LETTERS

Eisosomes mark static sites of endocytosis

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Endocytosis functions to recycle plasma membrane components, to regulate cell-surface expression of signalling receptors and to internalize nutrients in all eukaryotic cells. Internalization of proteins, lipids and other cargo can occur by one of several pathways that have different, but often overlapping, molecular requirements[1–5]. To mediate endocytosis, effectors assemble transiently underneath the plasma membrane, carry out the mechanics of membrane deformation, cargo selection and vesicle internalization, and then disassemble. The mechanism by which endocytosis initiates at particular locations on the plasma membrane has remained unknown. Sites of endocytosis might be formed randomly, induced by stochastic protein and/or lipid clustering. Alternatively, endocytosis might initiate at specific locations. Here we describe large immobile protein assemblies at the plasma membrane in the yeast *Saccharomyces cerevisiae* that mark endocytic sites. These structures, termed eisosomes (from the Greek '*eis*', meaning into or portal, and '*soma*', meaning body), are composed primarily of two cytoplasmic proteins, Pil1 and Lsp1. A plasma membrane protein, Sur7, localizes to eisosomes. These structures colocalize with sites of protein and lipid endocytosis, and their components genetically interact with known endocytic effectors. Loss of Pil1 leads to clustering of eisosome remnants and redirects endocytosis and endocytic effector proteins to these clusters.

Examination of a library of yeast proteins tagged with green fluorescent protein (GFP) identified two closely related cytoplasmic proteins, Pil1 and Lsp1, that share 72% sequence identity and localize in a marked pattern at the cell cortex[6] (Fig. 1). The amino acid sequences of Lsp1 and Pil1 do not contain any recognizable functional domains. Fluorescence and confocal microscopy of cells expressing GFP-tagged Pil1 or Lsp1 showed 20–45 bright dots, termed 'eisosomes', at the cell periphery underneath the plasma membrane (Fig. 1a and Supplementary Information). Fluorescently tagged Pil1 and Lsp1 colocalize in these structures (Fig. 1c). Moreover, co-immunoprecipitation showed that Pi1l and Lsp1 are the main proteins in these complexes and are present in roughly equimolar proportions (Fig. 1b).

To find additional eisosome components, we further inspected the yeast GFP database and identified a previously characterized family of structurally related transmembrane proteins with a similar localization pattern[6–8]: Sur7 (Fig. 1a), Ydl222c and Ynl194c. Further analysis showed colocalization of Sur7–GFP with Pil1 tagged with the haemagglutinin A (HA) epitope (Fig. 1d).

Quantification of the fluorescence intensity of individual eisosomes revealed a tight distribution of GFP-tagged molecules per eisosome (Fig. 1a, bottom). Thus, different eisosomes contain similar numbers (within a factor of two) of constituent molecules. As estimated by quantitative western blot of tandem affinity purification (TAP)-tagged proteins, there are roughly 100,000 molecules of both

Pil1 and Lsp1 per cell[9]. We detected no perceptible pool of soluble Pil1–GFP. We therefore estimate that each eisosome comprises about 2,000–5,000 molecules of each protein.

To test whether eisosomes show dynamic behaviour, we observed single cells in time-lapse movies. Eisosomes are unexpectedly stable structures that do not disassemble or move noticeably even over the 30-min time course of the experiment (Fig. 1e). Eisosomes also do not exchange subunits with a cytoplasmic pool: fluorescence recovery after photobleaching (FRAP) analysis of Pil1–GFP-labelled eisosomes showed no detectable recovery of fluorescence to bleached eisosomes over a 20-min time course (Fig. 1f, g). Thus, eisosomes are stable ultrastructural assemblies in cells that, akin to centrosomes, could be considered as unique organelles.

SUR7 has been previously characterized as an overexpression suppressor of the conditional growth defect of a null mutation in *RVS161*, encoding the yeast homologue of mammalian endophilin, an endocytic effector[8,10]. This observation suggested to us that eisosomes might have a role in endocytosis. We therefore monitored endocytosis of HA-tagged Ste3 (the **a**-factor mating pheromone receptor) by following its degradation after delivery to the vacuole[11]. In this assay, deletion of *PIL1* ($t_{1/2} = 27 \pm 3$ min), *LSP1* ($t_{1/2} = 24 \pm 3$ min) or both ($t_{1/2} = 32 \pm 5$ min) substantially decreased the rate of endocytosis compared with wild-type cells ($t_{1/2} = 18 \pm 2$ min; see Supplementary Information).

To test whether *PIL1* and *LSP1* interact genetically with *RVS161*, we monitored endocytosis of Ste3 in strains lacking these genes in combination. In agreement with previous work[11], we observed a strong endocytosis defect in cells deleted for *RVS161* ($t_{1/2} = 112 \pm 37$ min). This defect was almost completely suppressed by deletion of *LSP1* ($t_{1/2} = 30 \pm 8$ min), suggesting that Lsp1 negatively regulates endocytic function in the *rvs161Δ* mutant. This suppression requires Pil1 because strains lacking all three components show no suppression ($t_{1/2} = 74 \pm 10$ min; not significantly different from *rvs161Δ*). Together, the data show that *LSP1* and *RVS161* interact genetically and support the notion that Pil1 and Lsp1 affect endocytosis, having overlapping but separable functions.

Deletion of *RVS161* can also be suppressed by deletion of genes involved in sphingolipid biosynthesis, suggesting that endocytosis can be promoted by perturbing sphingolipid abundance in the cell[12]. Moreover, Pil1 and Lsp1 have been described as substrates of two sphingolipid-regulated kinases, Pkh1 and Pkh2 (ref. 13), and mutations in these kinases[14,15] or in sphingolipid biosynthetic enzymes[16] block endocytosis. To determine whether loss of Pil1 or Lsp1 affects endocytosis by altering the lipid composition of the cell, we determined the relative abundance of ceramides and long-chain bases in *pil1Δ*, *lsp1Δ* and *pil1Δ lsp1Δ* strains and found that they were not measurably altered (see Supplementary Information). Therefore, the genetic interactions between *PIL1*, *LSP1* and *RVS161* described
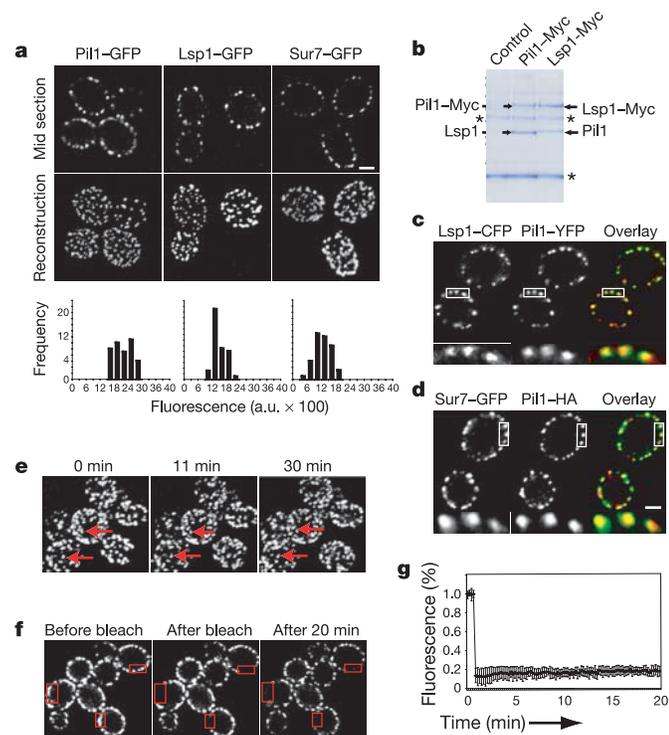
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above do not indirectly result from effects on the lipid pools in the mutant cells.

Microscopic analyses of *pil1Δ* cells showed that Pil1 is required for proper localization of both Lsp1–GFP (Fig. 2a) and Sur7–GFP (Fig. 2b): in the absence of Pil1, Lsp1–GFP localized to only a few bright clusters (less than four per cell) at the cell periphery and to a diffusely staining cytoplasmic pool. Analogously, in the absence of Pil1, Sur7 localized to a few bright clusters and a pool diffusely distributed over the plasma membrane. By contrast, deletion of *LSP1* did not affect the localization of Sur7–GFP (Fig. 2b) or Pil1–GFP (not shown), and deletion of *SUR7* (alone or in combination with its orthologues Ydl222c and Ynl194c) did not affect the localization of Lsp1–GFP or Pil1–GFP (data not shown). These results identify Pil1 as the fundamental component of eisosomes required for their construction or maintenance and further support the notion that, despite their high degree of sequence identity, Pil1 and Lsp1 have distinct nonredundant functions.

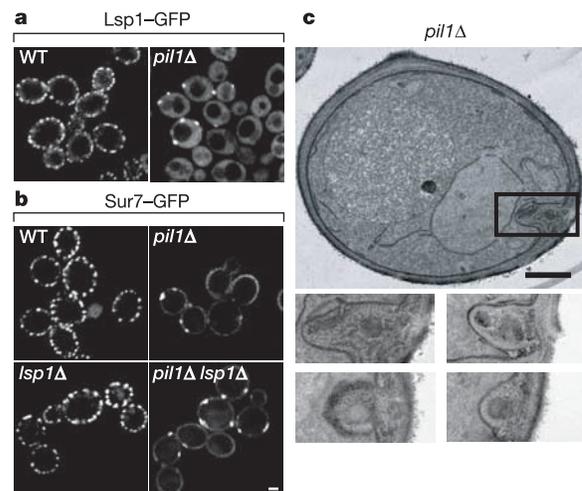To study the relationship of eisosomes to sites of endocytosis, we monitored the uptake of the fluorescent dye FM4-64, a lipid marker for endocytosis[1,3,4], in cells expressing Pil1–GFP. We labelled cells with FM4-64 and blocked endocytosis either during labelling or shortly after labelling by poisoning the cells with azide and fluoride on ice as described[1]. As expected, when endocytosis was blocked during labelling (Fig. 3a, $t = 0$), we observed FM4-64 staining of only the plasma membrane. When endocytosis was blocked after a 20-s incubation at room temperature, the dye concentrated into discrete foci, representing early endocytic intermediates[17]. Strikingly, all of the FM4-64-labelled foci localized to eisosomes (Fig. 3a), although not all eisosomes nucleated FM4-64 foci.

Movies following FM4-64 uptake in living cells also showed the formation of FM4-64 foci that always colocalized with eisosomes. As expected for *bona fide* endocytosis intermediates, FM4-64 labelling of eisosomes was transient and consumed within 5–10 min as the dye was transported to endosomal and vacuolar membranes (Fig. 3b, 600 s). Thus, at any given time, only a subset of eisosomes are active for endocytosis as monitored by this method. Monitoring the time course of FM4-64 internalization in strains bearing the *pil1Δ*, *lsp1Δ* or *rvs161Δ* mutation either alone or in combination showed kinetic delays of FM4-64 uptake consistent with the delays observed for Ste3 internalization (data not shown).

To test whether eisosomes also colocalize with sites of protein uptake, we made use of the regulated endocytosis of the hexose transporter Hxt2. Cells grown in low-glucose media express Hxt2 and transport it to the plasma membrane. On shift to high-glucose media, Hxt2 synthesis is arrested and the protein is removed from the plasma membrane by endocytosis[18]. Similar to FM4-64, Hxt2–GFP accumulated in discrete foci on the plasma membrane. The foci were consumed as Hxt2 was delivered to endosomes and then to vacuoles. However, endocytosis of Hxt2–GFP occurred more slowly than that of FM4-64 (Fig. 3d). Thus, to determine whether these presumed endocytic intermediates were the same, we added FM4-64 at different times after shift to high glucose. Under such conditions, all Hxt2–GFP foci colocalized with early FM4-64 endocytic intermediates (Fig. 3d, left), and thus with eisosomes. Together, these results indicate that eisosomes have a role in endocytosis of both protein and lipid cargos.

If eisosomes mark sites of endocytosis, then their redistribution would be expected to change the sites of endocytosis. To test this prediction, we made use of the clustering of eisosomal remnants

Figure 1 | **Eisosomes are large protein assemblies at the cell cortex. a,** Pil1, Lsp1 and Sur7 colocalize to patches beneath the plasma membrane. GFP-tagged Pil1, Lsp1 and Sur7 were localized in live cells by acquiring confocal *z*-stacks. Top, mid section and projection of a three-dimensional reconstruction of the cell. Bottom, distribution of the fluorescence intensities of Pil1–GFP, Lsp1–GFP and Sur7–GFP patches plotted in classes (*y* axis, number of spots) of arbitrary fluorescence units (*x* axis, 12 bit). **b,** Pil1 and Lsp1 interact physically. Myc-tagged Lsp1 and Pil1 were immunoprecipitated from cell extracts and labelled bands were identified by mass spectrometry. Asterisks indicate IgG heavy and light chains. **c,** Pil1 and Lsp1 colocalize in live cells. A representative mid section is shown. Insets show magnification of the boxed region. **d,** Sur7 colocalizes with Pil1. Sur7–GFP and Pil1–HA were visualized by indirect immunofluorescence and confocal microscopy. A representative mid section is shown. Scale bar, 2 μm. **e,** Eisosomes are immobile. Cells expressing Pil1–GFP cells were followed by time-lapse confocal microscopy for 30 min. Projections of three-dimensional reconstructions of cells at 0, 11 and 30 min are shown. Arrows mark two eisosomes as visual reference points. **f,** FRAP of Pil1–GFP. Images of cells before, immediately after bleaching, and after 20 min of recovery are shown. Bleached areas are boxed. **g,** Quantification of fluorescence recovery over time averaged between three independent experiments.

Figure 2 | **Pil1 is required for Lsp1 and Sur7 localization. a,** Confocal image of Lsp1–GFP expressed in wild-type and *pil1Δ* cells. A representative field of mid sections is shown. **b,** Confocal images of Sur7–GFP expressed in wild-type, *pil1Δ*, *lsp1Δ* and *pil1Δ lsp1Δ* strains. Scale bar, 2 μm. **c,** Top panel, electron micrograph of *pil1Δ* cells fixed with glutaraldehyde and stained with uranyl acetate and permanganate. Bottom panels, magnified large aberrant membrane invaginations seen only in mutant cells. Scale bar, 0.5 μm.

observed in *pil1Δ* strains (Fig. 2). Notably, after FM4-64 uptake in *pil1Δ* cells expressing Sur7–GFP, we observed colocalization of all early FM4-64 foci with the clusters (Fig. 3c). Similarly, foci defining Hxt2–GFP endocytosis were redirected to the clusters in *pil1Δ* cells (Fig. 3d, right).

Analysis of *pil1Δ* cells by electron microscopy revealed large, aberrant plasma membrane invaginations (Fig. 2c) not observed in wild-type cells. From their frequency of appearance in the cell sections, we estimate that every *pil1Δ* cell contains at least one such structure (Supplementary Information), consistent with the frequency of clustering of endocytic sites found in *pil1Δ* cells by FM4-64 and Hxt2–GFP fluorescence. Thus, the aberrant plasma membrane invaginations seen in the electron micrographs are likely to be sites of clustered eisosome remnants. These results suggest that eisosomes are required for the proper spatial distribution of endocytic events. In the absence of Pil1, the remaining eisosomal components collapse into clusters, representing large and aberrantly structured endocytic sites. Endocytosis at the aberrant sites functions with reduced capacity but is efficient enough to sustain cell viability.

The mechanics of endocytosis are carried out by effector proteins that assemble at the membrane, where they mediate vesicle formation, pinching-off, and movement into the cell's interior. Several parallel endocytic pathways are thought to use subsets of endocytic effectors, may select different cargos, and, indeed, may be localized to discrete domains of the cell surface[2]. Genetic and microscopic analyses indicate that the transient formation of actin patches at the cell cortex is an important step in the process[2]. Mutations in numerous genes encoding cortical actin patch components lead to defects in endocytosis[2], and most of these proteins have homologues in mammalian cells, where they likewise participate in endocytosis. The dynamic assembly of actin patches at the cell cortex is thought to promote the formation of endocytic vesicles by propelling them from the plasma membrane into the cell interior through a defined coordinated sequence of molecular events.

The sequential recruitment of endocytic effectors has been visualized directly[19,20]. The assembly occurs in several phases, each of which is marked by the transient ($\sim$20–30 s) presence of specific actin patch proteins. Colocalization of one such protein, Abp1, with FM4-64 in early endocytic intermediates showed that these patches are indeed sites of endocytosis[17]. To examine the relationship between eisosomes and cortical actin patches, we first tested the effects of latrunculin A (Lat A), a drug that prevents actin
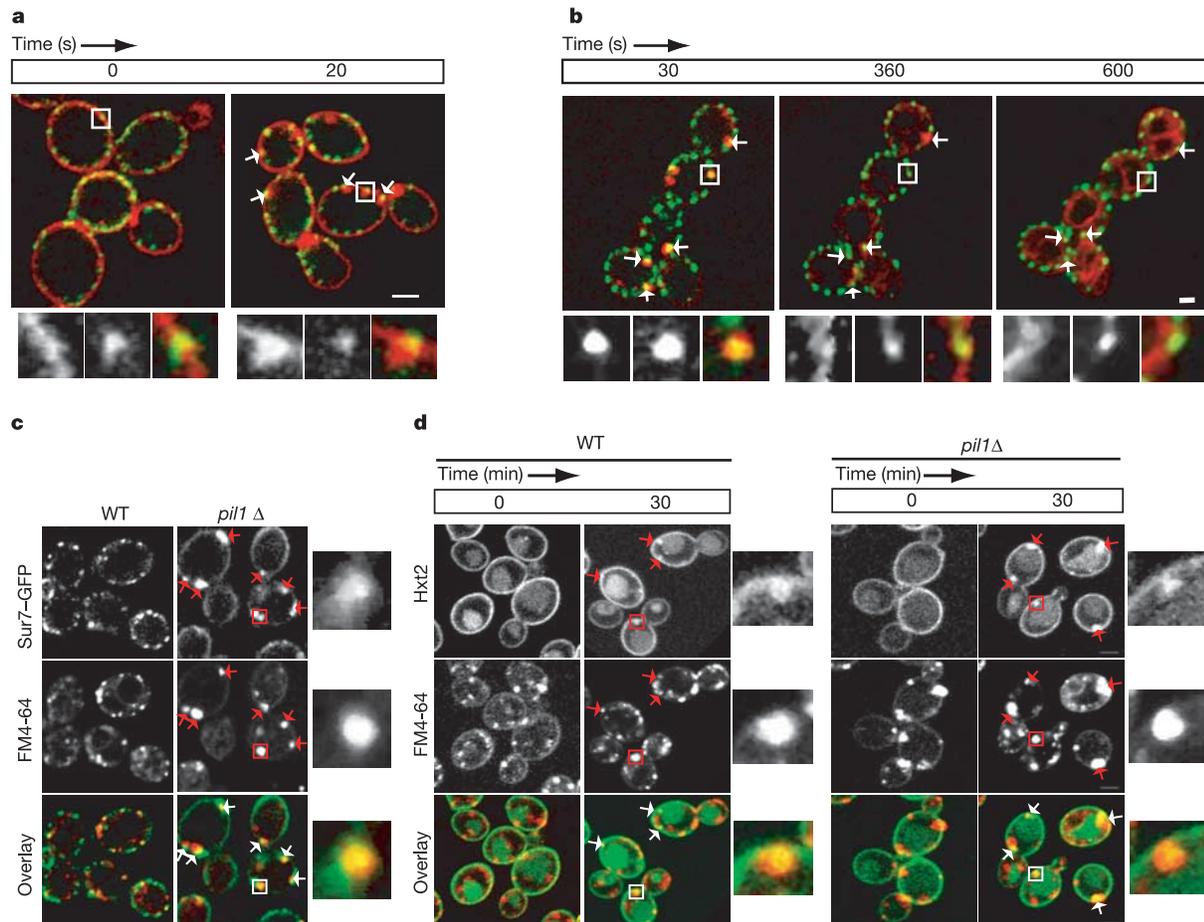
Figure 3 | **Eisosomes colocalize with sites of endocytosis. a**, Cells expressing Pil1–GFP were labelled with FM4-64 on ice. To remove excess dye, cells were washed with ice-cold YPD and subsequently resuspended in room-temperature YPD. Endocytosis was stopped by transfer to ice and the addition of 10 mM NaN$_3$ and 10 mM NaF either immediately or after 20 s. Top, confocal microscopy images. Bottom, magnification of single eisosomes (boxed above), showing, from left to right, FM4-64 staining, GFP staining and overlay. Arrows indicate representative examples of FM4-64 and Pil1–GFP colocalization. Scale bar, 2 μm. **b**, Cells labelled as in **a** and imaged at the indicated times after warming to room temperature. **c**, Endocytosis can be redirected to mislocalized eisosome remnants. Uptake of FM4-64 was visualized in wild-type or *pil1Δ* cells expressing Sur7–GFP. Arrows indicate representative examples of FM4-64 and Sur7–GFP colocalization. Right images show close-ups of a representative eisosome (boxed). **d**, Hxt2 is taken up through eisosomes. Wild-type or *pil1Δ* cells expressing Hxt2–GFP were grown in 0.1% glucose for 6 h and then shifted to 5% glucose. Uptake of Hxt2 was much slower than that of FM4-64; thus, the number of foci at any given time was lower. At the indicated time points, cells were pulse-labelled with FM4-64 and imaged after a 30-s pulse to catch early FM4-64 endocytic intermediates. Arrows indicate representative examples of FM4-64 and Hxt2–GFP foci colocalization. Right images show close-ups of a representative (boxed) eisosome (WT) or eisosome remnant (*pil1Δ*).

polymerization and inhibits endocytosis[21]. Lat A did not block the concentration of FM4-64 at eisosomes, but did prevent Abp1–GFP from assembling into cortical actin patches (Fig. 4a), thus indicating that the Lat A treatment was effective. In contrast to untreated cells, however, FM4-64 foci in Lat-A-treated cells were not consumed even at late time points. Together, these results show that the formation of FM4-64 foci at the plasma membrane is actin-independent, but that their consumption, namely the depletion of FM4-64 from eisosomes, is actin-dependent[17].

Imaging of both Abp1–GFP and FM4-64 revealed two populations of actin patches: one that colocalized with FM4-64 foci (and thus with eisosomes) and another that appeared at unrelated positions near the cell cortex. To confirm that Abp1 patches colocalizing with FM4-64 were functionally related to eisosomes, we observed Abp1 patches in *pil1Δ* mutant cells in which eisosome remnants are clustered. As expected, Abp1 patches formed in *pil1Δ* mutant cells more frequently at the clustered sites (Fig. 4b and Supplementary Information). Owing to crowding at the cell cortex, images of both eisosome and non-eisosome-localized Abp1 patches were difficult to interpret. We therefore exploited the transient nature of the actin patches and integrated the Abp1–GFP signal over time. A clear peak of the Abp1–GFP-containing patches coinciding with clustered eisosome remnants emerged over time, whereas the population of patches that appeared at unrelated and apparently random positions became averaged into a continuous rim at the cell periphery (Fig. 4b). Another actin patch protein, Sla2, showed a very similar behaviour (Fig. 4c).

Our results show that actin patches form at eisosomes, but Abp1- and Sla2-containing actin patches also formed at unrelated sites at the cortex. Furthermore, whereas Pil1–GFP labelled eisosomes are not visible in small buds (T.C.W. and P.W., unpublished data), Abp1- and Sla2-containing patches formed frequently in small buds (see Fig. 4c). Assuming that formation of these actin patches in each case marks productive endocytic events, they probably represent eisosome-independent endocytic events that are not monitored by FM4-64 uptake. Actin patches in buds, for example, did not collapse into clusters in *pil1Δ* cells (Fig. 4c), indicating that eisosome-mediated endocytosis may be especially important in mother cells. Thus, like metazoan cells, yeast may have parallel endocytic pathways that have different molecular requirements. According to this view, one

pathway, localized to eisosomes and defined by its sensitivity to eisosome disruption, primarily operates in the mother cell, whereas others, less sensitive to eisosome disruption, primarily operate in the bud. These pathways share at least some common components, such as the actin patch proteins analysed here.

The notion that endocytic events initiating at eisosomes use actin patch proteins is further underscored by genetic interactions between eisosome components and endocytic effectors. For example, *pil1Δ* mutants show synthetic lethality with a conditional allele of *PAN1* (*pan1-20*) and synthetic growth defects with *sla1Δ* mutations (Supplementary Information). Such synthetic defects could indicate that two components closely interact. Alternatively, each mutation could compromise alternative but redundant pathways: when both mutations are combined, reciprocal compensation between the pathways would be lost. We also observed suppression of *end4-1* (*END4* is allelic with *SLA2*) mutants by deletion of *PIL1* and suppression of the endocytic defect of *rvs161Δ* by deletion of *LSP1*. Thus, although so far the complete matrix of possible interactions has been only explored superficially, there is an extensive web of genetic, biochemical and cytological interactions that solidly ties the three known eisosome components to the endocytic machinery (Fig. 4d).

Taken together, the notion that eisosomes have a role in endocytosis is supported by the following observations: first, early endocytic intermediates form initially at eisosomes; second, these are built in an actin-independent, but consumed in an actin-dependent, reaction; third, a subpopulation of cortical actin patches colocalizes with eisosomes; fourth, endocytosis of both lipid and protein cargo, and cortical actin patches can be redirected to clustered eisosome remnants in *pil1Δ* strains; fifth, *pil1Δ 1sp1Δ* mutant strains are impaired in endocytosis and show large, aberrant plasma membrane invaginations; and sixth, *LSP1* and *PIL1* connect through a web of genetic and biochemical interactions with known endocytic effectors, such as *RVS161*, *RVS167*, *PAN1*, *SLA1* and *SLA2*. Eisosomes consist of two main cytoplasmic components, Lsp1 and Pil1, the plasma membrane protein Sur7, and perhaps other undefined proteins, such as other members of the Sur7 protein family. By contrast to other previously described endocytosis components, eisosomes are immobile static structures. Eisosomes thus provide a stable, functional link between the cytoplasmic components that
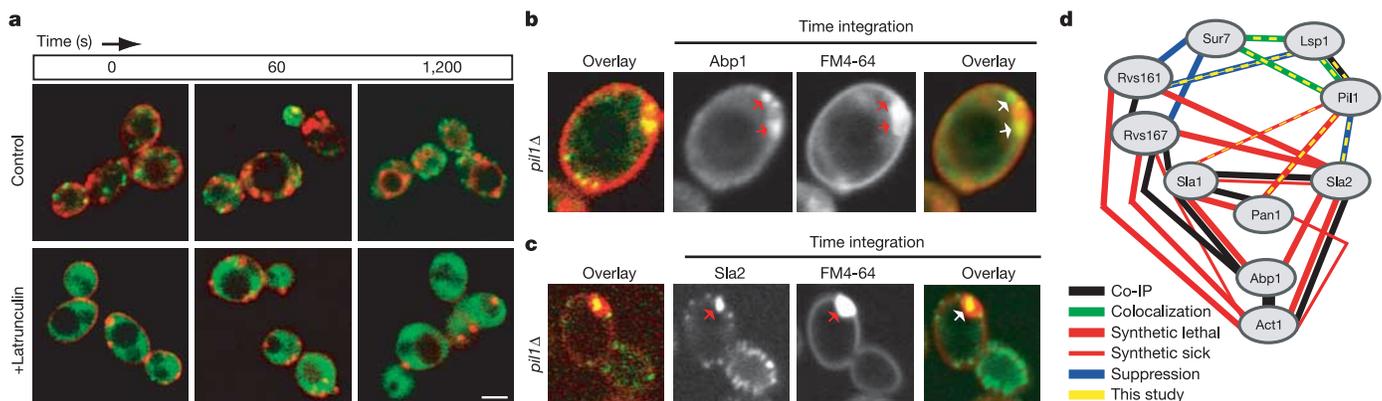
Figure 4 | Endocytic actin patches form at eisosomes. a, Formation of early endocytic intermediates is actin-independent. Cells expressing Abp1–GFP were treated with or without Lat A, pulse-labelled with FM4-64, incubated at room temperature for the indicated times, and imaged by confocal microscopy. **b,** Confocal time-lapse images of *pil1Δ* cells expressing Abp1–GFP and pulsed with FM4-64. An individual still frame of this time series is shown on the left. Three images integrated over a 4-min observation period are shown on the right. Note that the small sharp foci of Abp1–GFP staining in the still frame have disappeared in the time-averaged images because they did not recur at the same loci. By contrast, the Abp1–GFP signal at the eisosome cluster amplified during time-averaging (arrows).

c, *pil1Δ* cells expressing Sla2–GFP treated as in **b**. Note the frequent appearance of Sla2-containing actin patches in the bud (lower right). These patches do not relocalize to clustered eisosome remnants as observed in the mother cells and may represent a parallel, eisosome-independent pathway. **d,** Network of interactions of eisosome components with known endocytic effectors. Physical and genetic and biochemical interactions and colocalization are indicated by colour coding. Data for interactions reported in this study (dashed yellow lines superimposed) and references for previously established interactions are given in the Supplementary Information.

catalyse endocytosis and the plasma membrane, and may regulate when and where endocytosis occurs.

Many observations point to an eisosome–lipid connection. First, Sur7 has been suggested to function in endocytosis because it is an overexpression suppressor of a deletion of *RVS161* and *RVS167*, encoding yeast endophilin and amphiphysin, respectively[10]. In metazoan cells, these proteins are thought to modulate membrane curvature after invagination of endocytic pits. Other suppressors of *rvs161Δ* map to genes involved in sphingolipid biosynthesis. Second, the lipid-regulated kinases Pkh1 and Pkh2 phosphorylate Pil1 and Lsp1 (ref. 13). Pkh2 physically associates with Pil1 and Lsp1 and shows an eisosome-like localization[22,23]. Last, long-chain bases, the precursors of sphingolipids, inhibit the phosphorylation of Pil1 but stimulate the phosphorylation of Lsp1. Thus, differential regulation of Pil1 and Lsp1 could modulate the activity of eisosomes to recruit other endocytosis components and concentrate them beneath the plasma membrane where they participate in the mechanics of membrane uptake by endocytosis, perhaps ascertaining that the local lipid composition is compatible with a productive endocytic event. In the *PIL1* and *LSP1* mutants, a diminished local concentration of endocytosis effectors would result in a reduced rate of uptake. Their differential regulation by phosphorylation parallels the opposite effects that *PIL1* and *LSP1* deletions have on endocytosis in the context of *rvs161Δ*. Thus, Lsp1 could be a negative regulator or 'governor', restricting endocytosis to a few sites. Its deletion would then increase endocytosis under suboptimal conditions, providing a plausible explanation for its genetic interaction with the *rvs161Δ* mutation.

In many metazoan cell types, endocytosis and exocytosis are spatially segregated into mutually exclusive zones and both can occur repeatedly at the same sites[24,25]. In synaptic nerve terminals, endocytic activity is organized in membrane patches forming a ring surrounding the central zone of exocytosis, enabling the cell to coordinate efficiently bursts of exocytic activity with endocytic membrane recycling[26]. Endocytic effectors including endophilin, synaptojanin and AP180 are concentrated in the endocytic zone by scaffold proteins, such as Dap160 (also known as intersectin). In *Drosophila* mutants lacking intersectin, these effector proteins are no longer concentrated around the active zone. Intriguingly, like *pil1Δ* mutants, these mutant cells lacking intersectin also show only a modest kinetic delay in endocytosis, even though the structural organization of the endocytic zones is severely damaged[27]. Putative homologues of Pil1 and Lsp1 (ref. 13) and potentially other eisosome components might function similarly to organize endocytosis in higher eukaryotes.

A mechanism to regulate endocytosis spatially—that is, to choose which of several possible sites of endocytosis to activate—is likely to be important for maintaining organization of the plasma membrane. In the absence of such regulation, plasma membrane structure can be grossly affected, as seen here by the large aberrant invaginations formed in *pil1Δ* cells. A controlled imbalance between the two processes through local enhancement or inhibition of endocytosis could also be used to localize cell growth and to contribute to the establishment and maintenance of cell polarity. Eisosomes may thus have a profound role in regulating plasma membrane architecture.

METHODS

Yeast strains and plasmids. All yeast strains were derived from the W303 strain by using PCR-based modification[28].

Protein analyses. To immunopurify Pil1 or Lsp1 complexes, 50 ml of cells expressing Myc-tagged proteins were lysed in the presence of 1% Triton X-100 (150 mM NaCl and 20 mM HEPES; pH 7.4), incubated with agarose conjugated to anti-Myc antibodies for 2 h, washed, and eluted in 2% SDS. Proteins were identified by mass fingerprinting after SDS–PAGE using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Endocytosis assays. We monitored endocytosis by uptake of FM4-64 (Molecular Probes) as described[1]. In short, cells were labelled on ice (40 μM), washed three times with ice-cold YPD medium and resuspended at $t = 0$ in YPD (room

temperature). Samples were either stopped by the addition of 10 mM NaN$_3$ and 10 mM NaF or imaged continuously. For Lat A treatment, 50 μM was added for 15 min before start of the assay; Lat A was also included in subsequent media.

Microscopy. Light microscopy was done on an LSM510 confocal microscope (Zeiss). Images were processed using LSM (Zeiss) and ImageJ (W. S. Rasband: http://rsb.info.gov/ij/) software. Immunofluorescence and electron microscopy were done as described[29,30].

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