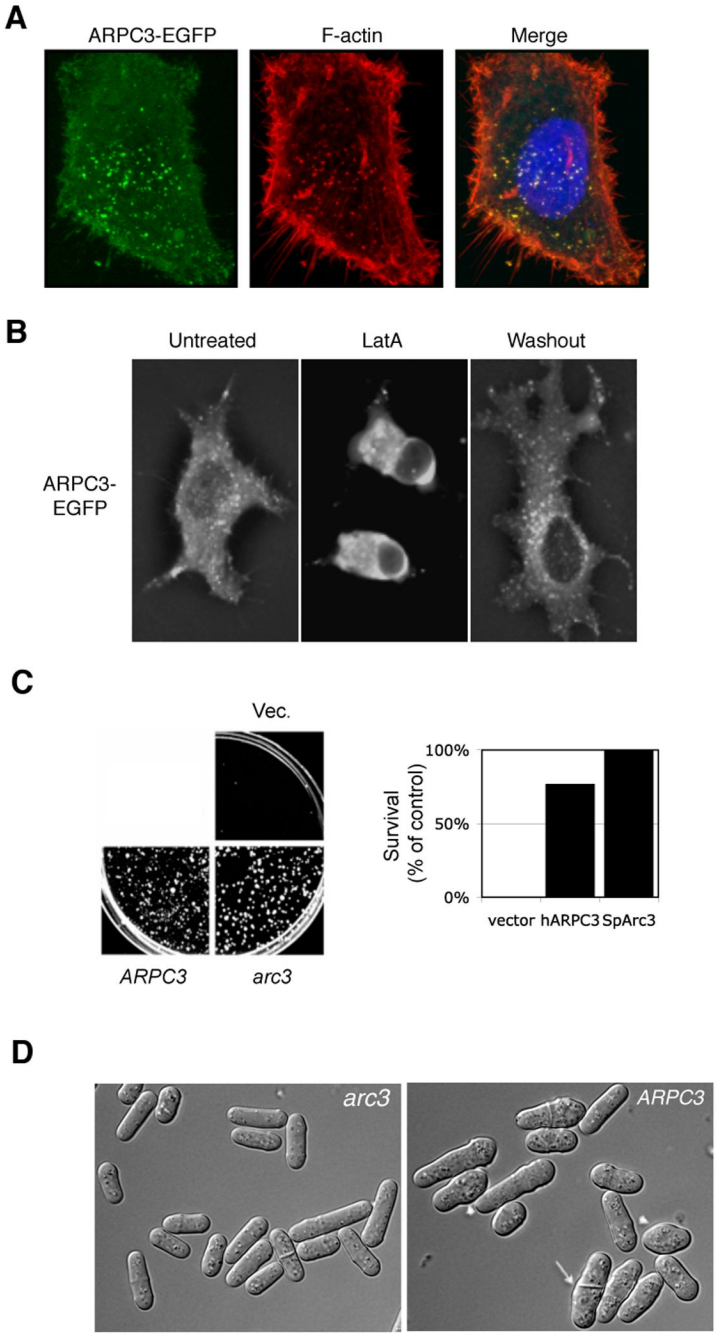

Figure 4.

arc3 function has been conserved throughout evolution. (A) HeLa cells expressing human *ARPC3-EGFP* were fixed and stained with rhodamine-phalloidin to visualize F-actin. We found that nearly every GFP dot overlaps with an F-actin dot. (B) HeLa cells expressing *ARPC3-EGFP* were treated with 10 μM Latrunculin A for 30 min to depolymerize F-actin. These cells were then washed and incubated in regular media for 1 hr before being photographed. (C) Diploid cells heterozygous for *arc3* deletion (+/*arc3::ura4*) were transformed with plasmids expressing *S. pombe arc3* (pREP41ARC3), human *ARPC3* (pREP1ARPC3) or the empty vector as indicated. Cells were then sporulated, and plated on media with selection for the *arc3* deletion. Relative survival compared to control was

quantified (right). (D) Colonies from cells transformed with *S. pombe arc3* (pREP41ARC3) or human *ARPC3* (pREP1ARPC3) obtained as in (C) were grown on MM to log phase and observed under the microscope. The arrowheads show cells with abnormal morphology and the arrow indicates a cell with an improperly positioned septum.

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