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Insights into eisosome assembly and organization

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Eisosomes, large protein complexes that are predominantly composed of BAR-domain-containing proteins Pill and its homologs, are situated under the plasma membrane of ascomycetes. A successful targeting of Pill onto the future site of eisosome accompanies maturation of eisosome. During or after recruitment, Pill undergoes self-assembly into filaments that can serve as scaffolds to induce membrane furrows or invaginations. Although a consequence of the invagination is likely to redistribute particular proteins and lipids to a different location, the precise physiological role of membrane invagination and eisosome assembly awaits further investigation. The present review summarizes recent research findings within the field regarding the detailed structural and functional significance of Pill on eisosome organization.

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1. Introduction: Central organizers of eisosome

The plasma membrane is a diverse structure that manages the traffic of materials in and out of the cell. It consists of dynamic compartments where different functions take place. These compartments exist over a wide range of spatial and temporal scales (Lingwood and Simons 2010). The budding yeast plasma membrane has been subdivided into three distinct membrane compartments: the membrane compartment of Can1 (MCC), the membrane compartment of TORC2 (MCT) and the membrane compartment of Pmal (MCP) (Young et al. 2002; Malinska et al. 2003; Grossmann et al. 2007; Berchtold and Walther 2009; Brach et al. 2011). MCC and MCT are found as distinct domains, whereas the MCP is found throughout the membrane except where a MCC or a MCT exists (Grossmann et al. 2007; Berchtold and Walther 2009). The cytoplasmic side of MCC appears to be closely associated with the protein cluster containing thousands of copies of Pill and its homolog Lsp1 (Grossmann et al. 2007; Frohlich et al. 2009). The cytosolic cluster was so named 'eisosome' (meaning portal for body in Greek) by Walther and coworkers because of its implication in endocytosis (Walther et al. 2006). It has been reported that in total 22 proteins (9 transmembrane and 13 cytoplasmic proteins) are members of MCC-associated proteins (Grossmann et al.

2008; Deng et al. 2009). Since the fluorescence microscope does not provide sufficient spatial resolution to differentiate between MCC and eisosome, Stradalova et al. (2009), using electron microscopy approaches, showed the ultrastructure of MCC that structurally resembles a furrow-like endocytic invagination. They showed that the transmembrane MCC marker Sur7 was found to localize to the superficial parts of the invaginated plasma membrane, while the cytosolic eisosome marker Pill was detected in the deeper parts of the furrow-like invagination mainly around the curved bottom of the structure (Stradalova et al. 2009). Upon deletion of budding yeast PIL1, GFP-fused eisosome (Lsp1, Slm1, Pkh1 and Pkh2) and MCC (Sur7, Nce102 and Can1) markers are localized to a few bright peripheral clusters called eisosome remnants, instead of localizing to the cell cortex evenly in a punctuated pattern (Walther et al. 2006, 2007; Frohlich et al. 2009; Grossmann et al. 2008; Kamble et al. 2011), indicating Pil1 is essential for the structural integrity of eisosome and MCC. Although Pil1 exhibits high levels of sequence homology among fungi, it seems that the function of Pill has not been completely conserved between budding yeast and other fungal species (Vangelatos et al. 2010; Kabeche et al. 2011; Reijnst et al. 2011; Seger et al. 2011). For instance, unlike budding yeast Pil1 (ScPil1) implicated in endocytosis (Walther et al. 2006; Murphy et al.

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2011), *Ashbya gossypii* Pil1 (*Ag*Pil1) and *Candida albicans* Pil1 (*Ca*Pil1) are required for polar growth and essential for cell growth, respectively (Reijnst *et al.* 2011; Seger *et al.* 2011). The fact that the fission yeast orthologs of Slm1 and Sur7 do not colocalize with the MCC/eisosome marker Pil1 or depend on Pil1 for their localization in *S. pombe* also suggests the functional divergence of Pil1 in organizing MCC/eisosome between budding yeast and fission yeast (Kabeche *et al.* 2011).

2. Up-to-date model for Pil1 assembly onto eisosome

Recent studies discovered that both Pill and Lsp1 contain a BAR domain, structurally most similar to N-BAR domain which is also found in amphiphysins (Olivera-Couto et al. 2011; Ziolkowska et al. 2011). BAR domains consist of a coiled-coil of three long alpha-helices that dimerize to form a banana-shaped domain with a positively charged concave surface. Cationic residues on the positive surface interact with anionic membrane lipids via electrostatic interactions (Frost et al. 2009). Owing to the rigid concave shape of the surface, the BAR domain can induce membrane curvature, required for a wide range of cellular processes such as endocytic invagination and cell motility (Gallop and McMahon 2005; Suetsugu et al. 2010). The BAR-domaincontaining eisosome proteins Pill and Lsp1 are capable of self-assembly, binding lipid membranes, preferably those containing $PI(4,5)P_2$ (or PIP_2), and deforming them into long tubules (Kabeche et al. 2011; Karotki et al. 2011; Olivera-Couto et al. 2011). As expected, the positive surface patch of theoretical Pil1 homology modelling on the structural template of Lsp1 turned out to be important for its membrane binding and normal eisosome organization (Karotki et al. 2011; Olivera-Couto et al. 2011; Ziolkowska et al. 2011). Karotki et al. (2011) further proposed that, in addition to the positive residues on the concave surface, an N-terminal segment of Pil1 or Lsp1 might facilitate their efficient membrane binding and/or bending.

3. Other protein factors implicated in organizing eisosome

Beside the essential eisosomal organizing factor Pil1, according to a genome-wide screen for deletion mutants that show an alteration of Pil1 localization, a wide range of genes (a total of 88 genes), functioning ranging from endocytosis and vesicle trafficking to metabolism, appear to be involved in the organization of the eisosome (Frohlich *et al.* 2009). Additionally, a similar genome-wide screen for deletion mutants that shows an altered Can1 (MCC marker) pattern identified 27 genes (Grossmann *et al.* 2008). Given eisosome and MCC are tightly associated with each other, it is striking

to note that there was such a low level of consistency between those screens that in both screens NCE102, SUR4 and MNN10 genes were the only overlapping ones that affect both MCC and eisosome organizations (Grossmann et al. 2008; Frohlich et al. 2009). One straightforward explanation for this would be that the recruitment of MCC and eisosome markers is differently regulated by non-overlapping factors in general, perhaps pointing out a subtle functional difference between MCC and eisosome. Except for Mnn10, the two other gene products, Sur4 and Nce102, are likely to influence eisosome organization through sphingolipid signalling by altering the level of sphingolipids and serving as a sphingolipid sensor in the plasma membrane, respectively (Han et al. 2002; Paul et al. 2006; Frohlich et al. 2009). Interestingly, it was found that the recruitment of Nce102, a bona fide MCC transmembrane protein, depends on the availability of sphingolipids at MCC where Nce102 acts as a negative regulator of Pkh kinases in Pill phosphorylation (Frohlich et al. 2009). Although more than 100 genes/proteins, to date, have been presented by the genetic screens to be important for MCC/eisosome organization, many more await further identification. This is because all essential genes (~18% of the yeast genome) and a considerable number of proteins that show functional redundancy with other proteins in the yeast genome were excluded from the lists. For the latter case, Slm1 and its homolog Slm2 share the same function in organizing the eisosome as shown by Kamble et al. (2011), but Slm2 is not included on either list of genome-wide screens. Slm1/2 were originally characterized as PIP₂ binding proteins through their C-terminal PH domain (Audhya et al. 2004; Fadri et al. 2005) and later found to be eisosome components (Grossmann et al. 2008: Kamble et al. 2011). A first clue to the targeting of Slm proteins to eisosome was provided by a recent microscopic study that clearly showed the central Slm (showing some sequence similarity to F-BAR) and PH domains are essentially required for eisosome targeting, but not by PH or F-BAR alone (Olivera-Couto et al. 2011), and thus underlining the significance of BAR domain on eisosome targeting.

4. Reversible phosphorylation and its consequences on eisosome organization

Another set of redundant genes that are not included in the genome list but important for eisosome organization are *PKH1* and *PKH2* (Walther *et al.* 2007), encoding the two mammalian PDK1 homologs Pkh1 and Pkh2 (Casamayor *et al.* 1999). The serine/threonine kinases Pkh1/2 are physically associated with the eisosome, and Pil1 and Lsp1 have been shown to be Pkh substrates *in vitro* (Zhang *et al.* 2004; Walther *et al.* 2007). Pil1 phosphorylation *in vivo* has been proposed to be an important regulator that affects eisosome assembly, since change in the phosphorylation level of Pil1

leads to defects in eisosome organization (Walther et al. 2007; Luo et al. 2008). However, controversy lies in the precise role of Pkh1/2-mediated phosphorylation of Pil1 on eisosome assembly. Walther et al. (2007) originally showed that hyperphosphorylation of Pill by elevated Pkh1 and Pkh2 protein levels leads to a severe defect in Pil1 assembly on eisosome (Walther et al. 2007). In support, they observed that the phospho-mimicking *pil1(4D)* mutant in which serines 45, 59 and 230, and threonine 233 were changed to Aspartic acid (D) was dispersed mainly into the cytoplasm, thereby concluding that Pill is dephosphorylated when eisosome-bound and released upon phosphorylation to the cytoplasm (Walther et al. 2007) (figure 1A). The purified recombinant *pil14(D)* protein indeed was less competent in self-assembly compared to wild-type Pil1, pointing to the role of Pil1 phosphorylation that leads to impairment of Pil1 assembly on eisosome (Karotki et al. 2011). In agreement, Deng et al. (2009) reported that the decreased Pill-GFP fluorescence level in the cytoplasm correlates with the dephosphorylation of Pil1, especially at the sites of Ser-230

(A) (Walther et al. 2007)



Figure 1. The controversy of the roles of reversible phosphorylation of Pill in eisosome organization. (A) The model suggests that Pill dephosphorylation promotes its recruitment to the eisosome. (B) The other model proposes that dephosphorylation of Pill is required for its disassembly from the eisosome.

and Thr-233. However, two lines of evidence argue against the notion that the dephophorylated Pill associates with eisosome. First, according to Luo et al. (2008), a nonphosphorylable Pil1 mutant in which multiple phosphorylation sites (up to six) were mutated to Alanine was mislocalized to the cytoplasm, suggesting that eisosome formation requires phosphorylation of Pill (figure 1B). Consistent with this observation, the addition of KP-372-1, which inhibits Pkh1/2 kinases, caused the increase of the pool of dephosphorylated Pil1, primarily situated in the cytoplasm, supporting the view that Pill phosphorylation is required for eisosome assembly (Baxter et al. 2011). At the moment it is not clear how different groups of researchers obtained two opposing results using similar Pil1 mutants. Nonetheless, the notion of reversible Pil1 phosphorylation and dephosphorylation controlling structural integrity of eisosome is highly acceptable. The existence of a phosphatase system involved in Pill dephosphorylation was suggested by Deng et al. (2009) as they observed an abrupt decrease in Pill and Lsp1 phosphorylation level during cell cycle. One potential candidate phosphatase for dephosphorylation of Pill would be calcineurin, which is known to be directly dephosphorylate another eisosome members Slm1/2 (Bultynck et al. 2006). If so, the consequence of Pill and Lsp1 dephosphorylation by calcineurin or other phosphatases yet to be identified on eisosome assembly should be further investigated to establish the biochemical recruitment mechanisms of Pil1.

5. Suggested roles of membrane lipids on eisosome

The plasma membrane is made up of several types of lipids, and these lipids play an important role in the recruitment of proteins. Sphingolipids consist of long saturated acyl chains that allow them to pack together tightly in the membrane (Brown and London, 2000). The micro-domain of the plasma membrane enriched in sphingolipids and cholesterol (Lemaire-Ewing et al. 2011) is often called a lipid raft, which is essential in cell signalling and protein trafficking (Staubach and Hanisch 2011). An early detergent lipid raft extraction assay showed that Can1, an integral MCC protein, localizes in lipid rafts and that a reduction in sphingolipids leads to the disruption of Can1 targeting (Malinska et al. 2003). The recruitment of another integral MCC protein Nce102 was also found to be dependent on a higher level of sphingolipids (Frohlich et al. 2009). Likewise, decreased content of sphingolipids in *lcb1-100* mutant (Zanolari *et al.*) 2000) or treatment of myriocin that inhibits sphingolipid synthesis resulted in a loss of the MCC-associating cytoplasmic eisosome carrying Pil1 (Walther et al. 2007; Luo et al. 2008; Frohlich et al. 2009). These results apparently support the notion that membrane lipids are required for correct intracellular targeting of raft-associated proteins (Hearn et al. 2003). $PI(4,5)P_2$ (PIP₂) is a minor yet dynamic phospholipid

component of the plasma membrane which assists in the recruitment of a wide range of proteins to the plasma membrane (Strahl and Thorner 2007). Indeed, this was the case for Can1 targeting to the periphery of the plasma membrane; in mss4^{ts} temperature sensitive mutant that has ~10% of normal amount of PIP₂ (Desrivieres et al. 1998), the targeting of MCC protein Can1 was found to be impaired (Daquinag et al. 2007). However, the phenotypic defect in Can1 targeting in the mutant appears to be a secondary effect, perhaps caused by an actin cytoskeleton defect. More recently, Karotki et al. (2011) established a direct role of PIP2 in organizing eisosome in vivo. In lower levels of PIP₂ (in mss4^{ts}), Pil1 progressively dissociated from the plasma membrane, mainly due to the fact that the probability of its direct interaction with PIP₂ significantly decreases while in an opposing condition (in *sjl1* Δ *sjl2* Δ) with higher chance, thus forming enlarged Pill puncta (Karotki et al. 2011) (E Murphy, unpublished). The fungal-specific sterol, ergosterol, enriched in lipid rafts, is another major lipid that plays a role in aiding the targeting of lipid-raft associating protein factors such as Gas1 and Pma1 (Bagnat et al. 2000). At least notable, but not complete, mistargeting of the MCC marker Can1 was observed in the condition where ergosterol levels were reduced ($erg6\Delta$ and $erg24\Delta$) (Malinska *et al.* 2003). In contrast, unpublished data from Walther laboratory (Frohlich et al. 2009), including ours, strongly argue for a negligible role of ergosterol in targeting of the eisosome protein Pil1, since GFP fused Pil1 in those sterol mutant strains was still properly targeted to the plasma membrane.

6. Physiological functions of eisosome

It is curious that the liquid phase endocytic marker FM4-64 shows partial colocalization with Pil1 (Walther et al. 2006) (E Murphy, unpublished), and that the extent of spatial overlap between the eisosome and FM4-64 appears to increase as aberrant eisosome aggregates are formed by the loss of Pil1. However, it is now generally accepted that the eisosome does not mark receptor-mediated endocytic (RME) sites, on the basis of several lines of evidence: (1) the plasma membrane MCC protein Sur7, which colocalizes to the eisosome (Malinska et al. 2004; Walther et al. 2006), does not colocalize with RME sites carrying Rvs161 and Ede1 (Grossmann et al. 2008); (2) none of Abp1- and Sla1-GFP endocytic sites colocalized with Pil1-mCherry (Brach et al. 2011) and (3) Slm1, an eisosome marker, displayed only rare colocalization with Abp1-GFP, raising the possibility of random colocalization between them (Kamble et al. 2011). Nevertheless, what has emerged clearly is that a stable eisosome structure at the cell cortex is required for efficient receptor-mediated endocytosis occurring in the vicinity of the eisosome. This notion is supported by the observation that the rate of Ste3-mediated (a factor receptor) endocytosis

to the vacuole in $pill\Delta$ and $lspl\Delta$ cells decreased significantly when compared to that of WT cells (Walther et al. 2006). Notably, the efficacy of receptor-mediated endocytosis dropped significantly in PIL1-lacking cells in which the synaptojanins (Sil1/2) were severely mislocalized to the cytoplasm (Murphy et al. 2011). In particular, Sjl2 is a major plasma membrane phosphoinositide phosphatase that hydrolyses phosphates of PIP₂ (Guo et al. 1999), and a transient reduction of PIP₂ or the change of PIP₂ levels in a temporal manner via Sil2 at endocytic sites is known to be critical for the efficiency of endocytosis (Sun et al. 2007; Toret et al. 2008). Thus, the failure of Sjl2 targeting to endocytic sites, most likely a side effect caused by loss of Pill (Murphy et al. 2011), might lead to a cascading failures in which an unsuccessful spatiotemporal regulation of PIP₂ levels, triggers the failure of endocytosis. At the moment it is plausible to propose that Pill is directly or indirectly involved in fine-tuning to regulate membrane phospholipid homeostasis. Furthermore, it appears that MCC/eisosome is a protective area that provides stability for the proteins localized there; the MCC component Can1 (transmembrane arginine transporter) was dissipated throughout the cell membrane and endocytosed at a much faster rate in *pill* Δ cells than it is in WT cells, most likely due to the lack of the protective barrier in the mutant strain (Grossmann et al. 2008). It is yet important to note that the protective role of eisosome for its protein component from endocytosis seems not to be unanimously supported, based on the finding that the endocytosis rates of Can1 in $pill\Delta$ and WT cells were essentially the same (Brach et al. 2011).

7. Concluding remarks

Work over the last 5-6 years has been focused on the contributions of Pil1 and its homologs in ascomycetes to MCC/ eisosome organization. As discussed, a new, and we believe potentially very important piece of information regarding the structure and function of Pill, has come with the recent finding that Pil1, containing a BAR domain, is able to selfassemble into filaments that serve as scaffold to reorganize membrane into an invagination. While evidence is mounting for a dynamic Pill assembly required for eisosome/MCC organization, precisely how these Pil1 fibres are used to help provide the force required for the invagination is not understood fully. In light of finding that inactivation of the PKC kinases Pkh1 and Pkh2 leads to the formation of extended net-like eisosome carrying Pil1, one can postulate that there must be a primary signal pathway with Pkh1/2 kinases that serve as a negative regulator of Pill assembly, as well as certain factors, including Nce102 that plays opposing regulatory roles. Therefore, in the future it is of great interest to understand the new and detail functions of already-known and yet-to-be-identified factors that influence Pil1 assembly *in vitro*, and to further elucidate the physiological relevance of the factors in eisosome assembly *in vivo*. Along with at least learning more details about Pil1-lipids interaction, it is highly likely in the next few years to gain a better understanding of how the interaction of Pil1 with transmembrane MCC and cytosolic eisosome proteins regulates MCC/eisosome organization. All together, these studies during the coming years should provide even greater insights to the understanding of biological membrane organization and function.

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