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The filament-forming protein Pil1 assembles linear eisosomes in fission yeast

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ABSTRACT The cortical cytoskeleton mediates a range of cellular activities such as endocytosis, cell motility, and the maintenance of cell rigidity. Traditional polymers, including actin, microtubules, and septins, contribute to the cortical cytoskeleton, but additional filament systems may also exist. In yeast cells, cortical structures called eisosomes generate specialized domains termed MCCs to cluster specific proteins at sites of membrane invaginations. Here we show that the core eisosome protein Pil1 forms linear cortical filaments in fission yeast cells and that purified Pil1 assembles into filaments *in vitro*. In cells, Pil1 cortical filaments are excluded from regions of cell growth and are independent of the actin and microtubule cytoskeletons. Pil1 filaments assemble slowly at the cell cortex and appear stable by time-lapse microscopy and fluorescence recovery after photobleaching. This stability does not require the cell wall, but Pil1 and the transmembrane protein Fhn1 colocalize and are interdependent for localization to cortical filaments. Increased Pil1 expression leads to cytoplasmic Pil1 rods that are stable and span the length of cylindrical fission yeast cells. We propose that Pil1 is a novel component of the yeast cytoskeleton, with implications for the role of filament assembly in the spatial organization of cells.

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INTRODUCTION

The plasma membrane is compartmentalized into domains that organize clustered signaling modules related to a wide range of cellular activities, including polarized growth, endocytosis, and sensing of nutrients (Lingwood and Simons, 2010; Hartman and Groves, 2011). *In vitro* experiments have shown the formation of nanoscale membrane rafts through the phase separation of self-organizing lipid species (Lingwood and Simons, 2010). In cells, many protein structures contribute to the regulated assembly and maintenance of membrane domains. Perhaps most notably, the cortical cytoskeleton comprises filaments that associate with the plasma membrane to generate and transmit force, as well as to provide spatial order. Traditional filament systems such as actin and septins comprise this

cortical cytoskeleton and have well-studied roles in regulating cortical rigidity and compartmentalization (Viola and Gupta, 2007; Chichili and Rodgers, 2009; Gilden and Krummel, 2010; Saarikangas *et al.*, 2010). In addition, microtubules form a cortical cytoskeleton in plant cells and contribute to cortical organization in a wide range of cell types (Wasteneys and Ambrose, 2009). Thus cytoskeletal filaments play a common role in organizing and stabilizing plasma membrane domains.

In budding yeast, a cytosolic structure dubbed the eisosome organizes plasma membrane domains that are called membrane compartment-containing Can1 (MCC) domains (Walther *et al.*, 2006; Grossmann *et al.*, 2008). Eisosomes/MCCs appear as puncta by light microscopy and correlate with invaginations of the plasma membrane that are observed by electron microscopy (Moreira *et al.*, 2009; Stradalova *et al.*, 2009). A large number of cytosolic proteins have been shown to associate with eisosome/MCC regions of the cortex (Grossmann *et al.*, 2008). The BAR domain-containing protein Pil1 forms the core of eisosomes, and both eisosomes and MCCs are disorganized in *pil1Δ* cells (Walther *et al.*, 2006; Grossmann *et al.*, 2007; Olivera-Couto *et al.*, 2011; Ziolkowska *et al.*, 2011). The ability of BAR domains to shape membranes may facilitate the link between Pil1/eisosomes and membrane invaginations, but the organizing principle that underlies the large-scale formation and organization of eisosomes is unclear.

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Abbreviations used: DIC, differential interference contrast; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; MCC, membrane compartment containing Can1.

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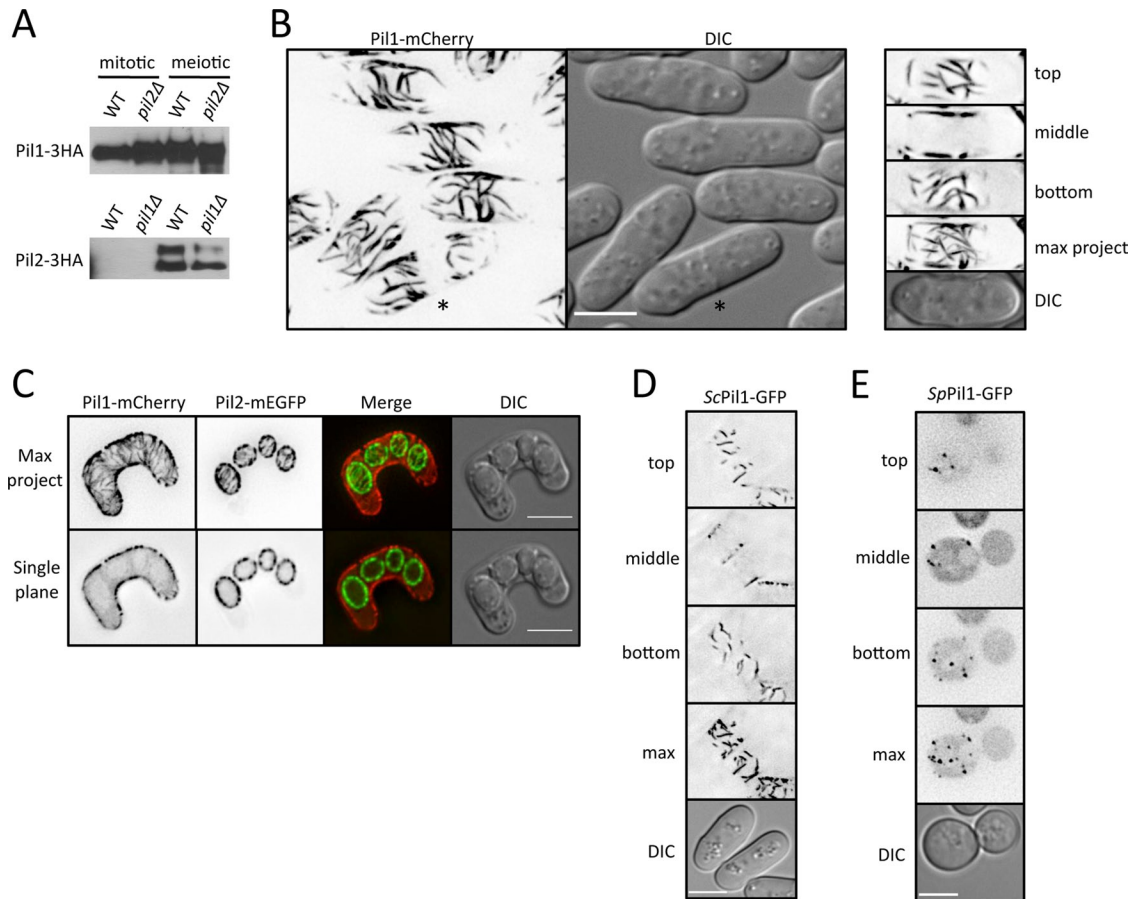


FIGURE 1: Pil1 forms filaments in fission yeast cells. (A) Western blot showing that Pil1-3HA is expressed in both mitotic and meiotic cells, whereas Pil2-3HA is expressed in meiotic cells but not mitotic cells. (B) Localization of Pil1-mCherry in vegetative cells. Left, inverted maximum projection for Z-planes in the top half of the cell. Asterisk, septating cell. Right, single focal planes and maximum projection for a single cell. (C) Localization of Pil1-mCherry and Pil2-mEGFP in mating cells. Top, inverted maximum projection from Z-planes in the top half of the cell; bottom, the middle focal plane. (D) Budding yeast Pil1 (ScPil1) forms filaments when expressed in fission yeast cells. Images show inverted single focal planes and maximum projection. (E) Fission yeast Pil1 (SpPil1) forms puncta when expressed in budding yeast cells. Images show inverted single focal planes and maximum projection. Scale bars: B–E, 5 μ m.

In this study, we report that fission yeast Pil1 forms filaments both *in vitro* and in cells. Our results indicate that eisosomes are stable linear structures and support a role for self-assembly of Pil1 filaments in the organization of cortical domains at the plasma membrane. These results have implications for the role of filaments in compartmentalization of the cell cortex and suggest that the cytoskeleton may be a more diverse network than previously considered.

RESULTS

Pil1 forms filaments in cells and *in vitro*

We identified two Pil1-related proteins in fission yeast, which we designated Pil1 (SPCC736.15) and Pil2 (SPAC3C7.02c). The two proteins are 46% identical and share 53% (Pil1) and 43% (Pil2) identity with budding yeast Pil1 (Supplemental Figure S1). We found that Pil2 is expressed during mating but not in vegetative cells, even in the absence of Pil1. In contrast, Pil1 is expressed in both vegetative and mating cells (Figure 1A). We examined the localization of Pil1 to determine its role in organizing eisosome-like structures in fission yeast cells. Pil1 localized to linear filaments that were confined to the cell cortex (Figure 1B), in contrast to punctate structures observed in budding yeast (Walther *et al.*, 2006; Grossmann *et al.*,

2008; Moreira *et al.*, 2009). These filaments did not show a uniform length or orientation relative to the cell growth axis but were excluded from sites of cell growth. Specifically, Pil1 cortical filaments were absent from growing cell ends during interphase and from the cell middle at septation. Consistent with exclusion from growth sites, Pil1 cortical filaments were present at the nongrowing ends of monopolar and branched mutants (Supplemental Figure S2). These filaments indicate the presence of a novel structure at the fission yeast cell cortex that is positioned by cell growth. We also examined the localization of Pil1 and Pil2 in mating cells, which generate an ascus containing four spores following meiosis. Pil1 localized to filaments along the ascus membrane, whereas Pil2 was found in filaments at the spore membrane (Figure 1C). Neither protein changed localization upon deletion of the other (Supplemental Figure S3). These results show that Pil1-related proteins localize to filaments at the fission yeast cell cortex.

Pil1 cortical filaments are reminiscent of plasma membrane invaginations that have been described by freeze-fracture electron microscopy (Takeo, 1984; Walther *et al.*, 1984; Konomi *et al.*, 2003). These linear invaginations extend 1–2 μ m and are excluded from regions of cell growth, similar to the localization of Pil1. Indeed,

linear invaginations in budding yeast are organized by Pil1 (Stradalova *et al.*, 2009) but in this case extend only ~200 nm and appear as spots by fluorescence microscopy (Walther *et al.*, 2006; Grossmann *et al.*, 2008; Frohlich *et al.*, 2009), likely due to the resolution limits of light microscopy. To examine this link between membrane invaginations and Pil1, we examined fission yeast Pil1 localization in stationary-phase cultures, when plasma membrane invaginations become more numerous (Walther *et al.*, 1984). Cortical Pil1 filaments became dense and were no longer excluded from the tips of stationary-phase cells, which are not growing (Supplemental Figure S4). These combined results suggest that eisosomes are linear structures that underlie invaginations in the plasma membrane.

The Pil1 cortical filaments that we observe in fission yeast differ from the spot-like structures that have been described by light microscopy for budding yeast Pil1 (Walther *et al.*, 2006; Grossmann *et al.*, 2008; Moreira *et al.*, 2009; Brach *et al.*, 2011). This could stem from differences in the two Pil1 proteins. Alternatively, differences at the cell cortex could allow extension of Pil1 filaments in fission yeast but not budding yeast. To distinguish between these possibilities, we first expressed budding yeast Pil1 (ScPil1) in *pil1Δ pil2Δ* fission yeast cells. ScPil1 formed linear filaments but not puncta at the cell cortex (Figure 1D). Next we expressed fission yeast Pil1 (SpPil1) in *pil1Δ* budding yeast cells and observed puncta but not filaments (Figure 1E). These SpPil1 puncta colocalized with Lsp1 in budding yeast cells (Supplemental Figure S5), supporting functional homology between SpPil1 and ScPil1. These results indicate that differences in the cellular environment, not the respective proteins, underlie the distinct appearance of Pil1 in budding yeast versus fission yeast. This also suggests that the budding yeast plasma membrane restricts elongation of cortical Pil1 filaments.

We next tested the possibility that Pil1 cortical filaments are related to known cytoskeletal structures. During interphase, filamentous actin in fission yeast is organized into endocytic patches at cell ends and linear actin cables (Kovar *et al.*, 2011). Pil1 did not colocalize with actin patches or cables, which were visualized using the F-actin marker GFP-CHD (Wachtler *et al.*, 2003). Furthermore, treatment of cells with the actin drug latrunculin-A disrupted all actin structures but did not affect Pil1 cortical filaments (Figure 2A). Similarly, Pil1 localization did not overlap with microtubules and was not affected by carbendazim (MBC; Figure 2B), which depolymerizes microtubules. We conclude that Pil1 cortical filaments are independent of the actin and microtubule cytoskeletons. This raises the possibility that Pil1 itself forms filaments at the cell cortex.

As an initial test of filament assembly, we expressed Pil1-GFP in *Escherichia coli* cells. Pil1-GFP formed linear filaments in these cells (Figure 3A), indicating that it assembles into filaments in both prokaryotic and eukaryotic cells. We next purified recombinant Pil1 from bacteria to test the ability of Pil1 to form filaments *in vitro* (Figure 3B). The purified Pil1 protein sedimented by high-speed but not low-speed centrifugation (Figure 3C), similar to other cytoskeletal polymers, such as actin and microtubules. Next purified Pil1 was negative stained and imaged by electron microscopy (Figure 3, D and E). This revealed the presence of Pil1 filaments that were 28.6 ± 2.2 nm wide ($n = 20$). Because budding yeast Pil1 can also form filaments *in vitro* (Olivera-Couto *et al.*, 2011), this appears to be a conserved feature of this family of proteins.

Pil1 cortical filaments are stable structures

We next examined the dynamics of Pil1 filaments at the fission yeast cell cortex using time-lapse microscopy. Fission yeast cells grow in a polarized manner at the cell tips. Pil1 filaments remained immobile in the cell middle and did not grow or shrink (Figure 4A).

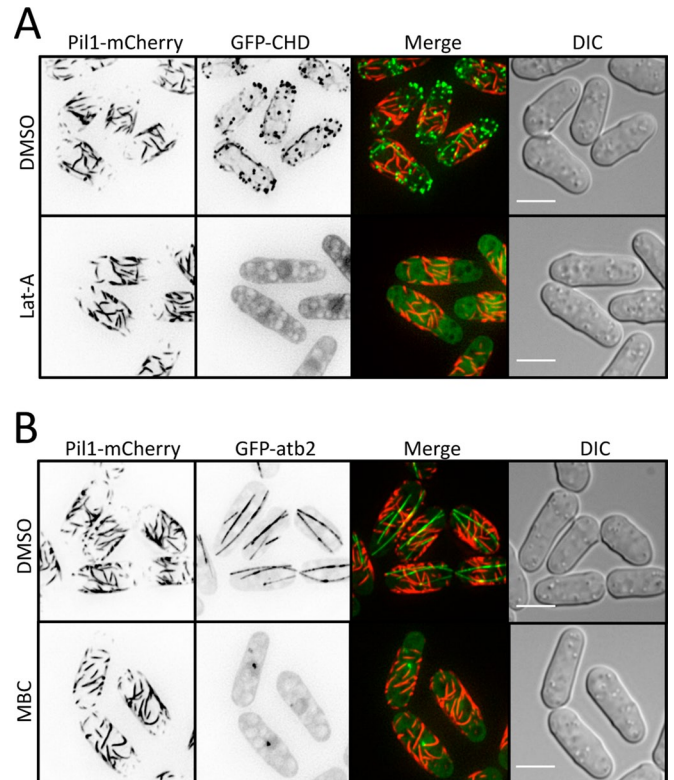


FIGURE 2: Pil1 filaments are independent of the actin and microtubule cytoskeletons. (A) Pil1 cortical filaments do not colocalize with or depend on the actin cytoskeleton. (B) Pil1 cortical filaments do not colocalize with or depend on microtubules. Images are maximum projections for Z-planes in the top half of the cell. Scale bars, 5 μ m. Cells were treated with the indicated drug or DMSO control for 20 min before imaging.

The distribution of Pil1 changed at cell division, when growth is redirected to the cell middle. At this site of septation, Pil1 filaments were dramatically cleared away through a process that involved both the movement and severing of filaments in the cell middle (Figure 4, B and C). This clearance appeared to correlate with changes in cell growth patterns because we also observed movement of Pil1 away from cell tips when they began growth following division. In some cases, individual Pil1 cortical filaments displayed directional movement away from the growth site (Supplemental Figure S6, A–D).

Segregation of Pil1 cortical filaments during cell division leads to the gradual depletion of Pil1 filaments during division cycles, raising the question of how new filaments assemble. We observed the *de novo* assembly of Pil1 filaments (Figure 4D), which always occurred at the cell cortex. New filaments appeared as a spot that elongated in a linear manner at a rate of 9.8 ± 2.4 nm/min ($n = 10$), which is remarkably slow compared with other yeast cytoskeletal structures, including interphase microtubule arrays and actin cables. We conclude that Pil1 cortical filaments are stable structures that assemble slowly and undergo little movement. Movement and remodeling of these structures are driven by the process of cell growth, which likely alters the composition of lipids and proteins at the cell cortex.

As an independent measure of Pil1 cortical filament stability, we used fluorescence recovery after photobleaching (FRAP) to examine the movement and exchange of Pil1 molecules in cortical filaments. Bleached regions of Pil1-mCherry in the cell cortex did not recover any fluorescence, indicating that there is no exchange between Pil1

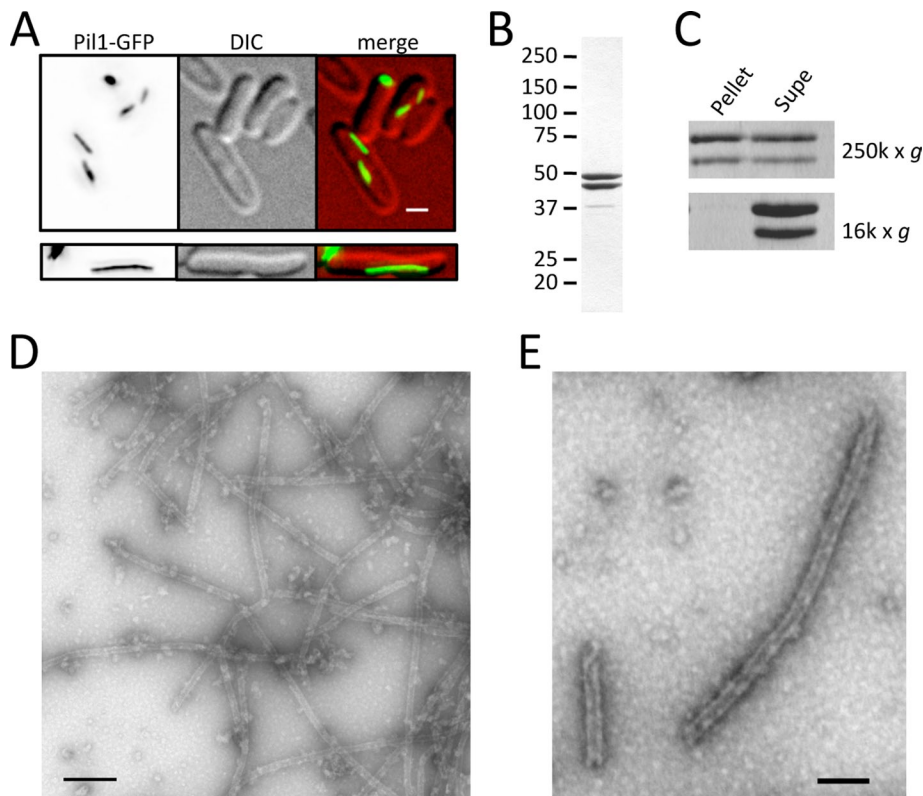


FIGURE 3: Pil1 forms filaments in bacterial cells and in vitro. (A) Localization of Pil1-GFP in *E. coli* cells. Fluorescence images are inverted maximum-intensity projections, and differential interference contrast (DIC) is red in the merged image. Scale bar, 1 μm . (B) Coomassie-stained SDS-polyacrylamide gel of purified Pil1, which migrates as a doublet similar to budding yeast Pil1 purified from bacteria. (C) Coomassie-stained SDS-polyacrylamide gel of purified Pil1 following centrifugation at high speed or low speed, as indicated. Pellet and supernatant fractions were separated and analyzed by SDS-PAGE. (D, E) Electron micrographs of purified Pil1 filaments. Samples were negatively stained and visualized by electron microscopy. Scale bar: D, 200 nm; E, 100 nm.

in cortical filaments and Pil1 in the cytoplasm (Figures 5, A and B). We next photobleached a spot within a single Pil1 cortical filament and observed neither recovery nor movement of this bleached region (Figure 5C). This demonstrates that Pil1 molecules do not move

in budding yeast. Surprisingly, the fission yeast orthologues of Slm1 (SPAC637.13c) and Sur7 (SPAC15A10.09c) did not colocalize with Pil1 or depend on Pil1 for their localization (Supplemental Figure S7), unlike their budding yeast orthologues (Walther et al., 2006; Grossmann

or treadmill within a single cortical filament. These combined data indicate that Pil1 cortical filaments are stable structures that form slowly, do not turn over, and remain static until there are changes in cell growth pattern.

We considered that association of Pil1 with the yeast cell wall could generate this stability. To test this possibility, we enzymatically digested away the cell wall and then monitored Pil1 cortical filaments. During this process, the cell often emerges from one cell end and leaves behind a cell wall ghost. At this stage, Pil1 filaments associated with the emerging spheroplast but not with the ghost (Figure 5D). Following complete cell wall digestion, Pil1 remained in filaments that were restricted to the cortex of spheroplasts and were not mobile (Figure 5E and Supplemental Figure S6E). We conclude that the stability of Pil1 cortical filaments is independent of the cell wall. The interaction of Pil1 with the plasma membrane may generate these stable structures, although other cellular factors could also contribute to this stability.

Pil1 cortical filaments require transmembrane protein Fhn1

Cytoskeletal polymers such as actin and microtubules are controlled by a wide range of cellular proteins that orchestrate their dynamic assembly and stability in cells (Sawin and Tran, 2006; Kovar et al., 2011). To identify factors that contribute to the stability of Pil1 cortical filaments, we examined proteins that have been shown to colocalize with Pil1

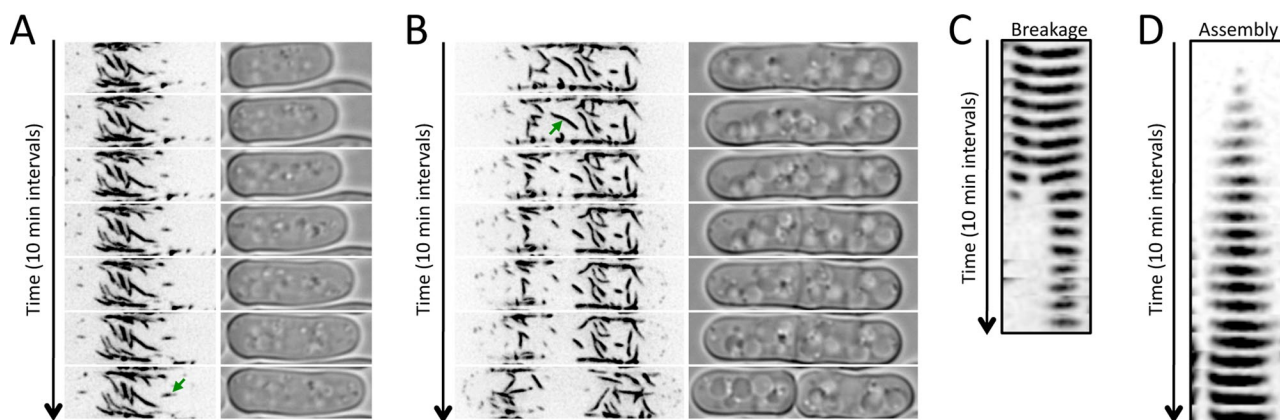


FIGURE 4: Pil1 cortical filaments are static structures that assemble slowly. (A) Time-lapse microscopy of Pil1-mCherry during polarized cell growth. Images are inverted maximum projections for Z-planes in the top half of the cell. The green arrow highlights a newly assembled filament. (B) Time-lapse microscopy of Pil1-mCherry during cell division. The green arrow marks filament breakage. Scale bars, 5 μm . (C, D) Kymograph showing breakage and disassembly (C) or assembly (D) of single Pil1 filaments in cells. Images are inverted single focal planes from time-lapse microscopy.

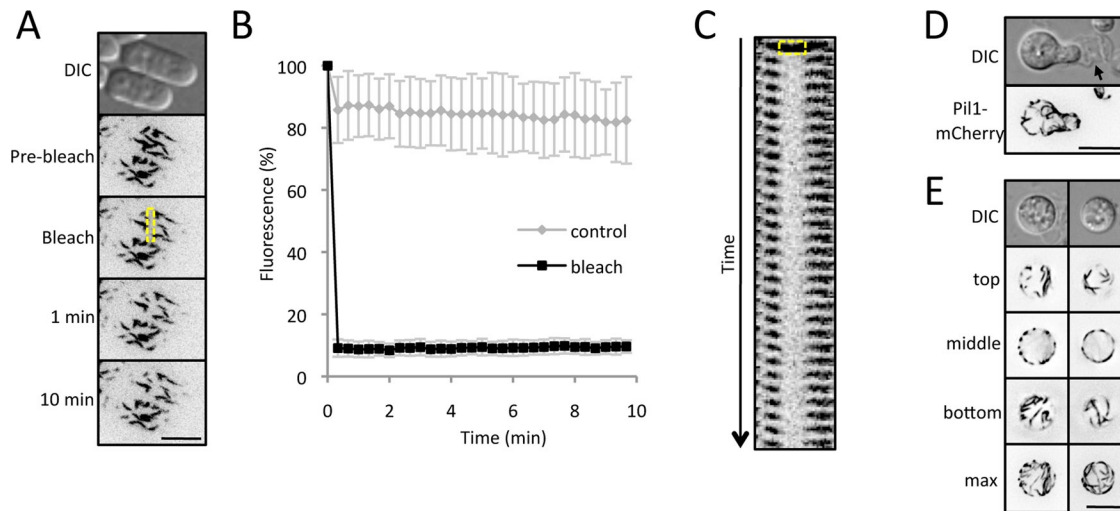


FIGURE 5: Pil1 cortical filaments are fixed structures that are independent of the cell wall. (A) FRAP of Pil1-mCherry. The yellow box denotes the bleached region, and images are inverted maximum projections for Z-planes in the top half of the cell. (B) Quantification of fluorescence recovery in bleached or control regions of FRAP experiments. Data points are the mean of 10 regions; error bars, SD. (C) Kymograph of single Pil1-mCherry filament bleached in the middle; each time point is 20 s. (D) Localization of Pil1-mCherry in emerging spheroplast. Image is inverted maximum projection; arrow, cell wall ghost. (E) Pil1-mCherry localizes to cortical filaments in spheroplasts. Images show inverted single focal planes and maximum projection, as indicated. Scale bars: A, D, E, 5 μ m.

et al., 2008; Kamble *et al.*, 2011). Instead, Sur7 and Slm1 localized to the cell tips, similar to the recently described Sur7-like protein Mug33 (Snaith *et al.*, 2011). Thus the role of Pil1 in organizing these proteins is not conserved between budding yeast and fission yeast.

We next examined the transmembrane protein Fhn1 due to its homology with budding yeast Nce102, which facilitates Pil1 localization in that organism (Grossmann *et al.*, 2008; Frohlich *et al.*, 2009; Loibl *et al.*, 2010). Fhn1 showed clear colocalization with Pil1 cortical filaments and in addition was present at growing cell tips and the division septum (Figure 6A). In *pil1* Δ cells, Fhn1 was absent from cortical filaments in the cell middle but remained at the growing cell tips (Figure 6B). Strikingly, Pil1 cortical filaments in *fhn1* Δ cells were less numerous and shorter (Figure 6C). We counted 2.2 ± 2.1 Pil1 filaments in *fhn1* Δ cells, compared with 15.7 ± 4.2 filaments in wild-type cells ($n = 200$; mean \pm SD). Furthermore, filament length was reduced from 2.3 ± 0.8 μ m in wild-type cells to 0.9 ± 0.3 μ m in *fhn1* Δ cells ($n = 100$; mean \pm SD). This indicates that Pil1 and Fhn1 are interdependent for localization to cortical filaments and that the ability of Pil1 to assemble into filaments is not sufficient for robust assembly of cortical filaments in cells. These results are consistent with the requirement of Nce102 for eisosome assembly in budding yeast (Grossmann *et al.*, 2008; Frohlich *et al.*, 2009; Loibl *et al.*, 2010) and suggest that the combined activities of these two proteins generate stable domains at the plasma membrane.

Assembly of cytoplasmic rods by overexpressed Pil1

The cellular function of Pil1 has been unclear. Early reports in budding yeast suggested a link between Pil1 and endocytosis (Walther *et al.*, 2006), although subsequent work found that Pil1 does not regulate actin patches or endocytosis (Brach *et al.*, 2011). A more striking phenotype is observed in the filamentous fungus *Ashbya gossypii*, where loss of Pil1 leads to cell polarity defects (Seger *et al.*, 2011). We did not observe phenotypic defects for *pil1* Δ (or *pil1* Δ *pil2* Δ) fission yeast cells. These mutant cells displayed a wild-type morphology and growth rate at temperatures between 25 and 36°C (unpublished data). Moreover, we did not detect colocalization of

endocytic actin patches with Pil1 cortical filaments (Figure 2A), and *pil1* Δ *pil2* Δ cells showed no defects in actin patch distribution (unpublished data). In contrast, fission yeast cells with increased Pil1 expression, driven by the P3nmt1 promoter, exhibited cell polarity and cytokinesis defects that led to severely reduced growth rates (Figures 7, A and B). Indeed, 81% of septa in Pil1-overexpressing cells were abnormal, compared with 2% of septa in control cells ($n = 200$ septa), and Pil1-overexpressing cells displayed abnormal cytokinetic structures (Supplementary Figure S8). Consistent with these phenotypes, cortical proteins that control cell polarity and cytokinesis, including Tea1 (Mata and Nurse, 1997), Pom1 (Bahler and Pringle, 1998), and Cdr2 (Almonacid *et al.*, 2009), were mislocalized

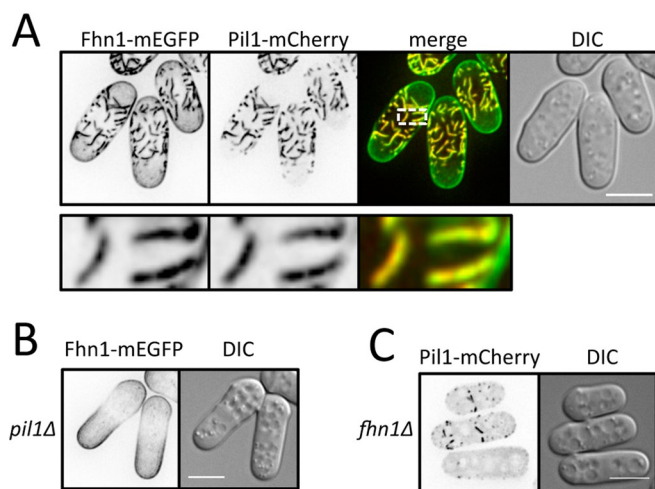


FIGURE 6: Pil1 and Fhn1 are interdependent for localization. (A) Colocalization of Pil1-mCherry and Fhn1-mEGFP in cortical filaments; region boxed in white is magnified in the bottom row. (B) Localization of Fhn1-mEGFP in *pil1* Δ cells. (C) Localization of Pil1-mCherry in *fhn1* Δ cells. Note decreased abundance and length of cortical filaments. All images are inverted maximum projections from Z-planes in the top half of the cell; scale bars, 5 μ m.

upon Pil1 overexpression (Figure 7C). This supports a role for Pil1 in maintaining spatial order at the cell cortex.

To determine the behavior of overexpressed Pil1, we visualized Pil1-GFP expressed from the intermediate-strength P41nmt1 promoter, which does not lead to the growth defects described previously. Strikingly, overexpressed Pil1 localized to long cytoplasmic rods that were not confined to the cell cortex and often spanned the length of the cell (Figure 8A and Supplemental Movie S1). The thickness of these rods suggests that they are composed of bundled Pil1 filaments. Similar to Pil1 cortical filaments, these Pil1 cytoplasmic rods were stable in the absence of other cytoskeletal elements, such as actin, microtubules, and septins (Figure 8B).

We next examined the stability of Pil1 rods using time-lapse microscopy and FRAP. These rods were mobile in the cytoplasm but did not exhibit rapid assembly or disassembly kinetics (Figure 8C and Supplemental Movie S2). The movement was not directional; instead, cytoplasmic rods appeared to float and twist randomly. In FRAP experiments, we detected slow fluorescence recovery of bleached areas, indicating addition of cytoplasmic Pil1 into rods, which might be expected for overexpressed protein (Figure 8D). However, bleached and unbleached regions did not move within Pil1 rods, indicating that Pil1 filaments within a rod do not move or treadmill in a polarized manner. We conclude that increased levels of Pil1 generate stable structures that are not confined to the cell cortex.

DISCUSSION

Much work in recent years has focused on identifying the mechanisms and structures that generate microdomains in the plasma membrane (Lingwood and Simons, 2010). We showed that the fission yeast eisosome protein Pil1 forms filaments *in vitro* and in cells.

These filaments underlie plasma membrane invaginations that generate spatial domains within the cell cortex. We envision that Pil1 cortical filaments in cells are made up of the filaments that we observed *in vitro* for purified Pil1, although other possibilities exist. Our work supports a model for eisosomes as linear structures and indicates the existence of factors that limit the elongation of eisosomes in budding yeast. One intriguing possibility is that the PKC kinases Pkh1 and Pkh2 impose this length restriction, as depletion of these kinases leads to elongated Pil1 structures in budding yeast (Brach *et al.*, 2011).

The role of Fhn1 in formation of Pil1 cortical filaments, combined with the appearance of cytoplasmic rods upon Pil1 overexpression, suggests the existence of cellular mechanisms that organize Pil1 filaments into higher-order structures. Several possibilities could explain the role of Fhn1 in this process. The transmembrane protein may interact directly with Pil1 to regulate filament dynamics and organization, similar to proteins that bind actin filaments and microtubules. Another possibility is that Fhn1 interaction with the plasma membrane “primes” Pil1–membrane interactions that stabilize Pil1 filaments in cells. Beyond Fhn1, the appearance of Pil1 cytosolic rods suggests mechanisms to bundle Pil1 filaments. More detailed analysis of Pil1 ultrastructure, both *in vitro* and *in vivo*, will distinguish between these and other models.

Pil1 family proteins have recently been shown to contain a BAR domain (Olivera-Couto *et al.*, 2011; Ziolkowska *et al.*, 2011), which can directly tubulate lipids. BAR-domain proteins remodel membranes in a wide range of eukaryotic organisms to facilitate endocytosis, cell motility, and phagocytosis (Suetsugu *et al.*, 2010). Our results raise the possibility that other BAR-domain proteins assemble into filaments, with the potential to couple or transmit force

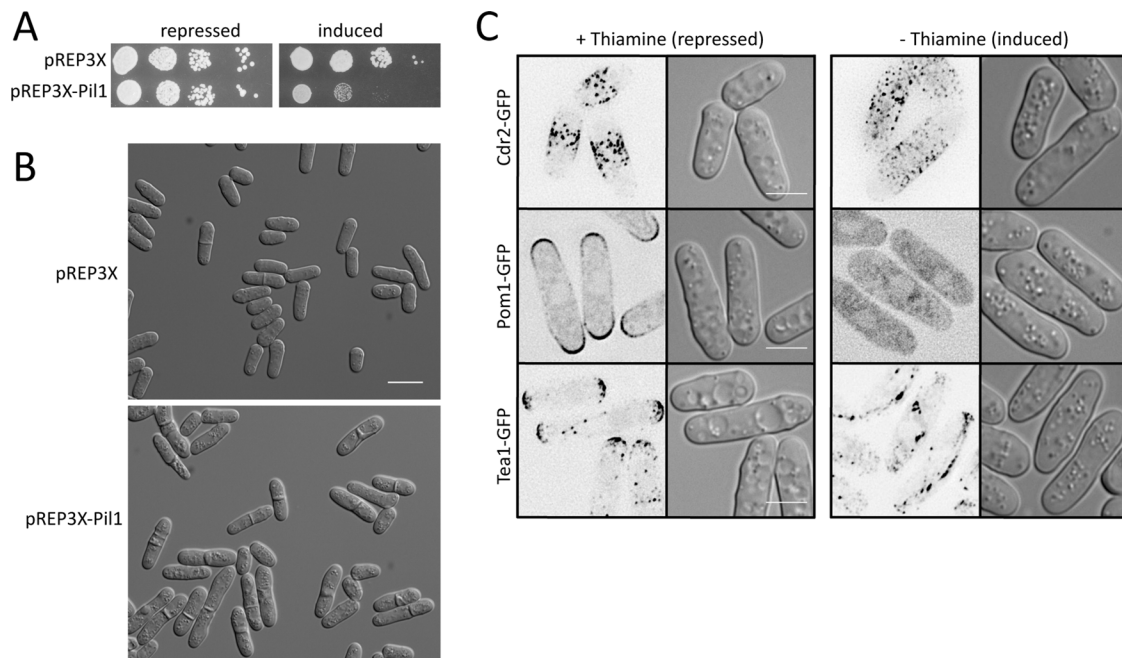


FIGURE 7: Pil1 overexpression impairs cell polarity and cytokinesis. (A) Tenfold serial dilutions of cells containing the indicated plasmids. Expression was induced by growth on media lacking thiamine. (B) DIC images of cells containing control plasmid or Pil1 overexpression plasmid. Expression was induced by removal of thiamine from growth media for 20 h at 32°C. Scale bar, 10 μm. (C) Pil1 overexpression disrupts the localization of mitosis/cytokinesis protein Cdr2 and cell polarity proteins Pom1 and Tea1. The indicated cells were grown in the presence of thiamine (repressed) or in the absence of thiamine for 36 h at 25°C to induce Pil1 overexpression. Pil1 overexpression disrupts the concentration of Cdr2 in the cell middle and leads to loss of Pom1 and Tea1 from cell ends. Images are inverted maximum projections from Z-planes in the top half of cells. Scale bars, 5 μm.

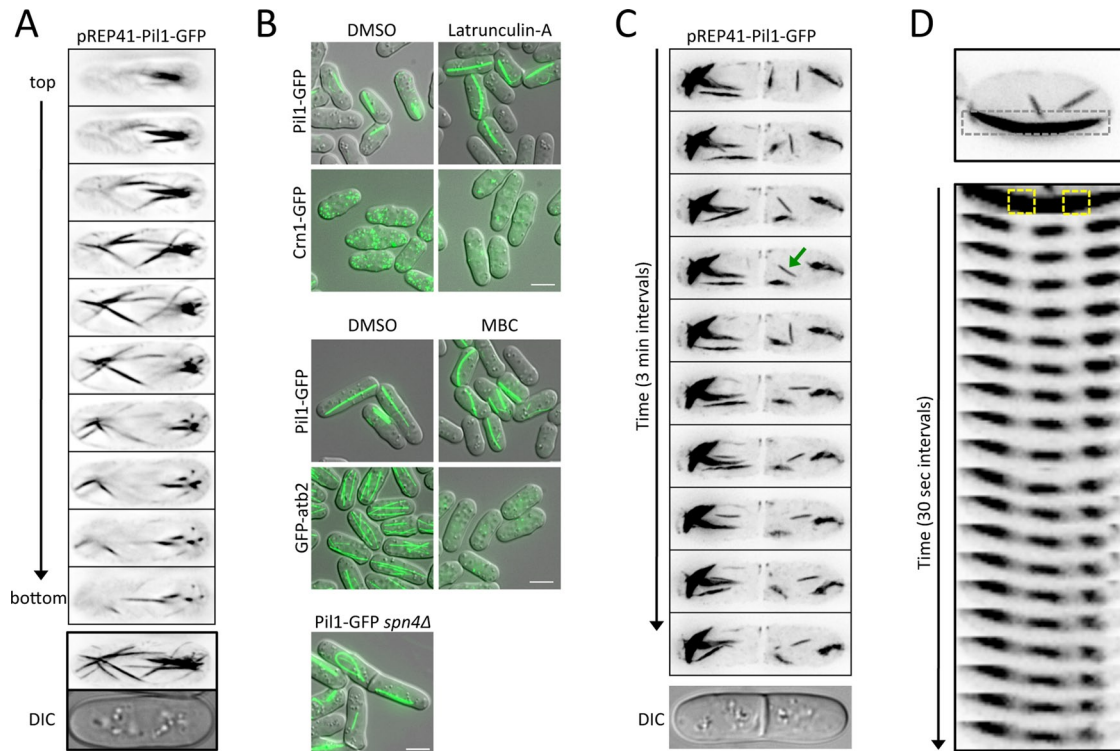


FIGURE 8: Formation of cytoplasmic rods by overexpressed Pil1. (A) Z-series and maximum projection of Pil1-GFP expressed by the medium-strength P41nmt1 promoter. (B) Pil1-GFP rods are stable in the absence of actin, microtubules, and septins. Cells were grown for 36 h at 32°C in the absence of thiamine and then treated with the indicated drug or DMSO control for 20 min. Images are maximum projections of Pil1-GFP overlaid on DIC image. Scale bars, 5 μm. (C) Time-lapse microscopy of cytoplasmic Pil1-GFP rods. Images are maximum projections; green arrow, mobile rod in cytoplasm. (D) FRAP analysis of cytoplasmic Pil1 rods. Kymograph shows time lapse for the rod outlined in gray. Yellow boxes in the prebleach image show sites of bleaching. Note the lack of movement for unbleached region in the center of the rod.

associated with membrane-remodeling events. Alternatively, filament assembly could be a mechanism to regulate lipid tubulation by the Pil1 BAR domain. We note that Pil1 filaments in vitro and in cells are not uniformly straight, which indicates that they are less stiff than actin filaments or microtubules. Thus individual Pil1 filaments may not resist or generate strong tensile forces, although their organization into higher-order structures could overcome this flexibility.

An increasing number of proteins have been shown to form filaments in both prokaryotic and eukaryotic cells (Bowman *et al.*, 2008; Ebersbach *et al.*, 2008; Ingerson-Mahar *et al.*, 2010; Noree *et al.*, 2010). This suggests that the cytoskeleton may be a more extensive network than previously imagined and that self-assembly of filaments may represent a common organizing principle for the generation of spatial information at the cell cortex. Filaments can play a structural role in signaling pathways by concentrating proteins in an ordered manner. In addition, the assembly and disassembly of filaments can generate and transmit force. We anticipate that the identification of novel filament-forming proteins and their biophysical activities will uncover new and unanticipated mechanisms for the regulated self-assembly of cellular structures.

MATERIALS AND METHODS

Yeast strains and methods

Standard *Schizosaccharomyces pombe* media and methods were used (Moreno *et al.*, 1991), and strains used in this study are listed in Supplemental Table S1. Gene tagging and deletion were performed

using PCR and homologous recombination (Bahler *et al.*, 1998), and integrations were verified by colony PCR. We confirmed that Pil1-mCherry is functional because it does not disrupt localization of Fhn1. All yeast strains were generated by tetrad dissection, when applicable. For plasmid-based expression, we used standard cloning techniques to generate plasmids pJM524 (pREP41-ScPIL1-GFP), pJM484 (pREP3X-6His-pil1), pJM512 (pREP41-pil1-GFP), pJM529 (pET24B-pil1-GFP), pJM533 (pRS316-ScPIL1-GFP), and pJM534 (pRS316-SpPil1-GFP). pJM533 and pJM534 contain the endogenous ScPIL1 promoter. Cloning details are available upon request.

Spheroplasts were generated by washing log-phase cells into 50 mM citrate-phosphate buffer, pH 5.6, with 1.2 M sorbitol and then incubating cells in the same buffer containing Zymolyase and Novozyme enzymes. Spheroplast emergence was monitored by microscopy. For latrunculin-A and MBC treatment, cells were treated with 50 μM Lat-A, 50 μg/ml MBC, or dimethyl sulfoxide (DMSO) for 20 min before imaging. No changes in Pil1 cortical filaments or Pil1 cytoplasmic rods were observed when treatment was continued for 1 h.

Microscopy

Cells were imaged on agar pads or in liquid medium under a coverslip with 0.4- or 0.5-μm step size using a DeltaVision Imaging System (Applied Precision, Issaquah, WA), comprising a customized Olympus (Center Valley, PA) IX-71 inverted wide-field microscope, a Photometrics (Tucson, AZ) CoolSNAP HQ2 camera, and an InsightSSI

(Applied Precision) solid-state illumination unit. Images were captured as Z-series and processed by iterative deconvolution in SoftWoRx (Applied Precision) and then analyzed in ImageJ (National Institutes of Health, Bethesda, MD). For Figures 1, B and C, 2, A and B, 4, A and B, and 6, A–C, maximum-intensity projections were generated using five or six focal planes from the top to the middle of the Z-series because it was difficult to follow individual Pil1 cortical filaments along the cell cortex in full maximum-intensity projections containing both the top and bottom planes. To image Pil1-GFP in bacterial cells, pJM529 was transformed into *E. coli* strain BL21(DE23). Transformed cells were grown at 37°C to log phase and shifted to 25°C for 30 min before imaging. Cells were not treated with isopropyl- β -D-thiogalactoside (IPTG), meaning that “leaky” expression of Pil1-GFP was sufficient to observe filaments in Figure 3A.

For FRAP experiments, cells were imaged on agar pads by spinning disk confocal microscopy on a Nikon (Melville, NY) Eclipse Ti equipped with a Yokogawa spinning disk, a Nikon 100 \times 1.4–numerical aperture Plan Apo VC objective, and a Hamamatsu (Hamamatsu, Japan) Imagem C9100-13 EM-CCD camera. This system was controlled by MetaMorph 7 (Molecular Devices, Sunnyvale, CA) and assembled by Performed Technologies (Guelph, Canada). Photobleaching was performed using a 405-nm laser at 75% power for 1 s guided by a Mosaic micromirror array system. Cells were imaged at time points (20 or 30 s) using a 561-nm laser at 10% power and 0.3- μ m step size. Z-series were projected and analyzed in ImageJ.

For transmission electron microscopy, 30- μ l drops of purified Pil1 (2.5 μ M) were spread onto carbon-coated grids (300 mesh), negatively stained with 2% aqueous uranyl acetate, and examined at 100 kV on a JEOL (Peabody, MA) 1010 transmission electron microscope equipped with an XR-41B digital camera and AMTV540 capture engine software.

Protein extraction and purification

For *S. pombe* whole-cell extracts, protein was extracted by the NaOH method (Matsuo *et al.*, 2006), separated by SDS–PAGE, and analyzed by Western blot using anti-HA antibody (F-7; Santa Cruz Biotechnology, Santa Cruz, CA). To purify recombinant Pil1, the plasmid pJM503 (pGEX6P1-pil1) was transformed into *E. coli* strain BL21(DE3). Cells were grown with shaking to OD₆₀₀ = 0.3 at 37°C and then shifted to 25°C for 1 h before induction by addition of IPTG. Cells were grown for an additional 4 h at 25°C and then harvested by centrifugation, washed into lysis buffer (1 \times phosphate-buffered saline [PBS], 250 mM NaCl, 1 mM dithiothreitol, Roche [Indianapolis, IN] complete protease inhibitors, and phenylmethylsulfonyl fluoride), and lysed by sonification. The lysate was clarified by centrifugation (15,000 \times g, 20 min, 4°C), and the resulting supernatant was incubated with glutathione–agarose (Sigma-Aldrich, St. Louis, MO) for 2 h at 4°C. Beads containing GST-Pil1 were washed extensively, and Pil1 was cleaved from glutathione *S*-transferase by overnight incubation with 3C protease (a gift from Dean Madden). Pil1 was further purified on a monoQ 5/5 anion exchange column and eluted using PBS with a linear gradient of 1 M NaCl from 1 to 80%. Peak fractions containing Pil1 were identified by SDS–PAGE with Coomassie staining, pooled, dialyzed overnight against PBS, and then stored at 4°C. This purified Pil1 in PBS buffer was used for pelleting and electron microscope imaging, indicating that no additional factors are required for assembly of Pil1 filaments. Purified Pil1 migrated as a doublet by SDS–PAGE, similar to ScPil1 that has been purified from bacteria (Olivera-Couto *et al.*, 2011). We used tandem mass spectrometry (Vermont Genetics Network Proteomics Facility, University of Vermont, Burlington, VT) to confirm that both

bands are Pil1. For Pil1 centrifugation assays, the purified protein was centrifuged at room temperature at the indicated speeds for 10 min and supernatant versus pellet fractions were analyzed by SDS–PAGE and Coomassie staining.

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