

## MINIREVIEW

# Sterol-Rich Plasma Membrane Domains in Fungi<sup>∇</sup>

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Concepts regarding the eukaryotic plasma membrane have been evolving in light of growing evidence that it is segregated into distinct lateral domains known as lipid rafts. These sterol- and sphingolipid-rich raft domains are thought to play important roles in dynamic processes, including protein sorting, cell polarity, and signal transduction. Because of this, it has been very intriguing that sterol-rich domains (SRDs) that are much larger than lipid rafts have been identified in the plasma membranes of fungi. The larger SRDs can be readily observed in cells stained with filipin, a naturally fluorescent sterol-binding antibiotic. These large SRDs are located at sites of active morphogenesis and are regulated in a developmental or cell-cycle-regulated manner. For example, SRDs were detected at the tips of mating projections in *Saccharomyces cerevisiae* and hyphae in *Candida albicans*, but not in budding cells. In the fission yeast *Schizosaccharomyces pombe*, an SRD forms in a cell-cycle regulated manner in the medial zone of the rod-shaped cells at the future site of cell division. SRDs have also been reported at sites of polarized morphogenesis in *Cryptococcus neoformans* and *Aspergillus nidulans*. These domains may be formed by the clustering of lipid rafts and may therefore also function as organizing centers in the membrane. In this review, we assess recent progress in the analysis of lipid rafts and large SRDs in fungi. Comparisons with other plasma membrane domains, including eisosomes and septin boundary domains, are made to examine how these specialized regions work together to contribute to polarized morphogenesis and cytokinesis.

### LIPID RAFT ORGANIZATION AND FUNCTION IN FUNGI

**Lipid rafts, liquid-ordered domains, and detergent-resistant membranes (DRMs)—what's the skinny on these fatty regions?** Plasma membranes differ from most subcellular membranes because they contain a high fraction of sterols and sphingolipids in addition to the glycerophospholipids. Studies on model membranes indicate that sterols and sphingolipids can cluster into liquid-ordered domains instead of forming homogeneous mixtures with glycerophospholipids (58). The underlying basis for this is that sterols are composed of a

four-ring structure and an aliphatic tail that can pack together in a favorable manner with sphingolipids, which are comprised of ceramide and long saturated acyl chains (57) (Fig. 1). The lipid acyl chains pack tightly with the rigid sterols to create a compacted zone of condensed bilayer that has been termed the liquid-ordered state (10, 60). Glycerophospholipids with unsaturated side chains do not pack in this manner and are excluded from the raft domains.

The possibility that these liquid-ordered domains may occur in vivo, where they would preferentially associate with certain types of proteins (e.g., acylated and glycosylphosphatidylinositol [GPI]-anchored proteins), gave rise to the idea that these protein-lipid domains would form lipid rafts in the plasma membrane (11, 71). Because of their unique lipid composition, the liquid-ordered domains are more resistant to extraction with nonionic detergents, which, as described below, is why these domains are sometimes referred to as DRMs. Although the terms lipid raft, liquid-ordered domain, and DRM are intended to represent similar concepts, they have distinct implications and should not be used interchangeably (50).

The pioneering studies on lipid rafts and their biological roles have been primarily carried out in mammalian cells (10, 11, 23, 38, 60). However, the general properties of plasma membrane organization and lipid raft function appear to be similar in fungi. For example, on a molar basis, the yeast plasma membrane lipid content is similar to that of mammalian cells in that it is about 30 to 40% sterols, 10 to 20% sphingomyelin, and typically a small fraction of glycosphingolipids (60, 76). The primary sterol found in fungi is ergosterol, which is a better raft former than cholesterol (97). In addition to lateral organization into lipid rafts, the inner and outer leaflets of the plasma membrane also contain distinct lipid contents. Raft-forming sphingolipids are found primarily in the outer leaflet, and some glycerophospholipids, like phosphatidylserine, are primarily present in the inner leaflet (32). Asymmetry is maintained by active transport, which is why phosphatidylserine is seen only on the outer leaflet in mammalian cells undergoing apoptosis.

**DRMs.** DRM analysis was one of the first indicators that raft domains could form in cellular membranes (12). In mammalian cells, plasma membrane proteins differ in their abilities to associate with DRMs, which is inferred to represent differential abilities to partition into rafts in vivo. Analysis of DRMs in *S. cerevisiae* has confirmed that they are enriched in ergosterol and sphingolipids, as expected (4, 5). In contrast to mammalian cells, it appears that most yeast integral plasma membrane proteins primarily partition with the DRM fraction (46, 53).

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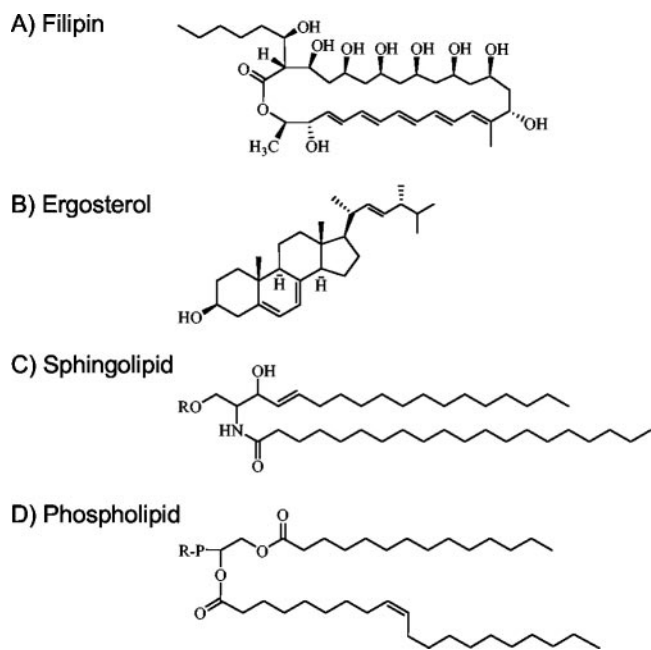


FIG. 1. Comparison of filipin and plasma membrane lipid structures. (A) Structure of filipin, a polyene antibiotic that binds sterols. (B) Structure of ergosterol, which contains a four-ring structure common to other sterols, including cholesterol. (C) Sphingolipids are based on a ceramide and contain saturated acyl chains that are variable in length (26 carbon atoms are shown) attached to the sphingosine base. The R group is phosphocholine in the case of sphingomyelin or sugar(s) in the case of the glycosphingolipids. (D) Glycerophospholipids are based on diacylglycerol and typically carry acyl chains of 16 to 18 carbon atoms. One of the acyl chains contains a *cis* double bond, which prevents close packing with saturated acyl chains present in lipid rafts.

Only three yeast plasma membrane proteins have been reported to partition primarily in the detergent-soluble fraction: Tre1 (Ypl176c), Gap1, and Hxt1 (5, 54). However, a recent study found that Gap1 and Hxt1 were in fact primarily present in DRMs when these proteins were localized to the plasma membrane, but they dissociate from DRMs during endocytic trafficking (46). It is not clear why there are discrepancies, given that control experiments in these studies indicated that the relevant proteins appeared to be present in the plasma membrane. Possibly, it has to do with differences in yeast strains or growth conditions, since the trafficking of Gap1 and Hxt1 is regulated by nutritional conditions. Alternatively, it could be due to differences in DRM extraction conditions, as discussed below. Although DRM analysis has not identified differences in the abilities of plasma membrane proteins to partition into lipid rafts in fungi the way it has in mammalian cells, it has played a very significant role in other areas, such as defining the stages of protein trafficking to the plasma membrane that will be described below.

DRM analysis has provided important new insights into the role of lipid rafts in biological membranes, but it has also been controversial, in part because of different experimental methods and interpretation of data. For example, some investigators consider a protein to be DRM associated if it is insoluble after detergent extraction at 4°C without doing further con-

trols. Since many proteins, such as components of the cytoskeleton, are insoluble after extraction with nonionic detergents, it is more reliable to float the DRMs on a density gradient before concluding whether a protein is in the DRM fraction. Even so, caution must be used, because other proteins may stick to the DRMs nonspecifically. In addition, the components of DRMs should be insoluble at 4°C but soluble at warmer temperatures. Another potential source of variability is that it is important to extract DRMs using the same ratio of protein to detergent in each sample (66). Also, different results can be obtained using different nonionic detergents. Most studies use 1% Triton X-100 to determine detergent insolubility, but other nonionic detergents, such as CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, will also work (77). Perhaps these experimental differences or other variables explain why the plasma membrane ATPase Pma1 was initially reported to be absent from DRMs in *S. cerevisiae* (44) but was subsequently found to be almost exclusively in DRMs in *S. cerevisiae* and *C. albicans* (4, 37, 55). Similar conflicting results for Gap1 and Hxt1 are described above. Another concern regarding the analysis of DRMs is that even if two different proteins are found in the DRM fraction, this does not necessarily imply that they were present in the same subset of lipid rafts *in vivo* (50).

**Lipid raft assembly in fungi.** Studies on yeast DRMs indicate that lipid rafts begin to assemble in the endoplasmic reticulum (ER) (4, 46). This differs from mammalian cells, where the association of proteins with DRMs takes place in the Golgi (11, 12). Ergosterol is synthesized in the ER and is present along the secretory pathway and in the plasma membrane (101). Interestingly, transport of ergosterol can occur by a process that is independent of the standard secretory pathway, because an *S. cerevisiae sec18<sup>ts</sup>* mutant does not affect ergosterol transport (56). Sphingolipid synthesis begins in the ER and continues in the Golgi, and then the sphingolipids are sent to the plasma membrane in secretory vesicles (27). Sterols and sphingolipids are also internalized during endocytosis and are present in endocytic vesicles. However, these raft constituents are presumably recycled back to the plasma membrane, as they are not found in the vacuole (68). Proteins such as the *OSH* gene-encoded oxysterol binding proteins also contribute to proper sterol distribution in cells (8). Other factors that mediate proper sterol distribution have been identified through the study of human genetic diseases that cause abnormal accumulation of sterols (52, 56).

**Lipid raft functions.** One presumed function of lipid rafts is to act as organizing centers by preferentially associating with specific proteins. Lipid-modified proteins, such as GPI-anchored proteins on the extracellular side and acylated proteins on the intracellular side, are thought to partition into rafts due to the favorable packing of their saturated acyl chains. It is not clear what targets transmembrane proteins into rafts. In yeast, lipid rafts have been implicated in protein sorting, secretion, endocytosis, and cell polarity (6, 91). Although it is difficult to obtain direct evidence for lipid rafts in these processes, ergosterol and sphingolipids are clearly involved. For example, transport of the GPI-anchored Gas1 protein from the ER to the Golgi is dependent on sphingolipid synthesis (36, 84). Also, a mutant form of Pma1 that failed to associate with DRMs was defective in trafficking, suggesting that proper association with lipid rafts is important for trafficking

TABLE 1. Comparison of sites of sterol-rich domains in different fungi

Parameter	Cell type					
	<i>S. cerevisiae</i> buds	<i>C. neoformans</i> buds	<i>C. albicans</i>		<i>A. nidulans</i> hyphae	<i>S. pombe</i> rods
			Buds	Hyphae		
Morphogenesis Site	Absent	Present Bud tips	Absent	Present Hyphal tips	Present Hyphal tips	Present Ends of rods
Septae	Absent	Present	Absent	Present	Present	Present
Pheromone-induced cells	Tips of mating projections	Tips of mating projections	Not reported	Not reported	Not reported	Tips of mating projections
Reference	5	62	55	55	67	92

(3, 31). Analysis of the amino acid permease Gap1 during exocytic and endocytic trafficking showed that DRM association correlated with plasma membrane localization (46). Ergosterol and sphingolipid synthesis are also important for endocytosis. Analysis of *ergΔ* mutants that accumulate different sterol intermediates showed that sterols function in the early stages of endocytosis and also in the later stages of vesicle trafficking (34, 59).

**Why are lipid rafts so controversial?** The small size of lipid rafts and consequent difficulties in visualizing them *in vivo* has made it necessary to rely on indirect methods to define their structure and function. Differences in how these indirect approaches are carried out and how they are interpreted has created controversy (60). In addition to the problems with the analysis of DRMs described above, other methods used to examine lipid rafts have also led to disagreements. For example, the depletion of cholesterol from membranes using methyl- $\beta$ -cyclodextrin has been used in many studies to infer the role of lipid rafts. However, cholesterol depletion is also expected to cause other effects, such as changes in bilayer properties and permeability (60). New approaches that are being developed will therefore play an important role in better defining the structure and function of these domains in the future (38). One new approach that is less controversial is single-molecule tracking, which maps the lateral mobility of single proteins and lipids in the plasma membrane. This approach has revealed that raft constituents become trapped in transient confinement zones that have properties that are consistent with those of lipid rafts (15, 18, 45). Other promising new approaches for studying rafts include atomic force microscopy (16) and mass spectrometry (43).

### SRDs IN FUNGI

Recent studies have identified SRDs in fungi that are much greater in size than lipid rafts are assumed to be. SRDs appear to range from about 3 to 15  $\mu\text{m}^2$ , depending on the organism and location. In contrast, recent estimates suggest that lipid rafts are about 75  $\text{nm}^2$  (23). So far, SRDs are unique to fungi and have not been reported to be present in mammalian cells. These large fungal SRDs are of interest for their potential roles in membrane organization, and they may therefore also have special significance for the presentation of virulence fac-

tors and as targets for antifungal therapy. The SRDs have been primarily visualized by staining cells with filipin, which is a polyene antibiotic that forms a complex with free 3- $\beta$ -hydroxy sterols (Fig. 1). Filipin was named for its identification as a compound produced by an actinomycete, *Streptomyces filipinensis*, found in soil from the Philippines (96). Filipin is naturally fluorescent with maximum excitation (360 nm) and emission (480 nm) properties that allow it to be detected using typical microscope filter sets used for DAPI (4',6'-diamidino-2-phenylindole) staining (21). It is a common histochemical stain for cholesterol and is used in the diagnosis of altered sterol storage caused by diseases such as type C Niemann-Pick disease. However, filipin can be tricky to work with; it is not stable in solution and should be freshly dissolved before use, it photobleaches rapidly, and cells should be visualized quickly after being stained, as it is toxic and can deform membranes. Earlier studies took advantage of the ability of filipin to promote deformation of sterol-containing membranes in order to compare the sterol compositions of membranes by electron microscopy (65, 88). More recent studies have analyzed filipin by fluorescence microscopy, since this can be carried out more quickly and is not subject to other limitations of the membrane deformation approach. Nonetheless, additional methods need to be developed to confirm sterol localization, such as new fluorescent probes or acyl-green fluorescent protein (GFP) (100), because filipin has the potential to cause deleterious effects on cell viability and membrane structure.

Little is known about how these large SRDs are formed or their functions *in vivo*. In fact, the mechanism of formation may be distinct in different organisms. Another question that also needs to be addressed is quantifying the degree of sterol enrichment observed by filipin staining of these domains. Since the overall plasma membrane is about 30 to 40% ergosterol, this presumably limits the enrichment that can occur in the SRDs. Therefore, the key observations regarding the identification of large SRDs in different fungi are summarized below to compare their properties. The sites of filipin staining in different fungi are also summarized in Table 1, and some examples are shown in Fig. 2. Models for the formation of SRDs and their potential roles in cell polarity and pathogenesis are then described.

***S. cerevisiae*.** In the budding yeast *S. cerevisiae*, filipin strongly stained the tips of cells induced with mating pheromone (5, 69). An example of this is shown in Fig. 2. Filipin staining was

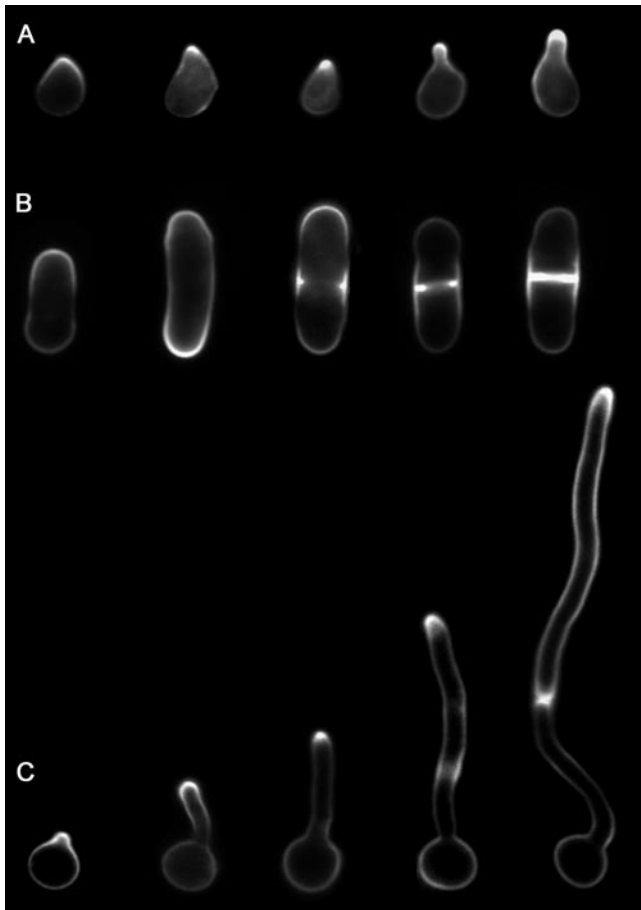


FIG. 2. Examples of filipin staining. (A) *S. cerevisiae* cells induced with  $\alpha$ -factor. (B) *S. pombe* cells grown to log phase. (C) *C. albicans* induced to form hyphae with serum. The cells were stained with filipin and then viewed by fluorescence microscopy as previously described (5, 55, 92).

not detected in vegetative cells, which polarize their growth into the new bud. The tips of pheromone-induced cells undergo highly polarized morphogenesis to form the conjugation tube that connects cells during mating. Disruption of sterol or sphingolipid production resulted in less polarized filipin staining. Other controls indicated that filipin staining is specific for sterols in *S. cerevisiae*, such as the decreased filipin staining of *erg9* $\Delta$  and *erg6* $\Delta$  mutants, which are defective in forming ergosterol (8, 52). Thus, it was proposed that this SRD at the tips of pheromone-induced cells plays an important role in cell polarization and proper localization of components needed for cell and membrane fusion during mating (5). Consistent with this, several proteins that localize to the tips of the pheromone-induced projections were present in DRMs.

The significance of the filipin staining at the tips of pheromone-induced cells was called into question for several reasons (90, 91). One is that this staining pattern was not detected in formaldehyde-fixed cells. However, it is possible that there could have been movement of the lipids postfixation (90). Another criticism was that uptake of the endocytosis tracer dye FM4-64 was perturbed in budding cells treated with filipin, and it accumulated in the same region of a nascent bud as filipin. It

is also not clear whether the SRD plays a role in localizing proteins to the tip, since some proteins achieve this localization through endocytic recycling and other proteins may be anchored by cytoskeletal interactions (69, 90). However, additional evidence for a polarized lipid domain at the tips of pheromone-induced cells was obtained by staining the cells with Laurdan, which is an environmentally sensitive dye that shifts its emission peak depending on whether it is in a liquid-ordered or -disordered lipid domain (69). The results demonstrated that the membrane was more ordered and condensed at the tips of the pheromone-induced cells than at the opposite sides. Similar results were observed in a strain that is deficient in endocytosis (*end4* $\Delta$ ) but not in a strain with a defect in sphingolipid synthesis (*lcb1-100*) (69).

***C. neoformans*.** The budding yeast *C. neoformans* is a member of the *Basidiomycota* division of the fungal kingdom, which makes it evolutionarily quite distant from the members of the *Ascomycotina* division that includes *S. cerevisiae* and the other fungi that are discussed below (39). Filipin showed intense staining of the leading edges of mating projections in *C. neoformans*, similar to what was observed in *S. cerevisiae* (62). In contrast to *S. cerevisiae*, filipin also stained the actively growing sites at bud tips and at sites of septation in *C. neoformans*. Staining at bud tips is so far unique to *C. neoformans*, but as described below, staining at septation sites was also observed in the fission yeast *S. pombe* (92), in the filamentous fungus *A. nidulans* (67), and in *C. albicans* hyphae (55). It has been proposed that lipid rafts may serve to concentrate virulence factors and regulate their release from *C. neoformans*, which is a human pathogen (80).

***C. albicans*.** The multimorphic fungus *C. albicans* is capable of growing in a variety of morphologies, including buds, pseudohyphae (chains of elongated cells), and hyphae (long filamentous chains of cells). Strong staining with filipin was detected at the tip of hyphal growth and was not seen in buds or pseudohyphae (55). Examples of filipin staining are shown in Fig. 2. In cells switching from budding to hyphal growth, strong filipin staining was observed at the earliest stage of hyphal growth (germ tube formation) and was maintained at the leading edge in mature hyphae. This filipin staining was observed only at the actively growing hyphal tips and not at inactive tips after cells switched to a new site of hyphal morphogenesis. Filipin stained the plasma membrane and was not associated with the subapical cluster of vesicles known as the Spitzenkörper (17). The formation of this SRD was prevented by treating cells with latrunculin A, indicating a role for actin (55). Inhibitors of ergosterol (ketoconazole) or sphingolipid (myriocin) synthesis diminished the formation of the SRD and also caused defects in polarized hyphal growth (55). Thus, the SRD may play a role in proper localization of cell polarity components that help restrict growth to the narrow zone at the tip of the hypha. In addition, as discussed below, the SRD at the hyphal tip may be important for proper presentation of virulence factors, many of which are GPI anchored (61, 72, 83). Proteomic comparison identified more than 40 hyphal DRM proteins that were not detected in buds, some of which are candidates for associating with SRDs (2).

Filipin staining in *C. albicans* hyphae was also more intense at sites of septum formation, although this staining was not as strong as that seen at hyphal tips (55). This contrasts with

septal sites in *C. albicans* buds or pseudohyphae, which did not show increased filipin staining. Prior to cytokinesis in hyphae, filipin staining appeared on the side of the septin ring toward the hyphal tip. At later stages after cytokinesis, filipin staining was detectable on both sides of the septum. It is not clear why filipin staining was detectable only at septation sites in hyphae and not in buds. Perhaps the septin ring captures sterol-rich membrane at the hyphal tip, since septin localization studies indicate that the ring initially forms at the tip (82, 94). Alternatively, as discussed below, there may be underlying similarities with the sterol-rich ring that forms at sites of septation in the fission yeast *S. pombe* (92). Either way, this distinct aspect of septum formation in hyphae is interesting in that it correlates with other differences in hyphal septation sites. For example, the hyphal septation site does not separate after cytokinesis and thereby shields  $\beta$ -glucans at this site from being recognized by macrophages (28, 35).

***A. nidulans.*** The filamentous fungus *A. nidulans* shows enriched filipin staining at both hyphal tips and sites of septation (67). The polarization of sterols to the hyphal tip correlated with the integrity of the actin cytoskeleton; a *mesA* mutant in which actin cables were not localized to hyphal tips showed depolarized filipin staining, including increased staining in subapical regions. Interestingly, staining at the septation sites was not affected in the *mesA* mutant. Similarly, the localization of the SepA formin was also rapidly lost from the hyphal tip in a *mesA* mutant, but SepA was still present at septation sites and septum formation was normal. Thus, the SRDs at hyphal tips and septation sites are regulated differently.

Subsequent studies demonstrated that addition of HSAF (heat-stable antifungal factor) (99) caused rapid loss of filipin staining at the hyphal tip (49). The cells treated with HSAF still grew, but the hyphal tips swelled, and numerous branches formed at apical and subapical sites. A *barA* mutant was identified that was resistant to HSAF, and at 42°C, it showed a phenotype similar to that of HSAF treatment, including defects in forming polarized SRDs and defects in polarized hyphal growth (49). The *barA* gene was found to encode a ceramide synthase that is involved in sphingolipid production. Reduced expression of a distinct second ceramide synthase, *lagA*, also caused defects in polarized hyphal growth, but the morphological defects were still exacerbated by HSAF. It was concluded that *A. nidulans* contains two distinct types of ceramide synthases, with LagA producing the bulk of the ceramide and BarA producing a specialized pool that is targeted by HSAF.

***S. pombe.*** SRDs have been observed at three sites associated with morphogenesis in the fission yeast *S. pombe*: at the ends of the rod-shaped cells, in a medial zone where cells undergo cytokinesis, and at the tips of mating projections (70, 92). Examples of this are shown in Fig. 2. The SRDs have been primarily studied by filipin staining but were also found to be enriched in the lipid raft marker protein acyl-GFP (87). The SRD in the medial region of the cell has been studied most extensively, because it forms in a cell-cycle-regulated manner as the cells prepare to undergo cytokinesis at this site. Temporal studies demonstrated that the cytokinetic actin ring forms first, then the SRD forms at the corresponding site in the plasma membrane around the medial zone, and then the actin ring contracts. Although the SRD colocalizes with the cytoki-

netic ring, actin and microtubules were not required to form the medial sterol-rich ring. Takeda et al. (87) further dissected the genetic requirements and found that several other genes related to the function of actin, septins, or septum formation were not required to form the medial SRD. However, a functional secretory pathway was needed, as brefeldin A blocked its formation (92).

Subsequent studies identified two proteins that were needed to form SRDs in *S. pombe*. One is the *cdc15* protein, which is a member of the PCH family that is involved in cytokinesis (87). *cdc15* mutant cells failed to form a medial ring and instead frequently formed spirals of SRDs in the plasma membrane. Overproduction of *cdc15* resulted in abnormal SRDs that appeared as patches and spirals in the plasma membrane. Since these mutant phenotypes cannot be attributed to the role of *cdc15* in actin organization, this suggests that *cdc15* may be directly involved in promoting the SRD. Consistent with this, Cdc15-GFP localized to the medial zone prior to the formation of the SRD in an actin-independent manner, and the *cdc15* protein was present in DRMs. Interestingly, *cdc15* interacts with the *cdc12* formin, similar to the interaction of *A. nidulans* MesA with the SepA formin (67, 87).

*cdc15* also interacts with *myo1*, which is the other protein identified as playing a role in forming the medial-zone SRD (86). The *myo1* protein localizes to the SRD and fractionates with DRMs, and its overproduction induces ectopic SRDs. Significantly, a *myo1* mutant did not form SRDs. In particular, SRD formation required the *myo1* TH1 domain, which contains a putative phospholipid-binding site that could potentially act as a membrane-organizing center (86). The *myo1* mutation lacking the TH1 domain that was used in these studies did not block endocytosis, further indicating that *myo1* may act directly to promote SRDs and not just indirectly through membrane-trafficking effects. Mutants lacking the TH1 domain were viable but exhibited partial defects in F-actin organization, septation, polarized growth, and viability under stress conditions.

These studies demonstrate that the *cdc15* and *myo1* proteins play key roles in the formation of the SRD, perhaps by binding to acidic phospholipids and promoting membrane organization. Although this helps to define the mechanisms of SRD formation, the biological role of SRDs in *S. pombe* is still not clear. It was initially assumed that the SRDs would provide a framework to facilitate interaction between proteins involved in the organization of processes such as cell polarity, cell wall synthesis, targeted membrane addition, and cytokinesis and septum formation (91, 92). However, the analysis of *cdc15* and *myo1* mutants indicates that the SRDs are not needed for viability, polarized growth, or cell division site placement (86, 87). Further study should provide insights into the links between actin assembly, endocytosis, and SRDs in the plasma membrane. In this regard, it is interesting that in addition to binding *myo1*, *cdc15* also binds to both the ARP2/3 complex and the formin *cdc12*, thus binding to both forms of actin nucleation involved in forming the cytokinetic ring (13). In mammalian cells, lipid rafts are thought to be platforms for membrane-linked actin polymerization stimulated by ARP2/3 (73).

## MODELS FOR THE FORMATION OF SRDs

The composition of SRDs is thought to be similar to that of lipid rafts, suggesting that a good starting point for developing models for the formation of SRDs involves the clustering of lipid rafts (5). In mammalian cells, clustering of lipid rafts is thought to be mediated by proteins that become cross-linked during processes such as virus budding and the activation of B-cell and T-cell receptors (1, 22, 78, 85). In an analogous fashion, protein-mediated clustering of lipid rafts could occur in *S. pombe*, since as described above, the *myo1* protein that is needed to form SRDs binds to *cdc15* and other proteins that could form a scaffold for stabilizing the SRD (86). The *cdc15* protein also binds to other proteins, including the ARP2/3 complex and the *cdc12* formin, which could contribute too.

It is not clear if *myo1* is involved in forming SRDs in other organisms, since the SRD at the medial zone in *S. pombe* forms in a cell-cycle-regulated manner that is independent of actin, whereas in other fungi the SRDs are present constitutively or are formed in a developmentally regulated manner in which actin is required for proper morphogenesis. However, there are potential similarities, since *C. albicans* mutants with defects in myosin I (*Myo5*) or the actin-related protein *Sla2* did not display SRDs, although these results are complicated by the fact that the mutants are also defective in forming hyphae (64). There are also similarities in the mechanisms of SRD formation in *S. pombe* and *A. nidulans*, in which the *MesA* protein is needed both to form SRDs at hyphal tips and for proper localization of the formin *SepA* (67).

Another model for clustering lipid rafts into larger SRDs involves GPI-anchored proteins. Since GPI-anchored proteins preferentially associate with lipid rafts (11), it has been suggested that the induction of GPI-anchored proteins during mating in *S. cerevisiae* and hyphal growth in *C. albicans* could promote clustering of lipid rafts (55). The GPI-anchored agglutinins that mediate intercellular adhesion are highly induced during mating in *S. cerevisiae* (51). Similarly, GPI-anchored adhesin proteins, such as *Hwp1* and *Als1*, are also highly induced during hyphal growth in *C. albicans* (25). A common feature of the agglutinins and adhesins is that their GPI anchors are ultimately cleaved off, and they become covalently linked to the cell wall. Thus, the older sites of deposition of these proteins would no longer be able to cluster lipid rafts. Only the recently synthesized GPI-anchored proteins that are deposited at the site of polarized growth would contribute to the formation of an SRD at the hyphal tip. Further studies will be required to determine the mechanisms of SRD formation.

## POTENTIAL ROLES OF SRDs IN VIRULENCE

The SRDs in pathogenic fungi are likely to play a role in pathogenesis by mediating the presentation of virulence factors and by influencing the biophysical properties of the plasma membrane. *C. albicans* produces many GPI-anchored virulence factors that are induced along with the SRD during hyphal growth. These include the members of the adhesin protein family (e.g., *Hwp1* and *Als1*), which mediate adhesion to host cells and biofilm formation, and also the secreted aspartyl protease family, which is needed for virulence (i.e., *Sap9*

and *Sap10*) (9, 40, 63, 75, 83). Other GPI-anchored proteins that contribute to virulence include *Eap1*, *Dfg5*, and *Phr1*, which are involved in epithelial adhesion, cell wall biogenesis, and proper hyphal growth (48, 74, 81). Bioinformatics approaches have also predicted other likely GPI-anchored proteins whose functions are not yet known (47). In addition, acylated proteins on the intracellular side are also likely to be present in the SRD, such as the *Ras1* protein that is needed for signaling hyphal growth and for virulence (24). Similarly, it has been suggested that special membrane domains in *C. neoformans* regulate membrane localization and release of the virulence factors phospholipase B1 and superoxide dismutase (80).

The SRDs are also expected to have distinct biophysical properties, which could contribute to virulence. For example, tight packing of lipid acyl chains with the sterols creates condensed zones that cannot be deformed to allow movement of small molecules across the membrane. This could be important at sites of polarized growth, such as hyphal tips, to provide additional strength while these regions are undergoing rapid cell wall remodeling. It is also possible that specialized membrane domains could influence signaling that occurs via lipids and their derivatives, such as farnesol, diacylglycerol, and eicosanoids in pathogenic fungi (79).

## IMPLICATIONS OF SRDs FOR ANTIFUNGAL THERAPY

The identification and analysis of SRDs provides new insights into the actions of the most commonly used antifungal drugs, amphotericin and fluconazole, which target ergosterol (29). Amphotericin binds to ergosterol and forms pores in membranes. Fluconazole blocks ergosterol synthesis by inhibiting lanosterol 14- $\alpha$ -demethylase (*Erg11*). However, these types of drugs have additional effects on cells by altering morphogenesis and interfering with the formation of SRDs (55). Thus, in addition to their previously defined roles, these antifungal agents may also decrease pathogenicity by altering the presentation of virulence factors, as described above. Furthermore, defects in morphogenesis caused by these drugs may weaken the ability of pathogens such as *C. albicans* to grow invasively or form biofilms. Even subtle defects in morphogenesis can influence pathogenicity by causing abnormal cell wall synthesis that exposes  $\beta$ -glucans and allows them to be recognized by the immune system (95).

Several possibilities exist for developing new drugs that target lipid rafts and SRDs. For example, amphotericin analogs could be linked to inhibitor molecules in order to target novel therapeutic agents to neutralize virulence functions at hyphal tips. It may also be possible to identify inhibitors that disrupt SRDs. An example of this is the disruption of the SRDs in *A. nidulans* by the HSAF dihydromaltophilin, which prevents stabilization of the axis of polarized hyphal morphogenesis (49, 67). This agent does not affect *S. cerevisiae*, which does not encode a homolog of the *BarA* protein. Therefore, similarly selective agents could have value for human fungal pathogens.

## RELATIONSHIP OF SRDs TO OTHER PLASMA MEMBRANE DOMAINS IN FUNGI

Many eukaryotic cells contain other types of plasma membrane domains that play important roles in plasma membrane

organization and function, such as those defined by tight junctions in epithelial cells. In the case of fungi, studies on *S. cerevisiae* have identified at least two other types of plasma membrane domains, septin barriers and eisosomes. Septin barrier domains are formed by septin proteins during budding and cytokinesis. Eisosomes are static sites of endocytosis that appear as punctate structures distributed across the plasma membrane. These specialized types of plasma membrane domains in *S. cerevisiae* are described below to compare their functions with those of SRDs.

**Septin barrier domains at the bud neck and the cytokinetic ring.** The septin proteins were identified in *S. cerevisiae* as peripheral membrane proteins that form a ring on the inner surface of the plasma membrane at the bud neck (20, 30). The septin ring acts as a scaffold to recruit proteins needed for cytokinesis and cell septation. Septins were subsequently shown to mediate a barrier function between the daughter and mother cells that blocks diffusion of integral membrane proteins, actin patches, exocyst and polarisome components, and Lte1, which is the guanine nucleotide exchange factor for the TEM1 GTPase that promotes exit from mitosis (7, 14, 89). Later in the cell cycle, the septin ring splits, and the two rings act as barriers to restrict proteins involved in cytokinesis and septum formation to the neck of the bud (19). A more diffuse type of septin ring also forms at the neck of pheromone-induced mating projections (26, 41, 42), but it has not been reported whether this ring plays a role in forming the SRDs at these sites. Interestingly, SRDs form at sites of septin rings in rod-shaped *S. pombe* and in hyphae of *C. albicans* and *A. nidulans* (55, 67, 92). In *C. albicans*, the SRD at the septation site was initially present on the side toward the actively growing hyphal tip, suggesting perhaps that septins can act as a barrier to prevent diffusion of lipids. In *S. pombe*, septin mutants did not affect the formation of the SRD, indicating that they are not necessary for its formation or localization in fission yeast (87). Thus, further work will be needed to determine the relationship between the septins and SRDs.

**Eisosomes.** *S. cerevisiae* plasma membrane proteins are present in distinct lateral subdomains, which indicates another form of plasma membrane organization (33, 53, 54). One domain has been termed the eisosome, which refers to large immobile complexes in the plasma membrane that are sites of endocytosis (93). The eisosome proteins are present in a punctate pattern that appears as dots distributed throughout the plasma membrane. Three proteins were identified in eisosomes, Pil1, Lsp1, and Sur7. Two of them, Pil1 and Lsp1, are closely related cytoplasmic proteins that function in endocytosis. Sur7 is a transmembrane protein that influences sphingolipid levels, which are also important for endocytosis (98). Additional studies have shown that Fur4 (H<sup>+</sup> symporter), Can1 (arginine permease), and Tat2 (tryptophan permease) colocalize with Sur7 in punctate spots (33, 53). These punctate spots, which correspond with eisosomes, are thought to represent a distinct lateral domain, since Can1 and Pma1 were found in nonoverlapping regions of the plasma membrane (54). Both Can1 and Pma1 are present in DRMs, so it was suggested that these proteins occupy distinct lipid raft domains. Interestingly, filipin staining was stronger at sites of Sur7-GFP localization, and this staining pattern was dependent on the Pil1 protein that is needed for eisosome formation

(33). However, it is not known if there is any relationship between eisosomes and SRDs.

## CONCLUDING COMMENTS AND FUTURE QUESTIONS

The discovery of SRDs in fungi is intriguing because of their potential to play important roles in plasma membrane organization, structure, and function. However, further work is needed to determine the specific function of these domains in vivo. At present, it is not clear if they act as organizing centers for proteins, as lipid domains with special biophysical properties, or both. Further progress in studying these domains will greatly benefit from the development of new approaches for studying membrane domains, such as those being developed for the analysis of lipid rafts. It will also be interesting to determine whether SRDs play similar roles in different fungi in view of the differences in the formation of SRDs at sites of septation in *S. pombe* versus the sites of polarized apical growth in *S. cerevisiae* mating projections and at hyphal tips in *C. albicans* and *A. nidulans*. Other significant avenues of study for these SRDs are to determine their roles in presenting virulence factors during fungal pathogenesis and as possible targets for the development of new antifungal therapies. The discovery of these SRDs in a broad range of evolutionarily diverse fungi also suggests that it is likely similar domains will be found in other organisms.

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