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Pil1 cytoplasmic rods contain bundles of crosslinked tubules

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Cytoskeletal polymers are organized into a wide variety of higher-order structures in cells. The yeast BAR domain protein Pil1 self-assembles into tubules *in vitro*, and forms linear polymers at cortical eisosomes in cells. In the fission yeast *S. pombe*, over-expressed Pil1 forms thick rods that detach from the plasma membrane. In this study, we used thin-section electron microscopy to determine the ultrastructure of these cytoplasmic Pil1 rods. We found that cytoplasmic rods contained crosslinked Pil1 tubules that displayed regular, hexagonal spacing. These bundles were stained by filipin, a sterol-binding fluorescent dye, suggesting that they contained lipids. Cytoplasmic Pil1 rods were present but less abundant in *sle1Δ* and *fhn1Δ* mutant cells. We also found that endogenous Pil1 formed thick rods under saturated growth conditions. Taken together, our findings suggest the presence of cellular mechanisms that assemble Pil1 tubules into higher-order structures.

The assembly and organization of cytoskeletal polymers determines many aspects of cell shape, as well as the formation of diverse subcellular structures. These cytoskeletal polymers self-assemble from monomeric subunits, and a host of polymer-binding proteins then organize polymers into more complex architectures. For example, bundles of crosslinked actin filaments drive the assembly of filopodia,[1] and the mitotic spindle contains a dense array of interdigitated microtubules.[2] Filament bundles and other higher-order assemblies appear to represent a common organization of cytoskeletal polymers in cells.

Recent studies have identified new filament-forming proteins in both prokaryotic and eukaryotic cells.[3-5] In many cases, the organization of these new filaments into higher-order structures remains unexplored. The yeast BAR domain protein Pil1 self-assembles into linear polymers both *in vitro* and *in vivo*.[6-8] These polymers form the core of linear eisosome structures at the yeast plasma membrane. *In vitro*, Pil1 assembles into tubules, and these polymers can tubulate liposomes due to a lipid-binding surface inside the tubules, similar to other BAR domain proteins.[7-10] These tubules are approximately 30 nm wide in the presence or absence of lipids, based on extensive structural studies *in vitro*.[7] However, these 30 nm tubules have not been observed in cells. Rather, Pil1 forms cortical half-tubules that have been visualized by different types of EM.[7,11,12] Thus, it has remained unclear if the closed 30 nm Pil1 tubules that have been extensively characterized *in vitro* are capable of forming in cells. In addition, the assembly of Pil1 tubules into higher-order structures, such as those seen for other cytoskeletal filaments, has remained undetected.

In the fission yeast *S. pombe*, endogenous Pil1 forms linear eisosome filaments that are confined to the plasma membrane.[6] When overexpressed by the medium-strength P41nmt1 promoter, Pil1 localizes to thick cytoplasmic rods that are independent of the actin, microtubule, or septin cytoskeletons.[6] We used thin-section electron microscopy to examine the ultrastructure of Pil1 cytoplasmic rods. In long-axis sections, we observed bundles of tubules that bore striking resemblance to the closed tubules formed by Pil1 *in vitro* (**Fig. 1**). Cross-section views revealed the organization of tubules within a bundle. Cytoplasmic rods contained hexagonal arrays of bundled tubules, and each tubule was approximately 30 nm wide (**Fig. 1**). We did not observe any tubules in control cells expressing an empty plasmid pREP41-GFP alone (data not shown). This suggests the possibility that cytoplasmic rods are comprised of crosslinked, closed Pil1 tubules.

We next tested for the presence of Pil1 and lipids in these crosslinked tubules. First, we performed immuno-electron microscopy on cells over-expressing Pil1-GFP. Thin sections were probed with anti-GFP primary antibody and 6 nm gold-conjugated secondary antibody, and then imaged by electron microscopy. Gold particles were strongly enriched at crosslinked tubules in both long-axis and cross-section views (**Fig. 2A**). This result indicates that the crosslinked tubules observed by electron microscopy are the same structures as Pil1-GFP cytoplasmic rods seen by fluorescence microscopy. Next, we incubated cells over-expressing Pil1-GFP with filipin, a sterol-binding fluorescent dye. Filipin colocalized with Pil1-GFP in cytoplasmic rods (**Fig. 2B**), suggesting the strong possibility that bundled Pil1 tubules contain lipids. We conclude that Pil1 can form closed tubules in cells, and these tubules are not confined to the plasma membrane. Our findings also reveal the existence of cellular

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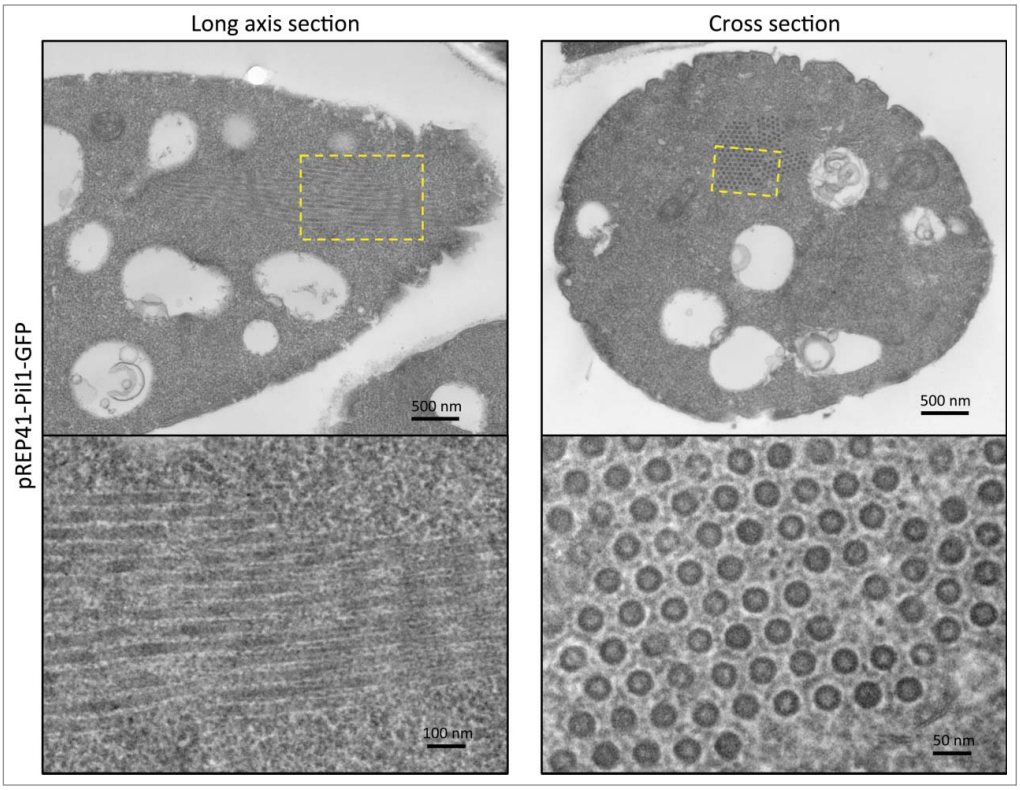

Figure 1. Thin-section electron microscopy reveals bundled tubules. Long-axis section (left) and cross section (right) views of *S. pombe* cells expressing Pil1-GFP from the medium-strength P41nmt1 promoter. Boxed areas in the upper panels are zoomed in the lower panels.

mechanisms that organize Pil1 tubules into highly ordered bundles with hexagonal spacing.

The proper assembly of endogenous Pil1 into linear eisosome filaments at the plasma membrane requires the peripheral membrane protein Sle1 and the transmembrane protein Fhn1, which both localize to eisosome filaments.[6,11] We next tested if Sle1 and Fhn1 are required for Pil1 cytoplasmic rods. Using the pREP41-Pil1-GFP over-expression plasmid, we found that Pil1 cytoplasmic rods were present in both *fhn1Δ* and *sle1Δ* cells (**Fig. 3A**). The rods appeared less abundant in both mutants, particularly in *sle1Δ*. This indicates that neither protein is essential for cytoplasmic Pil1 rods, but may contribute to their assembly and/or stability.

Finally, we considered the physiological significance of thick Pil1 rods, which had only been observed upon Pil1 overexpression. Under most conditions, endogenous Pil1 does not form thick rods, but instead assembles thin cortical filaments. Unexpectedly, we found that endogenous Pil1 forms thick rods after cells have grown to saturation in rich media (YE4S) (**Fig. 3B**). By imaging multiple focal planes, we observed endogenous Pil1 rods that had detached from the cell cortex, similar to over-expressed Pil1 bundles (**Fig. 3B**). This suggests the possibility that environmental signals control the assembly of high-order Pil1 structures such as rods. We note that additional work will be required to determine if the endogenous Pil1 rods also contain bundled tubules, as we observed for overexpressed Pil1. Nonetheless, our observation of endogenous Pil1 rods suggests the possibility that nutrient status and/or environmental signals trigger the assembly of high-order Pil1 structures such as bundled tubules.

Taken together, our studies have revealed the presence of bundled Pil1 tubules in cells. It is important to note that Pil1 tubules assembled *in vitro* do not appear to be bundled or crosslinked.[6-8] This means that bundling is not an inherent activity of Pil1 polymers, rather cellular mechanisms actively assemble these bundles *in vivo*. The proteins and/or lipids that generate this ultrastructure are unknown, but might be identified through biochemical isolation in future work. It will also be important to understand how bundles relate to Pil1 function in regulating phosphoinositide levels,[9] and what membrane lipids they might contain. The formation of higher-order bundled structures supports the notion that Pil1 may act as a cytoskeletal polymer in cells, and raises the possibility that other new cytoskeletal filaments also form complex architectures as part of an expanded cytoskeleton.

Materials and Methods

Yeast strains and methods: Standard *S. pombe* methods and media were used. Strains in this study were JM837 (*leu1–32 h-*), JM1262 (*pil1-mCherry::natR h-*), JM1293 (*fhn1Δ::kanMX6 ura4-D18 leu1–32 ade6-M210 h*$^+$) and JM1461 (*sle1Δ::kanMX6 ura4-D18 leu1–32 ade6-M210 h*$^+$). Expression from plasmid pJM512 (pREP41-pil1-GFP) was induced by growth in the absence of thiamine for 36 hours at 32°C. To observe endogenous Pil1-mCherry rods, strain JM1262 was grown on YE4S plates at 32°C for 3 d. Cells were scraped from the plate and immediately imaged.

Electron Microscopy: For thin sectioning, cells carrying plasmid pJM512 (pREP41-pil1-GFP) or control plasmid pJM211 (pREP41-GFP) were grown in the absence of thiamine for 36 hours at 32°C, and then prepared and imaged as previously described.[9] No tubules were observed in cells carrying the control plasmid pJM211. For immunolabeling of Pil1 bundles, fixations and digestion were the same except for the following changes: initial fixation of 3% PFA/1% GTA was used instead of 3% GTA/

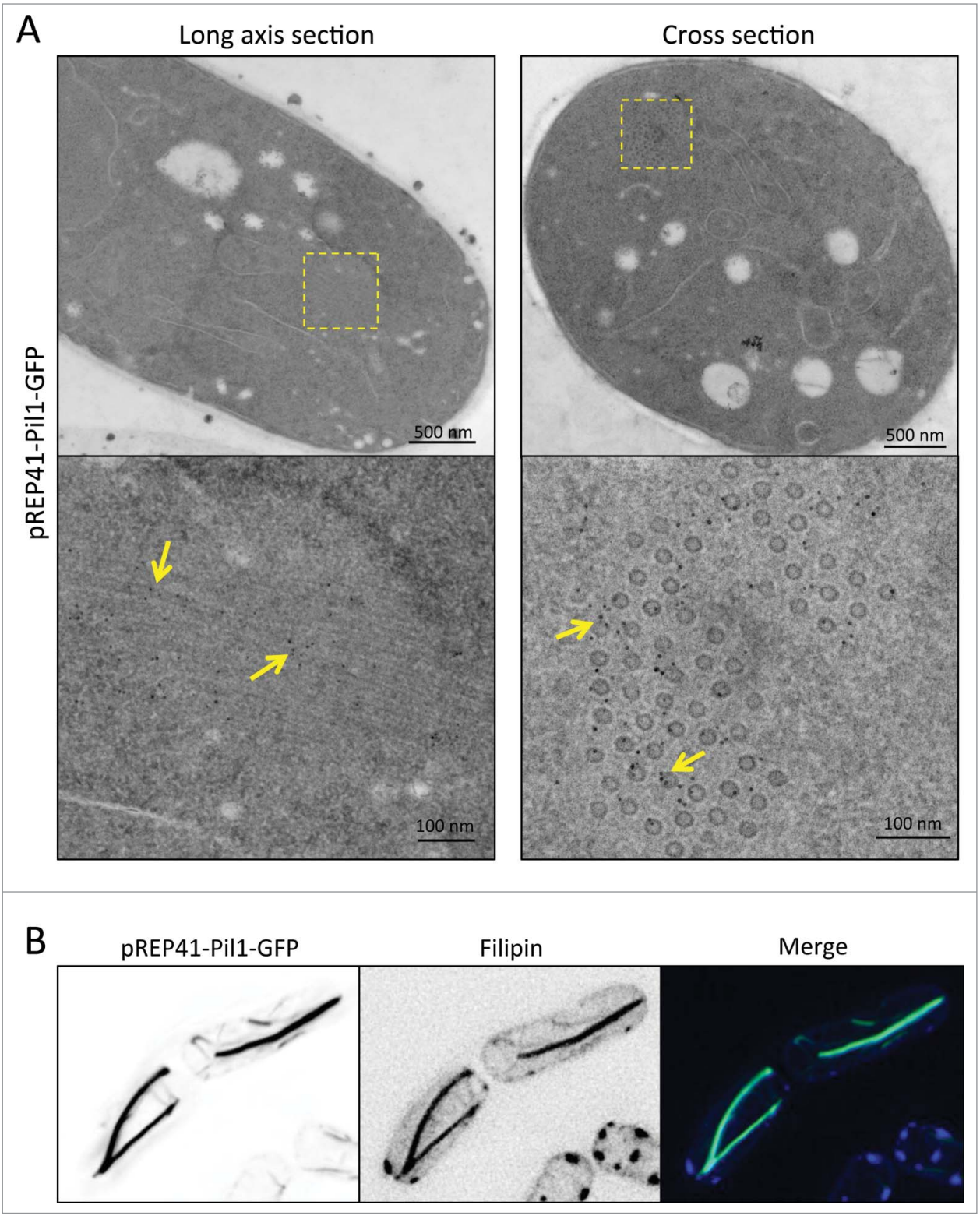

Figure 2. Bundled tubules contain Pil1 and lipids. (**A**) Immuno-electron microscopy of long-axis section (left) and cross section (right) views of *S. pombe* cells over-expressing Pil1-GFP. Boxed areas in the upper panels are zoomed in the lower panels, and yellow arrows denote enrichment of antibody-conjugated gold particles in bundled tubules. (**B**) Colocalization of Pil1-GFP rods and filipin, a sterol-binding dye. Images are inverted maximum projections taken from deconvolved serial Z-sections in the cell middle.

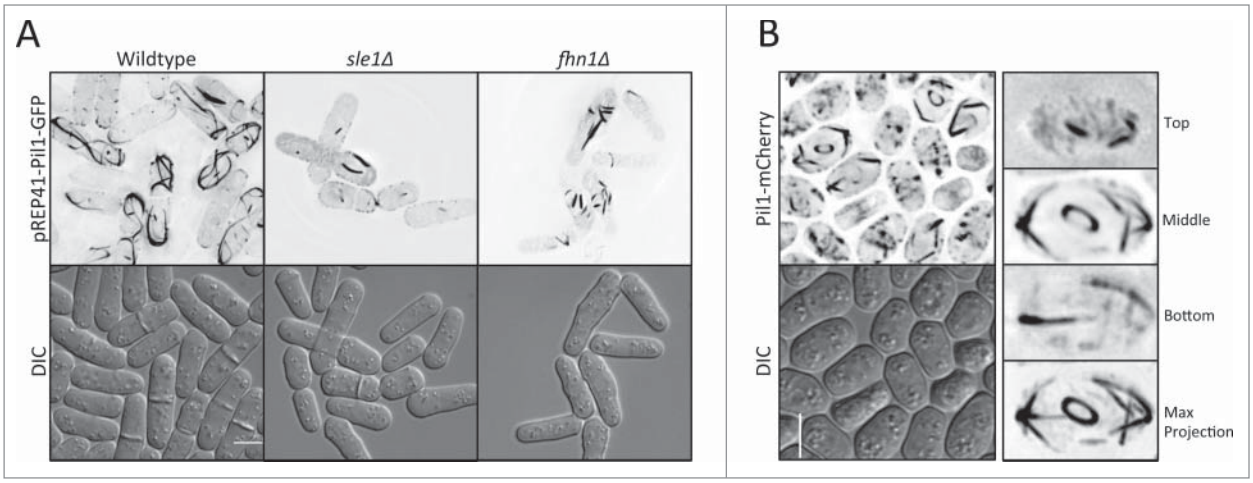

Figure 3. Control of Pil1 cytoplasmic rods by regulatory factors and environmental cues. (**A**) Localization of over-expressed Pil1 in the indicated strains. Images are inverted maximum projections from deconvolved serial Z-sections. Scale bar, 5 μm. (**B**) Localization of endogenous Pil1-mCherry to thick rods. Cells were grown on YE4S plates at 32°C for 3 d before imaging. Left, images are inverted maximum projection from deconvolved serial Z-sections in the top half of the cell (upper panel) and differential interference contrast (DIC) image (lower panel). Scale bar, 5 μm. Right, single deconvolved focal planes and maximum projection for a single cell.

1% PFA. Osmium postfixation was omitted. Samples were incubated in 0.05 M glycine in NaCac buffer for 30 min to remove excess aldehydes. Additionally, 1% p-phenylenediamine (PPD) was included in each EtOH solution. Four additional 85% EtOH/1% PPD rinses over 1 hour were performed, and then pellets were resuspended in a 2:1 mixture of 85% EtOH/1% PPD:LR white medium resin. This suspension was placed on a rotator for 2 hours at room temperature and then resuspended in 1:1 85% EtOH/1% PPD:LR white medium resin, rotated for 1 hour, and then kept at 4°C overnight. The following day, samples were resuspended in 1:2 85% EtOH/ 1% PPD:LR white medium resin rotated for 1 hour at room temperature and then resuspended in 100% LR white medium resin. Solution was changed until clear, and then 3 more changes over 6 hours rotating at room temperature were performed before placing at 4°C overnight. Samples were warmed to room temperature and 4 changes of 100% LR White medium resin were made with 1 hour each change. Samples were transferred to gelatin capsules and left for 4 h at room temperature, then polymerized at 50°C for 24 h. Thin sections (70–80 nm) were placed on G300 nickel-coated grids (Electron Microscopy Sciences). Grids were floated in a moist chamber with solutions spotted on clean parafilm. Grids with sections were washed in PBS for 5 min, put in block solution of PBS + 5% BSA (Sigma-Aldrich) for 15 min, incubated for 1 hour in 1/25th dilution of rabbit anti-GFP,[13] washed 6 times for 2 min, incubated for 30 min in a 1/25th dilution of goat anti–rabbit 6 nm gold-conjugated secondary antibody (25104; Electron Microscopy Sciences), then washed 6 times for 2 min with 2 additional washes in PBS and 2 final washes in distilled water. Washes and antibody dilutions were in PBS + 0.1% BSA (Sigma-Aldrich) at RT. Grids were stored at room temperature and stained with 2% aqueous uranyl acetate for 4 min and Reynold's lead citrate for 10 sec.

Light Microscopy: Fluorescence microscopy was performed as previously described[6] using a Deltavision Imaging System (Applied Precision). For filipin staining, cells expression from plasmid pJM512 was induced by growth in the absence of thiamine for 20 hours at 32°C; filipin (Sigma F9765) was added at a final concentration of 5 μg/mL to live cells. Cells were imaged in the DAPI channel immediately following staining.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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