Single-molecule imaging of the BAR-domain protein Pil1p reveals filament-end dynamics

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ABSTRACT Molecular assemblies can have highly heterogeneous dynamics within the cell, but the limitations of conventional fluorescence microscopy can mask nanometer-scale features. Here we adapt a single-molecule strategy to perform single-molecule recovery after photobleaching (SRAP) within dense macromolecular assemblies to reveal and characterize binding and unbinding dynamics within such assemblies. We applied this method to study the eisosome, a stable assembly of BAR-domain proteins on the cytoplasmic face of the plasma membrane in fungi. By fluorescently labeling only a small fraction of cellular Pil1p, the main eisosome BAR-domain protein in fission yeast, we visualized whole eisosomes and, after photobleaching, localized recruitment of new Pil1p molecules with ~30-nm precision. Comparing our data to computer simulations, we show that Pil1p exchange occurs specifically at eisosome ends and not along their core, supporting a new model of the eisosome as a dynamic filament. This result is the first direct observation of any BAR-domain protein dynamics in vivo under physiological conditions consistent with the oligomeric filaments reported from in vitro experiments.

INTRODUCTION

The eisosome is a multimolecular assembly on the cytoplasmic face of the plasma membranes of fungi, a structure similar to caveolae in mammals. It consists of a stable assembly of proteins clustered on a small invagination of membrane (Malinská et al., 2003; Walther et al., 2006; Strádalová et al., 2009; Douglas and Konopka, 2014), whose various functions in cell membrane organization and lipid regulation remain questions of study (Aguilar et al., 2010; Fröhlich et al., 2014; Kabeche et al., 2015a,b). Fission yeast eisosomes are highly stable, linear domains (50 nm wide and 1–2 µm long), whereas budding yeast eisosomes appear as diffraction-limited puncta. The main protein component of the eisosome, Pil1p in fission yeast, contains a Bin/amphiphysin/Rvs (BAR) domain, which facilitates its organization in vivo (Oliveira-Couto et al., 2011; Ziolkowska et al., 2011) and its oligomerization into filaments in vitro (Kabeche et al., 2011; Karotki et al., 2011), features conserved in budding yeast Pil1. Other BAR-domain proteins, common throughout eukaryotes, play critical roles in membrane-remodeling events and similarly form filaments in vitro, but the extent of oligomerization in cells remains unclear (Suetugu, 2016). Because Pil1p is closely related in structure to classical N-BAR proteins such as endophilin (Ziolkowska et al., 2011) and the fission yeast eisosome is highly stable and observable at nanometer to micrometer length scales, it provides an interesting model to study BAR domain oligomerization dynamics in live cells.

Methods such as fluorescence recovery after photobleaching (FRAP) have been invaluable for characterizing cellular organization and dynamics at the micrometer scale. However, detecting spatial heterogeneities at the nanometer scale and dynamics within multimolecular assemblies in cells is still challenging. In physiological conditions, eisosomes are essentially immobile and exhibit no dynamics in FRAP experiments on time scales up to 20 min (Walther et al., 2006; Kabeche et al., 2011), and are therefore considered to be static microdomains.
Here we present a strategy to monitor nanometer-scale single-molecule dynamics within dense macromolecular assemblies in live cells called single-molecule recovery after photobleaching (SRAP). By labeling only a small fraction of Pil1p molecules, we visualized whole eisosomes, and after photobleaching, we observed isolated Pil1p molecules binding to existing eisosomes. This strategy allows us to measure with high precision the positions and the on- and off-rates of dynamic Pil1p molecules in eisosomes in live cells. We show that binding and exchange of Pil1p occurs specifically at the ends of eisosomes and not along the filament body. By comparing our data with computer simulations, we reject simple models of the eisosome as a static or uniformly dynamic microdomain and show that our data support a model of the eisosome as a dynamic filament. This result is, to our knowledge, the first report of a BAR-domain protein as a membrane-bound oligomeric filament in normal cellular conditions. We expect that our studies of the eisosome will enable further insights into BAR protein oligomerization and function in other organisms.

RESULTS AND DISCUSSION

Quantitative analysis of number of Pil1p molecules and density at eisosomes

We used quantitative microscopy (Wu and Pollard, 2005; Wu et al., 2008) of live fission yeast to directly determine the cellular concentration of Pil1p as well as the local density of Pil1p at eisosomes in cells for the first time. By comparing the fluorescence intensity of cells expressing Pil1p fused to monomeric enhanced green fluorescent protein (mEGFP) to a calibrated standard strain expressing Fim1p-mEGFP, we determined the total expression of Pil1p-mEGFP to be 619,000 ± 60,300 molecules/cell, or 38.2 ± 3.7 µm² global concentration (mean ± SD across six images, 150 cells). This result is comparable to that reported by mass spectrometry experiments (Carpy et al., 2014), confirming that Pil1p is one of the most highly expressed proteins in fission yeast. In addition, we determined the cytoplasmic concentration of Pil1p-mEGFP to be 22.8 ± 4.7 µM; ~40% of the total protein is bound to the membranes in eisosomes. The local density of Pil1p-mEGFP at eisosomes is 2890 ± 680 molecules/µm along the linear eisosome axis, or approximately seven dimers per 5-nm length, remarkably consistent with the lattice dimensions of in vitro–reconstituted filaments (Karotki et al., 2011). Assuming a hemicylindrical geometry as seen in electron microscopy (Danuser and Waterman-Storer, 2006), we determined the total expression of Pil1p-mEGFP (Figure 1, A and B, and Supplemental Movie S1). Because only a small fraction of Pil1p molecules were fluorescently labeled, fluorescence reappeared as isolated spots. Intensity traces of recovery spots over the length of the movie revealed stepwise increases and decreases (Figure 1C) characteristic of single fluorescent molecules binding and unbinding or photobleaching. Although it is conceivable that fluorophore blinking could also give rise to recovery events, such events would appear uniformly along the eisosome. Our observation that recovery is localized at eisosome ends implies that the fraction of recovery events due to blinking is negligible, a conclusion supported by the fact that SiR647 has been shown to be very stable and usually requires high laser intensity or additives to enhance blinking (Uno et al., 2014).

In addition, SRAP spots at the sites of eisosomes were immobile, suggesting that they were not freely diffusing on the membrane surface and indeed corresponded to fluorescent Pil1p-SiR molecules in the cytoplasm or on the membrane beyond the TIRF field may diffuse into the illumination field in later frames of the movie (Figure 1, A and B, and Supplemental Movie S1). Because only a small fraction of Pil1p molecules were fluorescently labeled, fluorescence reappeared as isolated spots. Intensity traces of recovery spots over the length of the movie revealed stepwise increases and decreases (Figure 1C) characteristic of single fluorescent molecules binding and unbinding or photobleaching. Although it is conceivable that fluorophore blinking could also give rise to recovery events, such events would appear uniformly along the eisosome. Our observation that recovery is localized at eisosome ends implies that the fraction of recovery events due to blinking is negligible, a conclusion supported by the fact that SiR647 has been shown to be very stable and usually requires high laser intensity or additives to enhance blinking (Uno et al., 2014).

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Pil1p recruitment is not uniformly distributed

Further inspection of the recovery events suggested that eisosome ends are hot spots of Pil1p exchange (Figure 1D). Kymographs of lines drawn along eisosomes showed that fluorescence signal at eisosome ends persisted longer and recovered after photobleaching more frequently than along the interior (Figure 1E). To calculate precisely the distance of SRAP spots to the eisosome end, we determined the position of each spot with superresolution localization and determined the position of each eisosome end by fitting a sigmoidal curve to the intensity profile of the eisosome end extracted from initial frames (see Materials and Methods and Figure 2A). We found that 92% of SRAP spots were within 250 nm from their corresponding eisosome end, with an average position of 97 ± 119 nm (mean ± SD, 191 spots in 20 cells; Figure 2B).

To interpret more clearly this distribution of positions, we simulated data sets based on hypothetical models for Pil1p dynamics, including any possible sources of experimental noise or errors. In a first model (referred to as the uniform model), we assumed that...
The simulations included noise terms to mimic the uncertainty in the localizations for spots and eisosome ends (Figure 2, C and D, and Supplemental Figure S2) and took into account the observed distribution of eisosome lengths (Figure 1G). The simulated positions were broadly distributed, with an average position of 346 ± 254 nm, clearly disagreeing with our SRAP data (Figure 3).

In a second model (referred to as the end model), we assumed that binding of new Pil1p occurs only at eisosome ends, as in a dynamic oligomeric filament. The simulated distribution followed a shape more similar to our experimental data but with a mean position of 0 ± 67 nm (Figure 3, dashed magenta). The slight offset of our SRAP spot localizations toward the interior of the eisosome (97 ± 119 nm) seems to contradict a model of dynamics strictly confined to the end. This shift cannot be explained by our spot localization precision, as the SD of recurrent localizations at a given SRAP site was 27.9 ± 15.9 nm (Figure 2C). We wondered whether the offset could be explained by the accuracy of our localization of eisosome ends and whether a dynamic end could introduce a systematic bias.

**Localization accuracy of sparsely labeled, dynamic eisosome ends**

We evaluated the accuracy of our eisosome-end localizations by fitting simulated data mimicking linear filaments labeled with low density, similar to our experimental data (Supplemental Figure S2A). First, we found that fitting the intensity profile with an error function—a model that assumes a continuous distribution of emitters—overestimates the end position beyond the true position by a significant distance, depending on the number of fluorophores present. In simulations corresponding to 3% labeling efficiency, the average error of the fitted eisosome-end position is 38.6 ± 61.8 nm (Supplemental Figure S2C).

However, because we extracted intensity profiles from an image averaged over a short time, if Pil1p recruitment is indeed localized to the eisosome end, then any new labeled molecules that bind during the recording time would skew the intensity profile toward the end (Supplemental Figure S2B). Indeed, in kymographs of sparsely labeled eisosomes (Figure 1E), the signal at eisosome ends persisted longer than the signal along the eisosome body, likely due to additional Pil1p-SiR molecules binding before the initial labeled molecules have photobleached. We simulated this effect by adding extra emitters at the true end position before fitting the intensity profile. Simulations with three or six extra emitters (numbers as expected based on our estimates of Pil1p binding rate; see later discussion) resulted in net fitting errors of 67.8 ± 56.1 or 84.6 ± 52.1 nm, respectively (Figure 2D and Supplemental Figure S2C), mirroring the offset in our measured SRAP spot positions.

**Eisosome ends are specific sites of single-molecule recovery events**

We repeated simulations of the end model for Pil1p-SiR recovery incorporating this biased localization error for the eisosome end. Based on the more conservative bias estimated from simulations of three extra fluorophores in the initial fluorescence trace, the result resembled our SRAP data (70 ± 63 nm; Figure 3, solid magenta). Of importance, the only assumption of this model is that the eisosome end is the specific site of Pil1p binding; the bias in the eisosome-end localization arises from the sparse labeling of the sample.

As a putative alternative hypothesis, we considered a model in which Pil1p binding occurs on a “ragged end” or a dynamic region at the eisosome end rather than a flat end. Simulations of a ragged-end model using various sizes for the dynamic region showed that a 125-nm region at the eisosome end was necessary to produce a result similar to our SRAP data (97 ± 76 nm; Supplemental Figure S3). However, a ragged or tapered filament end is difficult to quantify from electron micrographs (Karotki et al., 2011) but might span...
only a few nanometers—not sufficient to cause the distribution of localizations we observed experimentally. In addition, the SD of recurrent localizations at the same SRAP site (27.9 ± 15.9 nm; Figure 2C) indicated that binding events occur at a fixed position on each eisosome, in contradiction with a large dynamic region. We conclude that the measured distribution of SRAP data is consistent with Pil1p binding only at the ends of eisosome filaments, but the sparse labeling introduces a slight error in conventional fluorescence image-fitting models.

Characterization of Pil1p kinetics using SRAP data

In addition to localization, a variety of other single-molecule analyses can be applied to SRAP spots, such as lifetimes analysis to determine rates of binding and unbinding. We first measured the lifetimes of SRAP spots and fitted the distribution with an exponential curve to determine an off-rate. The apparent off-rate, 2.4 ± 0.2 s⁻¹, was much faster than the overall rate of photobleaching in the images, 0.48 ± 0.03 s⁻¹ (fitted value ± 95% confidence intervals; Figure 4A), suggesting that Pil1p-SiR molecules are not only photo-bleaching but also unbinding from the eisosomes. By subtracting the photobleaching rate from the spots’ disappearance rate, we estimate the unbinding rate of Pil1p to be 2.0 ± 0.2 s⁻¹, consistent with the findings of Olivera-Couto et al. (2015).

We then measured the distribution of wait times between SRAP events to determine the apparent binding rate of Pil1p-SiR to an eisosome end (Figure 4B). Assuming mass-action kinetics, the distribution can be fitted to a single exponential. We noticed that our spot localization algorithm caused the bins for very short wait times (0.1–0.3 s) to be artificially overpopulated because it occasionally missed a localization for a spot that actually persisted over many frames. We excluded these bins and fitted the distribution of wait times between binding events with an exponential curve; we found an apparent on-rate of 1.2 ± 0.2 s⁻¹ (Figure 4B). This apparent on-rate is the product of a binding rate constant and the concentration of Pil1p-SiR (0.9 µM, i.e., the product of the labeling efficiency [4%] and the cytoplasmic Pil1p concentration [22.8 µM]). Therefore the binding rate constant for Pil1p binding to the end of an eisosome is 1.3 ± 0.9 µM⁻¹ s⁻¹. We used single-exponential fits for binding and unbinding because we did not expect multiple populations of different rates, such as in a polar filament with unique kinetics at each end. Pil1p exists primarily as a symmetric dimer, which would result in a filament with no polarity (Karotki et al., 2011; Olivera-Couto et al., 2011), and indeed we observed a number of filaments with Pil1p recruitment at both ends (Figure 1D). Taken together, our data indicate that Pil1p is undergoing fast single-molecule exchange at eisosome ends, even in the absence of large-scale eisosome remodeling.

SRAP reveals heterogeneities at the nanometer scale in vivo

Pil1p exchange at the eisosome has previously been unobservable using conventional imaging approaches. FRAP experiments (Walther et al., 2006; Kabche et al., 2011) were unable to observe this dynamic subpopulation because detecting single fluorescent proteins is challenging when fully labeled structures are imaged in the same frame. One study using fluorescence fluctuation techniques detected a subpopulation of Pil1 oligomers exchanging between the cytoplasm and plasma membrane (Olivera-Couto et al., 2015), but this method lacked the spatial resolution to determine the precise location and role of dynamic Pil1p molecules relative to the nanoscale structure of the eisosome.

Our SRAP method was critical for revealing the behavior of individual protein molecules in the context of the larger eisosome structure. We expect that our SRAP method will be easily and broadly applicable to reveal localized single-molecule dynamics and heterogeneities within other multimolecular assemblies because it requires only sparse labeling and a TIRF microscope with single-molecule detection capabilities. Although similar sparse fluorescence
conditions might be achieved by partial photobleaching of the sample (Brameshuber and Schütz, 2012) or photoswitching of fluorescent proteins (Manley et al., 2009), our SRAP protocol has several advantages over existing methods. We avoid high-intensity laser illumination required for FRAP methods, which can be damaging to cells. We use organic fluorophores that are brighter and more photostable than fluorescent proteins, enabling better localization precision. By using a single fluorophore to characterize both the initial structure and the recovery dynamics, we avoid challenges of multichannel imaging and alignment. Of importance, we demonstrated that sparse labeling is sufficient to determine the overall shape of a macromolecular assembly but may require a minor adjustment from conventional fluorescence image-fitting models. Future applications of SRAP imaging for large cellular assemblies should consider this factor when modeling a structure of interest.

**Filament model for the eisosome**

Our results demonstrate that the eisosome is highly dynamic, with continuous and fast exchange of Pil1p at its ends, even in the absence of perturbation. Models of the eisosome as a membrane microdomain (Walther et al., 2006; Kabeche et al., 2011; Karotki et al., 2011) would predict Pil1p exchange to occur uniformly around its edges. Instead, our data support a new model for the eisosome as a membrane-bound filament with a stable body and dynamic ends (Figure 5).

Pil1p and other BAR-domain proteins have been observed to oligomerize and form filaments and membrane tubules in vitro, but it has been unclear to what extent this oligomerization exists in vivo or whether instead BAR proteins are loosely clustered on patches of curved membranes (Adam et al., 2015; Daum et al., 2016; McDonald and Gould, 2016; Suetsugu, 2016). Recent in vitro studies of BAR proteins propose that binding at low or moderate surface density is sufficient to generate membrane tubes (Simunovic et al., 2016). Our quantitative analysis of Pil1p-mEGFP eisosomes indicates that Pil1p exists at extremely high density, consistent with the lattice structure of filaments reconstituted in vitro (Karotki et al., 2011). Although a polymer filament model has been previously hypothesized for eisosomes (Moseley, 2013), our results are the first experimental evidence of dynamic behavior that supports a filament model.

Reconsidering the eisosome as a membrane-bound oligomeric filament enables several predictions and poses new questions for future investigation. Of interest, electron micrographs of eisosomes in cells show that the membrane adopts a hemicylindrical furrow instead of a full tube as observed in vitro (Karotki et al., 2011). The physical or biochemical means by which a hemicylindrical scaffold of proteins is stabilized remain open questions, but our results clearly indicate that the filament body and long edge do not provide suitable binding sites for new Pil1p molecules.

Our measured rate constants predict a net growth of eisosome filaments of ∼28 Pil1p molecules/s, or ∼0.6 µm/min. This high rate of polymerization is surprising, considering that eisosomes grow very slowly throughout the cell cycle, ∼1 µm/h, and we do not observe large distances between successive Pil1p-SiR spots (Figure 2C). A likely reason for this discrepancy is that some eisosome ends might be capped, limiting the number of actively polymerizing filaments in the cell at any time. Indeed, we
be avoided by enzymatically digesting the cell wall, deleting the multidrug exporter genes (McMurray and Thorner, 2008), or using electroporation to allow a large amount of dye to enter the cells (Stagge et al., 2013). However, such approaches may be problematic if the structure of interest is sensitive to cell integrity, as is the case with the eisosome. To avoid these difficulties, we used a minimally disruptive approach, adding a low concentration of SNAP substrate fluorophore in the medium for a long incubation.

To label SNAP-tag protein in live cells, 0.5 ml of cells at OD_{OD_{595nm}} of 0.5 were incubated at 25°C on a rotator in liquid EMMSS containing 0.1, 0.5, or 2.5 µM silicon-rhodamine benzylguanine derivative SNAP-SiR647 or SNAP-Alexa 647 (SNAP-Cell 647-SiR and SNAP-Surface Alexa Fluor 647; New England Biolabs) for 0.5, 5, or 15 h. For samples incubated for 15 h, the cells were initially diluted to OD_{OD_{595nm}} of 0.1 to avoid overgrowing during the incubation time. Cells were washed three times by centrifuging at 900 × g for 3 min and resuspending in 0.5 ml of EMMSS, and then additionally incubated at 25°C for 1 h in 0.5 ml of EMMSS, washed three times again by centrifuging at 900 × g for 3 min, and resuspending in 0.5 ml of EMMSS. Cells were finally resuspended in 50–100 µl of 0.22-µm filtered EMMSS to achieve suitable cell density for imaging.

We estimated the extent of labeling by dividing the total intensity of cells in the first frame by the mean pixel intensity of the late-appearing single-molecule spots to determine the number of fluorophores per cell. We then determined the fraction of labeled Pil1p-SNAP molecules by dividing the number of fluorophores per cell by the expected visible membrane-bound fraction of total number of Pil1p molecules determined by our quantitative microscopy analysis. The samples we used for SRAP analysis (labeled 15 h at 0.5 µM SNAP-Sir647) consistently had labeling efficiencies between 3 and 5%. Future applications of SNAP imaging should aim for a similarly low labeling efficiency, but the precise value is not critical as long as the overall shape of the structure is visible and a single-molecule regime can be reached after a short time of illumination and photobleaching. This protocol is the first reported use of SNAP-tag in live fission yeast, but similar protocols should be easy to adapt in other organisms, especially those lacking a cell wall.

Our protocol still requires use of a cell-permeable fluorophore conjugate, as incubation with SNAP-Alexa 647 yielded poor labeling (Supplemental Figure S1C). Incubation with 2.5 µM Sir647 for 15 h achieved a higher density of labeled Pil1p-SiR (>15–20%; Supplemental Figure S1A), but short incubations yielded only sparse labeling with greater cell-to-cell variability (Supplemental Figure S1A).

Microscopy
Live cells were imaged on 25% gelatin pads in 0.22-µm filtered EMMSS with coverslips that had been washed in ethanol for 20 min and plasma treated for 2 min to avoid nonspecific attachment of dyes and other autofluorescent particles on the surface. Cells were imaged with an inverted fluorescence microscope (Ti Eclipse; Nikon) equipped with a 60×/1.49 numerical aperture (NA) objective (Nikon), illuminated with a 642-nm laser (for imaging Sir647 samples) or 488-nm laser (for imaging mEGFP samples) directed through the objective to achieve TIRF, and recorded with an electron-multiplying charge-coupled device (EMCCD) camera (iXon DU897; Andor). Samples labeled with Sir647 were imaged under low illumination intensity, ∼20 W/cm². Movies were recorded at a single focal plane near the cell base at 10 frames/s.

For quantitative microscopy of mEGFP-tagged proteins, cells were imaged on an inverted fluorescence microscope (Ti Eclipse; Nikon) equipped with a 60×/1.4 NA Plan Apochromat Lambda objective (Nikon), coupled with a CSU-W1 spinning-disk confocal...
system (Yokogawa), illuminated with 488-nm laser, and recorded with an EMCCD camera (iXon Ultra888; Andor). Cells expressing Pil1p-mEGFP or Fim1p-mEGFP were imaged in z-stacks spanning the entire cell height, with 21 z-slices in 500-nm steps.

**Image analysis and quantification**

Image analysis was carried out in the Fiji distribution of ImageJ (Schindelin et al., 2012; Schneider et al., 2012), and further quantification was performed in Matlab (MathWorks), using built-in tools as well as self-written macros and scripts (see the Supplemental Material).

We first measured the lengths of filaments in the average intensity projection of frames 1–5 (AVG1–5) of Pil1p-SiR and Pil1p-mEGFP movies by drawing a line along the full length of visible fluorescence for each filament. We then identified SRAP spots in the maximum intensity projection of frames 50–200 (MAX50–200), after labeled eisosomes had photobleached. We first generated a preliminary list of SRAP spot positions from the MAX50–200 image by using the Find Maxima command and determining the brightness-weighted centroid of a 3-pixel-diameter circle at each point.

To determine the end position of the underlying eisosome for each point in this list, we manually traced the eisosome filament in the AVG1–5 image with a 3-pixel-wide line spanning past the spot position to extend beyond the end of the filament (Figure 2A) and analyzed the intensity profile along this line in Matlab. Spots >4 pixels away (280 nm) from the nearest eisosome were discarded (<10% of detected spots). To find the position of the end of the eisosome underlying the diffraction-limited image, we fitted the intensity profile with the following step-like function:

\[
I(x) = \frac{1}{2} A \left[ 1 - \text{erf} \left( \frac{x - x_0}{\sqrt{2} \sigma} \right) \right]
\]

This equation is equivalent to the cumulative intensity of a continuous distribution of Gaussian emitters, where \(I(x)\) is the intensity along the line coordinate \(x\), \(A\) is the amplitude, \(\text{erf}\) is the error function, \(x_0\) is the position of the underlying step corresponding to the end of the labeled structure, and \(\sigma\) is the SD of the diffraction-limited Gaussian spot. Measured intensity profiles were fitted in Matlab using a nonlinear fitting algorithm, with \(A\) and \(x_0\) as independent variables and \(\sigma\) fixed to 1.85 pixels (130 nm), representing the diffraction-limited spot width.

We used the PeakFit plug-in for Fiji (www.sussex.ac.uk/gdsc/ intranet/microscopy/image/smlm_plugins) to determine superresolution localizations of the spots that appeared in frames 50–200, calibrated with pixel size 70 nm, wavelength 642 nm, objective NA 1.49, objective proportionality factor 1.4, and electron-multiplying gain 37.7, resulting in an estimated point-spread function width of 1.837 pixels. This generated a list of localizations with precision <40 nm.

By the line profile extracted earlier, we expect 980 possible Pil1p-SiR molecules per cell. We therefore fitted a subset of the data with a single-exponential curve, excluding events <0.4 s. We considered fitting with alternative models, accounting for photobleaching of the limited pool of free Pil1p-SiR or multiple rates or other processes. However, more complex analysis yielded little improvement and would require much larger data sets to be justified.

To quantify the number of Pil1p-mEGFP, we used quantitative microscopy approaches (Wu and Pollard, 2005; Wu et al., 2008; Berro and Pollard, 2014). We first corrected the raw z-stacks for camera offset noise and uneven illumination. We measured the integrated intensity of sum projections of z-stacks spanning whole cells expressing Fim1p-mEGFP or Pil1p-mEGFP or wild-type cells. We subtracted the autofluorescence intensity of wild-type cells and calibrated the brightness per mEGFP molecule in cells expressing Fim1p-mEGFP (using 86,500 ± 9100 molecules of Fim1p-mEGFP per cell as reported in Wu and Pollard (2005)) to determine the total number of Pil1p-mEGFP molecules per cell. To determine the local density of Pil1p-mEGFP at eisosomes, we used sum projections of z-stacks spanning only the lower half of the cell. We measured the integrated intensity of rectangular ROIs drawn across eisosomes and subtracted the local cytoplasmic background intensity as measured in an adjacent ROI. Using the intensity per molecule calibrated from Fim1p-mEGFP stacks, we converted these intensities to number of Pil1p-mEGFP molecules per ROI. We calculated a linear density along the length of the eisosome axis (without assuming any geometry for the structure), as well as the membrane surface area density (assuming the geometry of a half-cylinder with radius 16 nm, as determined from electron micrographs; Karotki et al., 2011).

**Characterization of eisosome-end localization**

We performed simulations to estimate the precision of our method of fitting an error function to the intensity traces of sparsely labeled eisosomes to localize their ends. Indeed, this continuum model might not fit the eisosome ends accurately when the structures are only sparsely labeled. From our quantitative microscopy of Pil1p-mEGFP filaments, we estimated that there are ~2.8 Pil1p proteins per nanometer of eisosome lattice. Therefore, for a 350-nm region (equivalent to the typical 5-pixel region of eisosome body covered by the line profile extracted earlier), we expect 980 possible Pil1p sites. With our estimated 3% labeling efficiency, there are most likely between 20 and 50 fluorescently tagged Pil1p-SiR in this region. For
each simulation, we first calculated a set of expected numbers of emitters according to a binomial distribution and then simulated each number of “emitter positions” on a uniform distribution along a 350-nm line. We added a Gaussian profile of intensity at each emitter position (mean $x_e$, SD 135 nm, peak height of 1 AU) to mimic the point spread function of the microscope, added signal from emitters outside the simulated region to account for other fluorophores on the rest of the eisosome body, and also added noise to the sum traces (random value of mean 0, SD 1 AU at each x value, -10–20% of the simulated fluorescence signal). We fitted the resulting intensity profile (10 pixels long, including the 5-pixel region of simulated fluorophores plus 5-pixel tail region) with the error function model described earlier. We determined the distance from the fitted end position (position $x_0$) to the true end of the eisosome (position 350 nm) in each simulation.

To determine a full population average of these errors, we simulated 1000 filaments. We repeated a similar set of simulations with a number of added fluorophores at the end position to account for the possibility of additional Pil1p-SiR binding during the imaging time, which leads to a characteristic bias in fitting (Figure 2D and Supplemental Figure S2C).

Eisosome dynamics model simulations

We compared the distribution of our experimentally measured distances to data sets simulated under different hypotheses. In one model (referred to as the uniform model), Pil1p SRAP events occur uniformly along the eisosome; in a second model (referred to as the end model), events occur exclusively at the end of the filament (Figure 3). For all models, each simulation was initialized by picking one of the eisosome lengths experimentally measured in Pil1p-SiR cells (10,000 runs with each of 275 filaments; Figure 1G). For the uniform model, the true SRAP spot positions were simulated by picking a number following a uniform distribution between zero and half the filament length, and for the end model, the true SRAP spot position was taken as the true end position of the eisosome end (position 0); a number following a Gaussian distribution (mean 0, SD 30 nm) was added to represent the spot localization uncertainty as measured experimentally (Figure 2C). For each simulation, we added a number following a Gaussian distribution with mean 0 and SD 60 nm to the true position of the eisosome end (position 0) to simulate the unbiased localization precision of the experimental fit of the eisosome end in our image analysis. Each simulated SRAP spot position was subtracted from the simulated end position to determine the relative distance from the end. In a second set of simulations to account for the fitting bias arising from a dynamic filament end, we used for the eisosome end position distribution a Gaussian distribution with mean position ~70 nm and SD 55 nm (as in Figure 2D).

We also simulated a third class of models (referred to as ragged-end models) in which events occur uniformly within a zone of defined length at the eisosome end. For the ragged-end models, the true SRAP spot position was simulated by picking a number following a uniform distribution between 0 and the length of the end zone (e.g., 200 nm), and the end position and noise terms were generated with unbiased Gaussian distributions as described earlier.

Statistics and reproducibility

Unless otherwise noted, all reported measurements are given as mean ± SD. Values derived from curve fitting are given as value ± 95% confidence interval, and the type of curve fit is noted in the text. Numbers of cells analyzed, data points, and simulation runs are given in the text and figure legends with each reported measurement. Imaging experiments used single biological replicates, and the number of technical replicates (images, cells) is given in the text and figure legends. Any statistical tests used are noted in Results and figure legends. Statistical tests were not used to compare data and simulations because the outcome of the simulations may vary significantly, depending on user-defined parameters.

Code availability

Matlab and ImageJ scripts we used to analyze data and generate simulated data sets are available as supplemental files, with brief descriptions in the Supplemental Material.

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