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Lipid Droplet–Peroxisome Connections in Plants

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Abstract

Lipid droplets (LDs) are the principal subcellular sites for the storage of triacylglycerols (TAGs), and in plants, TAG degradation requires metabolism in peroxisomes. This metabolic cooperation includes TAG hydrolysis by the sugar-dependent 1 lipase located on the LD surface and the transfer of fatty acids into the peroxisome matrix by the peroxisomal membrane ATP-binding cassette transporter, PXA1. During seed germination, this process fuels heterotrophic growth and involves the retromer-dependent formation of peroxisomal membrane extensions called *peroxules* that interact with LDs. Similar changes in membrane architecture are also observed during interactions of peroxisomes and LDs in yeast and mammalian cells, despite differences in the molecular components required for their connections. Proteins directly involved in LD–peroxisome membrane contact site formation in plants have not yet been identified, but the connection between these two organelles is dependent upon PXA1, which contains a cytoplasmic exposed FFAT (two phenylalanines in an acidic tract)-like motif capable of interacting with vesicle-associated membrane protein-associated proteins (VAPs). Indeed, the identification of several VAPs in plant LD proteomes supports the premise that a VAP-PXA1 connection might be part of a functional tethering complex that connects these two organelles, although other types of interactions are also possible. Overall, such connections between peroxisomes and LDs would allow for efficient transfer of lipophilic substrates from LDs to the peroxisome matrix in plant cells, similar to how VAPs participate in lipid transfer reactions between other subcellular compartments in eukaryotic systems.

Keywords

lipid droplet, lipid, FFAT (FFAT-like), peroxisome, vesicle-associated membrane protein-associated protein

The synthesis of lipids is one of the most efficient ways for cells to store energy; however, due to their physicochemical properties, storage lipids are incompatible with the aqueous environment of the cytoplasm. Consequently, eukaryotic cells (and some prokaryotes) have evolved compartments able to emulsify and stabilize storage lipids in the cytoplasm, namely, lipid droplets (LDs). In plant cells, these subcellular compartments were described initially in seeds as spherosomes, lipid bodies, oil bodies, or oleosomes (Huang, 1992), but all of these terms refer to the same LD compartment.

Unlike other organelles which are bounded by a bilayer of phospholipids, LDs are composed by a phospholipid monolayer surrounding a hydrophobic core of storage lipids formed mainly from triacylglycerols (TAGs) and sterol esters (StEs; Zweytick et al., 2000; Murphy, 2001; Chapman et al., 2012). In plants, LDs are most prevalent in oilseeds, pollen, and oleaginous fruits, but they are also found in essentially all cells/tissues and have functions beyond simply the storage of carbon and energy (Aubert et al., 2010, 2011; Brocard et al., 2017). Furthermore, depending upon

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the cell/tissue type, LDs are coated with a diverse array of proteins that promote their stability and/or otherwise mediate their cellular functions (Pyc et al., 2017; Chapman et al., 2019).

While lipids are stored primarily as TAGs in LDs, their utilization in plants involves their β-oxidation in peroxisomes (Chapman et al., 2012; Rinaldi et al., 2016). The peroxisomal compartment in plant (and yeast) cells is considered the major, if not the exclusive, location of fatty acid (FA) β-oxidation (reviewed in Poirier et al., 2006). The peroxisomal membrane separates the cytoplasm from the matrix of the peroxisomes, where several metabolic pathways take place, including FA β-oxidation, the glyoxylate cycle (in seedling endosperm and cotyledons, and senescing leaves), and photorespiration (in mature leaves; Titus and Becker, 1985; Nishimura et al., 1986; Nito et al., 2007). During seed germination and postgerminative seedling growth, the main role of peroxisomes (previously called glyoxysomes in seedlings, Pracharoenwattana and Smith, 2008) is to breakdown, via the β-oxidation pathway, FAs from TAGs to acetyl-CoA, which is subsequently converted by the glyoxylate cycle to succinate for the synthesis of carbohydrates (Gruber et al., 1970; Titus and Becker, 1985; Rinaldi et al., 2016). These carbohydrates are then transported to other parts of the seedling to fuel postgerminative growth prior to photosynthetic establishment.

The metabolic cooperation between LDs and peroxisomes for lipid mobilization is supported at the subcellular level by the intimate connections that are well known to exist between these two organelles in plant cells (Figure 1; Huang et al., 1983; Hayashi et al., 2001; Thazar-Poulot et al., 2015). Here, we describe these close contacts between plant LDs and peroxisomes and their requirement to support not only the energetics of seedling establishment in oilseeds but also broader roles in nonseed tissues. We suggest that these connections are likely stabilized by membrane contact sites (MCSs) with some similarities to those recently described in yeast and mammalian cells (Binns et al., 2006; Chang et al., 2019).

LD–Peroxisome Interactions

MCSs allow for communication and transfer of components between two organelles by bypassing vesicular trafficking (reviewed in Prinz, 2014; Wu et al., 2018). MCSs are well described in the interactions of endoplasmic reticulum/plasma membrane (ER/PM), ER/Golgi, ER/mitochondria, and ER/chloroplast but also occur between organelle membranes other than the ER, for example, peroxisome–mitochondria and mitochondria–chloroplast (Levine and Loewen, 2006; Prinz, 2014; Michaud and Jouhet, 2019). All of these MCSs occur between two membrane bilayers and involve similar mechanisms with protein complexes acting to tether the two compartments together (Prinz, 2014; Eisenberg-Bord et al., 2016; Wu et al., 2018). The contacts between membranes can be formed, for example, by a protein-conducting channel, like the TOM and TIM23 complexes that tether the outer and inner mitochondrial membranes (Chacinska et al., 2005; Mokranjac et al., 2005). Alternatively, MCSs can be stabilized through an enzyme-substrate complex, such as in the tethering of ER via protein tyrosine phosphatase 1B to the PM through a receptor tyrosine kinase (Haj et al., 2002; Hernández et al., 2006). MCSs can be also established by lipid transfer proteins complexed with vesicle-associated membrane protein-associated proteins (VAPs) tethering proteins, such as in the Golgi-localized ceramide transfer (CERT) protein and the *trans*-Golgi network (TGN)-localized oxysterol-binding protein (OSBP) that transfer ceramides and sterols, respectively (Hanada et al., 2009; Mesmin et al., 2017). Moreover, partial fusion, also called hemifusion, of membranes is also considered an MCS, as observed between the ER and chloroplasts (Mehrshahi et al., 2013; Michaud and Jouhet, 2019).

When considering MCSs that connect LDs to other organelles, the LD surface is uniquely composed of a phospholipid monolayer, so a connection between LDs and other organelles likely exhibits some differences compared with those between two membrane bilayer-containing organelles. Given their diverse functions, LDs might also interact with multiple organelles at the same time. Indeed, a recent study highlights how LDs are recruited to ER-PM contact sites by a protein called snazarus in *Drosophila melanogaster* (Ugrankar et al., 2019). Snazarus contains several distinct domains, including a Phox (PX) domain that binds to phosphatidylinositol 3-phosphate, a C-nexin domain, and a transmembrane domain (TMD). The TMD localizes snazarus in ER membranes, while the protein's PX domain binds to phosphatidylinositol 3-phosphate-enriched regions of the PM, and finally, the C-nexin domain contains an LD targeting signal (Ugrankar et al., 2019). Consequently, snazarus is able to interact with three different membrane surfaces at once, thereby tethering LDs, ER, and PM all together (Ugrankar et al., 2019).

Historically, microscopic evidence of connections between LDs and other compartments in plant cells has been known since the early 1970s when the use of the transmission electron microscopy became widespread (Gruber et al., 1970; Trelease et al., 1971, 1974). For example, Gruber et al. (1970) were among the first to describe peroxisomes (referred to at the time as microbodies) with crystalline inclusions that were closely associated with LDs in plants (see example in Figure 1a). They also localized the enzymes needed for

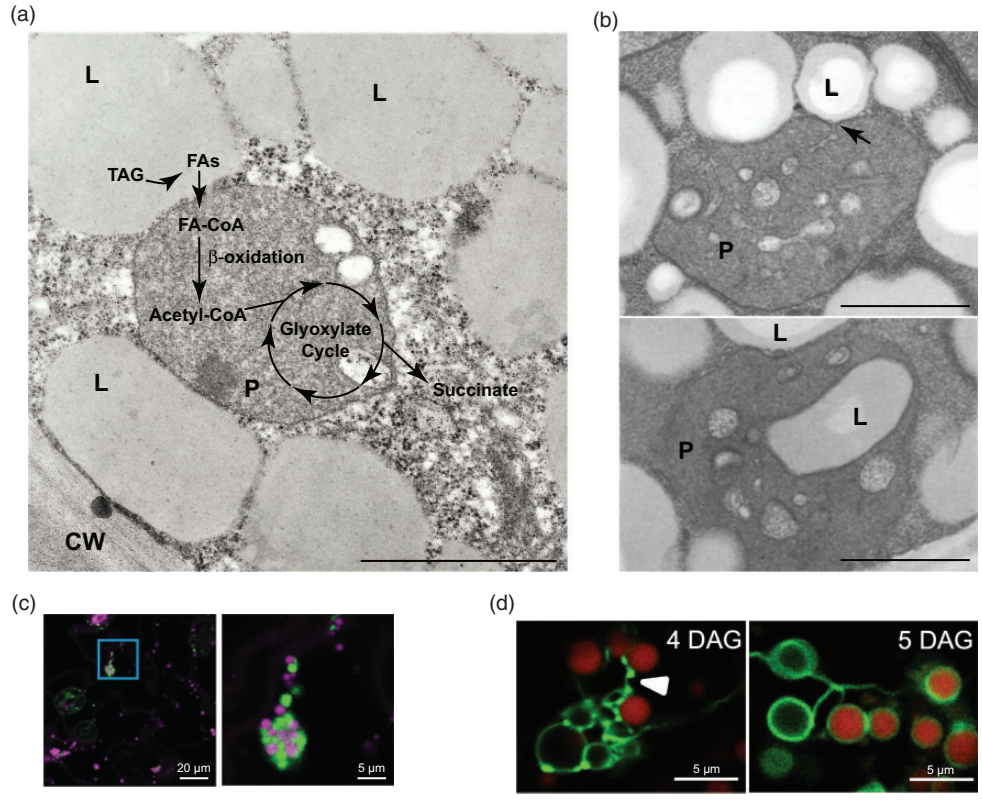

Figure 1. LD–Peroxisome Interactions in Plant Cells. Panel A: Electron micrograph conveying the interaction between LDs and peroxisomes (glyoxysomes) in a cotyledon cell of a cucumber seedling. CW = cell wall; FAs = fatty acids; L = lipid droplet; P = peroxisome; TAG = triacylglycerol. Scale bar = 1 μm. Courtesy of R.N. Trelease (Arizona State University). Panel B: Electron micrographs of LDs and peroxisomes in a cotyledon cell in an *Arabidopsis thaliana ped1* mutant seedling. Note the large, LD-like and membranous structures inside the peroxisome in the top and bottom panels; black arrow in the top panel indicates an example of an invagination of the peroxisomal membrane at the site of an LD–peroxisome connection. Scale bar = 1 μm. Reprinted with permission from Springer Publishing Company (figures from Hayashi et al., 2001). Panel C: Confocal micrographs of LDs and peroxisomes in a cotyledon cell of an *A. thaliana pex26* mutant seedling that is stably expressing the peroxisomal (matrix) marker protein GFP-PTS1 (Peroxisomal Targeting Sequence 1; green). LDs are stained with Nile red and false colored magenta. Box in left panel represents the portion of the cell containing the cluster of peroxisomes and LDs shown at higher magnification in the panel to the right. Reprinted with permission from John Wiley and Sons (figure from Gonzalez et al., 2017). Panel D: Confocal micrographs of peroxisomes, peroxules, and LDs (stained with Nile red) in *A. thaliana* seedlings (4 or 5 DAG) stably expressing GFP-SDP1. At 4 DAG, GFP-tagged SDP1 (green) is localized to the peroxisome membrane and peroxules (white arrowhead), which are reported to deliver SDP1 to LDs. At 5 DAG, note the localization of GFP-SDP1 which surrounds the Nile red-stained LDs. Reprinted with permission of corresponding author, Thierry Gaude, and the *Proceedings of the National Academy of Sciences of the United States of America* (figures from Thazar-Poulot et al., 2015). DAG = days after germination.

the glyoxylate cycle in the peroxisomal fractions of developing seedlings, a stage when peroxisomes were frequently observed to be in intimate association with LDs. Research over the next 40 years continued to elucidate the function of peroxisomes in plants, their protein/enzyme constituents, and the molecular mechanisms underlying their biogenesis and turnover, as well as their relationships with other organelles, including LDs during postgerminative seedling growth (reviewed in Hu et al., 2012; Reumann and Bartel, 2016; Kao et al., 2018). However, relatively few studies have explored the physical connections between peroxisomes and LDs in plants (Oikawa et al., 2019), and no MCS complexes at LD–peroxisomes connections have been described thus far.

One pertinent aspect of the LD–peroxisome connection in plants is the enlargement of peroxisomes (glyoxysomes) during lipid mobilization in seedlings, the membrane expansion of which is partly accommodated by the transfer of membrane lipids derived from the turnover of LDs (Chapman and Trelease, 1991), although the mechanism for this lipid transfer process is unknown. Also notable are the changes in peroxisome morphology that occur during LD–peroxisome interactions, which are more dramatic when the β-oxidation pathway for FA breakdown is disrupted (Hayashi et al., 2001; Shimada et al., 2018). For instance, in loss-of-function mutants of 3-ketoacyl-CoA thiolase (*ped1*), which plays an important role in peroxisomal β-oxidation, peroxisomes in seedlings become highly

vacuolated and contain in their matrix what appear to be membrane-bound LD remnants (see Figure 1b [top and bottom panels]; Hayashi et al., 2001). Peroxisomes in *ped1* mutant seedlings also possess unique, internal invaginations of the peroxisomal boundary membrane that are often evident at sites adjacent to LDs (Figure 1b [top panel]) and were proposed to represent a disrupted mechanistic exchange of lipid metabolites from LDs to peroxisomes (Hayashi et al., 2001). Similarly, the subcellular distribution of peroxisomes and their association with LDs is conspicuously altered in other mutants of FA β-oxidation enzymes (Rinaldi et al., 2016) or in mutants of the peroxisomal biogenetic protein factors, peroxin (PEX) 6 and PEX26 (Gonzalez et al., 2017). In the latter, peroxisomes often coalesce into aberrant clusters that surround LDs and, compared with wild type, the LDs are mobilized more slowly during postgerminative growth (refer to Figure 1c). Again, although no MCSs per se have yet to be identified in any of the aforementioned processes involving lipid delivery to peroxisomes, it seems likely that such mechanisms exist to help facilitate the large flux of FAs from LDs to peroxisomes during normal seedling growth.

Retromer-Dependent Re-localization of SDP1 From Peroxisomes to LDs

While the mechanisms participating in the interaction of LDs and peroxisomes in plants remain poorly understood, evidence from recent studies of the dynamic morphology of peroxisomes has revealed some interesting aspects of the process. During lipid mobilization associated with postgerminative seedling growth, extensions of peroxisomes were observed that associated with LDs (see Figure 1d; Thazar-Poulot et al., 2015). These peroxisome extensions, called *peroxules*, were first described based on live-cell imaging of fluorescent protein-labeled peroxisomes (Cutler et al., 2000; reviewed Muench and Mullen, 2003) and subsequently shown to emanate from peroxisomes in response to hydroxyl stress along paths defined by ER tubules (Sinclair et al., 2009). The underlying mechanism for these peroxule–ER connections is unclear. At the morphological level, peroxules resemble the dynamic extensions from other plant organelles, like stromules from chloroplasts (Scott et al., 2007) and matrixules from mitochondria (Logan, 2006), although only a few comparative reports exist (Mathur et al., 2012). Nonetheless, the formation of peroxules during germination is known to be dependent on the activity of a membrane-bound retromer complex (Thazar-Poulot et al., 2015). This complex is evolutionarily conserved in eukaryotes and composed of a heterodimer of sorting nexin and a trimer of vacuolar protein sorting (VPS) proteins, of which eight different sorting nexin proteins and six different VPS proteins exist in *Arabidopsis*. The overall composition of the retromer complex varies in order to mediate the trafficking of vesicles from endosomes to the TGN and/or alterations in membrane curvature (Heucken and Ivanov, 2018). In *Arabidopsis*, a loss-of-function mutant of one of the core retromer components, *vps29,* abolished the formation of peroxules in seedlings (Thazar-Poulot et al., 2015). Moreover, the *vps29* mutant exhibited an altered LD phenotype and a sucrose-dependent germination phenotype, demonstrating the importance of peroxules for lipid mobilization (Thazar-Poulot et al., 2015; Huang et al., 2019).

One of the roles of peroxules is proposed to be the delivery of the major plant TAG lipase, sugar-dependent 1 (SDP1), from peroxisomes to LDs. SDP1 is localized first on the cytoplasmic surface of the peroxisomal membrane, and then, during lipid mobilization in seedlings, SDP1 traffics to the LD surface via peroxules (Thazar-Poulot et al., 2015). SDP1 plays a key role in the initiation of TAG breakdown by hydrolyzing TAG to release free FAs (Eastmond, 2006). This represents the first step in conversion of stored lipids into carbohydrates (Eastmond, 2006; Quettier and Eastmond, 2009), and *Arabidopsis* seedlings lacking SDP1 are unable to grow in the absence of exogenously supplied sucrose (Eastmond, 2006; Kelly et al., 2011; Thazar-Poulot et al., 2015). Green fluorescent protein (GFP)-tagged SDP1 was localized to peroxisomes early in germination (i.e., 4 days after germination) and then observed to surround LDs during progression of postgerminative growth (at 5 days after germination; Figure 1d; Thazar-Poulot et al., 2015). However, in *vps29* mutant seedlings, there was a delay in the relocalization of GFP-SDP1 to the LD membrane, confirming that the retromer complex plays a role in the delivery of SDP1 from peroxisomes to LDs. *Arabidopsis sdp1* mutant seedlings displayed increased contacts between LDs and peroxisomes leading to the formation of aggregates of these organelles (Cui et al., 2016). Addition of sucrose to the growth media, however, reversed this LD–peroxisome clustered phenotype, emphasizing the importance of SDP1 and LD–peroxisome connections in the proper mobilization of FAs in support of postgerminative seedling growth (Cui et al., 2016).

Transfer of FAs From LDs to Peroxisomes

The hydrolytic activity of LD-localized SDP1 releases free FAs from LDs, and the next step requires transport of these FAs into the peroxisomal matrix for further metabolism through the β-oxidation and glyoxylate pathways (Huang et al., 1983; Poirier et al., 2006). Studies have demonstrated that the major transport

protein involved in the uptake of FAs into peroxisomes in plants is the peroxisomal membrane-bound ATP-binding cassette (ABC) transporter, PXA1 (also called comatose or peroxisomal defective 3 [PED3]; reviewed in Baker et al., 2015). *pxa1* loss-of-function mutants exhibit a sucrose-dependent seedling-establishment phenotype (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002) similar to the *sdp1* and *vps29* mutants described earlier. It also appears that PXA1 is a promiscuous protein capable of transporting a broad range of substrates, including FAs and signaling metabolites, into peroxisomes for their subsequent β-oxidation (Baker et al., 2015). Genetic evidence revealed that both *pxa1* and *sdp1* mutant seedlings have similar altered FA turnover phenotypes and display a reduction in TAG breakdown during postgerminative growth (Fan et al., 2017), emphasizing their potential cooperation in supporting a functional LD–peroxisome connection.

One interesting feature of PXA1 in plant peroxisomes is that the comparative gene identification-58 (CGI-58) protein was shown to interact directly with the nucleotide-binding domain 2 domain of PXA1 to modulate TAG breakdown and other PXA1-dependent signaling pathways (James et al., 2010; Park et al., 2013). CGI-58 is targeted specifically to peroxisomal membranes where it interacts with PXA1, and disruption of *CGI-58* gene expression (*cgi-58*) resulted in similar increases in TAG and LDs in leaves as that observed in leaves of *pxa1* mutant plants (James et al., 2010; Park et al., 2013). Notably, CGI-58 was not required for TAG breakdown during seedling establishment, unlike PXA1, which is essential for this process (Park et al., 2013). This latter observation suggests there might be differences in the interactions of LDs and peroxisomes in different plant cell types and/or that CGI-58 regulates PXA1 in leaves in manner that is not required in germinated seedling tissues. In fact, in guard cells, which control the stomatal aperture on the leaf surface, SDP1, CGI-58, and PXA1 cooperate together for the mobilization of TAGs from LDs, which provides the energy required for light-induced stomatal closure (McLachlan et al., 2016). Interestingly, in mammals, the CGI-58 homolog also modulates TAG homeostasis in a variety of cell types but does so, in part, by targeting to LDs and activating the adipose TAG lipase (Schweiger et al., 2006; Radner et al., 2010). Therefore, in plants (at least in leaves), CGI-58 appears to have evolved a different mechanism of action from animals that similarly results in regulation of lipid homeostasis. This mechanism includes an expanded role in modulation of substrate uptake by PXA1, likely accommodating the expanded substrate promiscuity of the plant peroxisomal ABC transporter.

Organismal Differences in LD–Peroxisome Connections

The specific molecular details for how LDs interact with peroxisomes in plants are unclear, but some information in yeast and mammalian cells suggests that different organisms may employ similar, but distinct mechanisms for mediating this important subcellular connection (summarized in Figure 2).

In yeast cells, the interaction between peroxisomes and LDs displays a unique morphology at the ultrastructural level (Binns et al., 2006). The LDs and peroxisomes in yeast form intimate connections that are thought to help mobilize lipids for carbon and energy production during periods of enhanced lipid breakdown (Kohlwein et al., 2013). These intimate associations are observed during TAG breakdown in yeast, where peroxisomes develop membrane domains called *pexopodia* that are enriched in β-oxidation enzymes and penetrate inside the TAG-filled LDs (Binns et al., 2006). In doing so, the outer leaflet of the pexopodia membrane is hypothesized to fuse with the LD monolayer and then form an extension that protrudes inside the LD (Figure 2). The morphology of these peroxisomal extensions into LDs were observed by transmission electron microscopy in yeast cells and proposed to be mediated by hemifusion between the peroxisome bilayer and the LD monolayer (Binns et al., 2006).

In animal cells, no pexopodia have been observed, and recent studies suggest instead that a pinching or tubulation of the LD membrane occurs where LDs and peroxisomes are tethered together through a process that requires the LD-localized M1 spastin protein and the peroxisomal FA transporter ABC transporter D1 protein (Figure 2; Chang et al., 2019). Furthermore, the LD pinching/tubulation process in animal cells requires endosomal sorting complex required for transport-III proteins, which overall facilitates the subsequent delivery of FAs from LDs to peroxisomes (Figure 2; Chang et al., 2019).

Mechanisms involving pexopodia or spastin/ABC transporter D1 protein and the endosomal sorting complex required for transport complex have not been described in plant peroxisome–LD connections. However, there do appear to be general similarities among eukaryotes that include alterations in LD and/or peroxisomal membrane architecture and dynamics. In plants, interactions of peroxisomal membranes with LDs occurs via peroxules enriched with β-oxidation enzymes, where the formation is dependent on the retromer complex (Figure 2; Thazar-Poulot et al., 2015; Shimada et al., 2018). When β-oxidation is disrupted, as in 3-ketoacyl-CoA thiolase (*ped1*) mutant plants, these membrane interactions are also disrupted and invaginations or LD remnants are observed inside

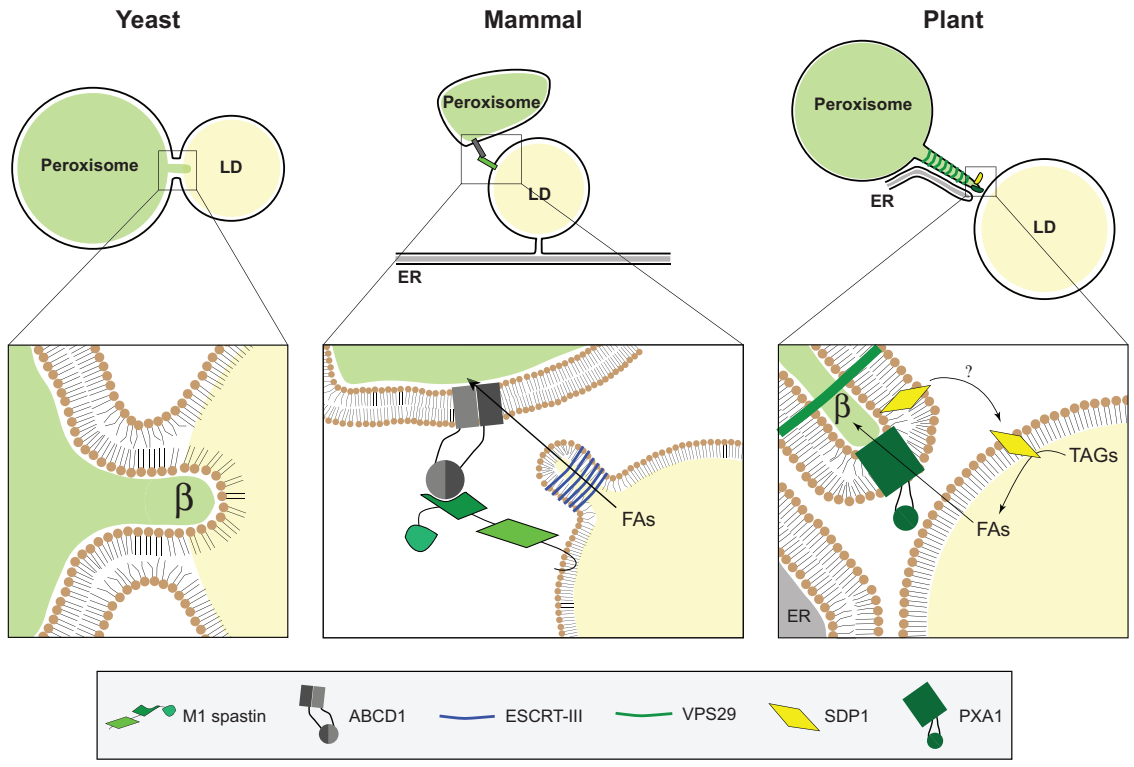

Figure 2. Models for Initiation of LD–Peroxisome Connections in Yeast, Mammals, and Plants. In yeast (left panel), the outer leaflet of the peroxisomal membrane is hypothesized to fuse with the LD monolayer and the inner leaflet of the peroxisome membrane forms a structure called a pexopodia, enriched in β-oxidation enzymes, that penetrates inside the LD (Binns et al., 2006). In mammalian cells (middle panel), the LD-localized protein, M1 spastin, acts as a tether by interacting with the peroxisomal ABCD1 transporter to stabilize the interorganellar interaction. This connection is believed to facilitate FA transfer from the LD to the peroxisome with assistance from ESCRT-III proteins (Chang et al., 2019). In plant cells (right panel), the peroxisome somehow delivers the lipase, SDP1, to the LD membrane via the formation of a peroxisomal tubule termed a peroxule. Peroxule extensions are reported to be guided by the ER (Sinclair et al., 2009). Peroxule formation and transfer of SDP1 requires the activity of VPS29, a core component of the retromer protein complex. After reaching the LDs, SDP1 hydrolyzes TAGs and the FAs are transferred into the peroxisome via PXA1 (Thazar-Poulot et al., 2015). The precise mechanism for the translocation of SDP1 from peroxisomes to the LDs or for the translocation of FAs from the LDs to the peroxisome is uncertain. ABCD1 = ATP-binding cassette transporter D1 protein; $\beta = \beta$-oxidation enzymes; ER = endoplasmic reticulum; FAs = free fatty acids or fatty acyl-CoA; LD = lipid droplet; PXA1 = peroxisomal ABC transporter protein 1; SDP1 = sugar-dependent protein 1; TAGs = triacylglycerols; VPS29 = vacuolar protein sorting 29; ESCRT-III: endosomal sorting complex required for transport-III.

peroxisomes (Figure 1b; Hayashi et al., 2001). While the pexopodia in yeast and the peroxules in plants may share some similarities, there are also important differences between these extensions. In the case of pexopodia in yeast, these structures penetrate into LDs, whereas peroxules in plants do not appear to enter LDs, but rather the reverse occurs where LDs become internalized into peroxisomes. Regardless, the interaction at the surfaces in both cases may include the formation of a hemifusion between the peroxisomal bilayer and LD monolayer, although this remains to be experimentally verified.

How peroxules mediate the relocalization of SDP1 to LDs or support the transport of FAs into peroxisomes by PXA1 remains unknown. Is there a hemifusion between peroxules and the LD monolayer, as in the pexopodia–LD connections observed in yeast, and is this process mediated by VPS29? Do the unusual intraperoxisomal structures observed in *ped1* mutants (Hayashi et al., 2001) suggest a mechanism similar to pexopodia that might be involved in LD mobilization in plants, especially during seedling establishment? A mechanism such as hemifusion would certainly help facilitate the passive diffusion of SDP1 to the LD surface, and it is possible that hydrolysis of TAGs to form membrane-disrupting free FAs by SDP1 might generate unusual, internalized structures that could become exaggerated if β-oxidation was slowed or disrupted, as in the *ped1* mutants (Hayashi et al., 2001). Isolation and identification of protein complexes that help connect these two compartments should shed light on the underlying mechanism of LD–peroxisome MCSs in plant cells, and this might be especially relevant, and best captured, during the process of rapid lipid mobilization observed during early postgerminative seedling growth.

Do VAPs Mediate LD–Peroxisome Tethering in Plants?

After their biogenesis, nascent LDs can either detach from the ER, by a mechanism(s) that is poorly understood, or remain associated with the ER by a lipidic bridge, where the outer leaflet of the ER membrane is continuous with the LD monolayer, and/or by tethering proteins that connect the two organelles (Schuldiner and Bohnert, 2017). The reason why LDs might remain attached to the ER is still unclear, but one possibility is to help facilitate the transfer of proteins and lipids from one compartment to the other and, in doing so, allow the cell to alter LDs in response to changes in growth conditions and/or a developmental or environmental cue. In yeast and animal cells, MCSs between LDs and other organelles are mediated by a diverse array of proteins, including the Rab18/NAG-RINT1-ZW10 complex and FA transporter protein/diacylglycerol acyltransferase (DGAT2) complexes (reviewed in Wu et al., 2018), as well as the previously mentioned snazarus protein (Ugrankar et al., 2019). However, none of these protein complexes have been identified yet in plants, and if they do not exist, perhaps other protein-mediated, LD–organelle connections have evolved as functional equivalents.

The VAPs have been extensively characterized in yeast and animals, and more recently in plants, where they are well established as being important for the formation of MCSs between the ER and various other organelles (Skehel et al., 1995; Wang et al., 2016; Stefano et al., 2018; Wang and Hussey, 2019). VAPs are ER-localized, tail-anchored membrane proteins that contain a cytoplasmic-facing major sperm domain (Loewen and Levine, 2005; Furuita et al., 2010). This domain interacts with FFAT motifs (two phenylalanines, FF, in an acidic tract), or variants thereof (i.e., FFAT-like motifs; Mikitova and Levine, 2012), found in several VAP-binding proteins (Loewen and Levine, 2005), and, in doing so, stabilize MCS formation. The VAP family of proteins in plants have elaborated considerably, with 10 VAP homologs reported in *Arabidopsis* based on the conserved major sperm domain (Wang et al., 2016). Some of the VAPs in plants have no TMD, and most VAP homologs in plants remain to be functionally characterized.

In several well-established examples in mammalian cells, VAP-mediated MCSs are involved in lipid transfer. For instance, the transfer of ceramide from the ER membrane to the Golgi is facilitated by the interaction of VAP with the Golgi-localized CERT (Hanada et al., 2009). Transfer of sterols between the ER and the TGN in mammalian cells is similarly mediated by interaction of ER-localized VAP with the cytoplasmic OSBP, which is also recruited to the TGN membrane through its interaction with phosphatidylinositol 4-phosphate (Mesmin et al., 2017). In both cases, CERT and OSBP interact with the VAP isoform A (VAP-A) protein to help stabilize the ER-Golgi or ER-TGN MCS (Kentala et al., 2015; Mesmin et al., 2017). In other studies, VAP-A and VAP-B were shown to interact with the peroxisomal membrane protein ACBD5, an acyl-CoA-binding protein that also contains an FFAT-like motif (Costello et al., 2017; Hua et al., 2017). This VAP-ACBD5 interaction plays an important role in peroxisome expansion in mammalian cells by facilitating the transfer of membrane lipids from the ER to peroxisomes but also contributes to cholesterol biosynthesis by transferring precursors from peroxisomes to the ER (Hua et al., 2017).

Taken together, these examples highlight the role of VAPs as tethers between organelles that also help facilitate lipid transfer between the two compartments. Could VAPs also play a role in the connections between LDs and peroxisomes in plants? No direct evidence for this possibility currently exists. However, the VAP family of proteins is extensive in plants, consisting of considerably more members than in yeast or mammals (Wang et al., 2016), suggesting a possible expansion of VAP function in organelle–organelle connections in plants. Indeed some plant VAPs, such as VAP27-1 and VAP27-3 in *Arabidopsis*, are described to participate in MCSs between the ER and PM (Wang et al., 2016) and in membranes during endocytosis (Stefano et al., 2018). VAPs have also been identified in proteomes of LDs isolated from germinated seedlings and senescing leaves (Brocard et al., 2017; Kretzschmar et al., 2018).

Possible Roles for VAPs in LD–Peroxisome Connections in Plant Cells

Two hypothetical models for VAP-mediated connections between plant peroxisomes and LDs are shown in Figure 3. In one model (Figure 3a), LD-localized VAPs would interact through their cytoplasmic-facing major sperm domain with the FFAT-like motif in the PXA1 transporter (discussed later) and, in doing so, tether LDs and peroxisomes together. In this model, the N-terminus of the tail-anchored VAP protein would face the cytoplasm, as usual, while the C-terminus would be oriented in a hairpin configuration. While there is no direct evidence for this topological orientation of VAPs in the LD monolayer, VAP family proteins were reported in several different studies of plant LD proteomes (Brocard et al., 2017; Kretzschmar et al., 2018) and some tail-anchored proteins do not always adopt a transmembrane topology (Kim et al., 2004; Brito et al., 2019). Alternatively, in the model presented in Figure 3b, the VAP proteins would remain in the ER

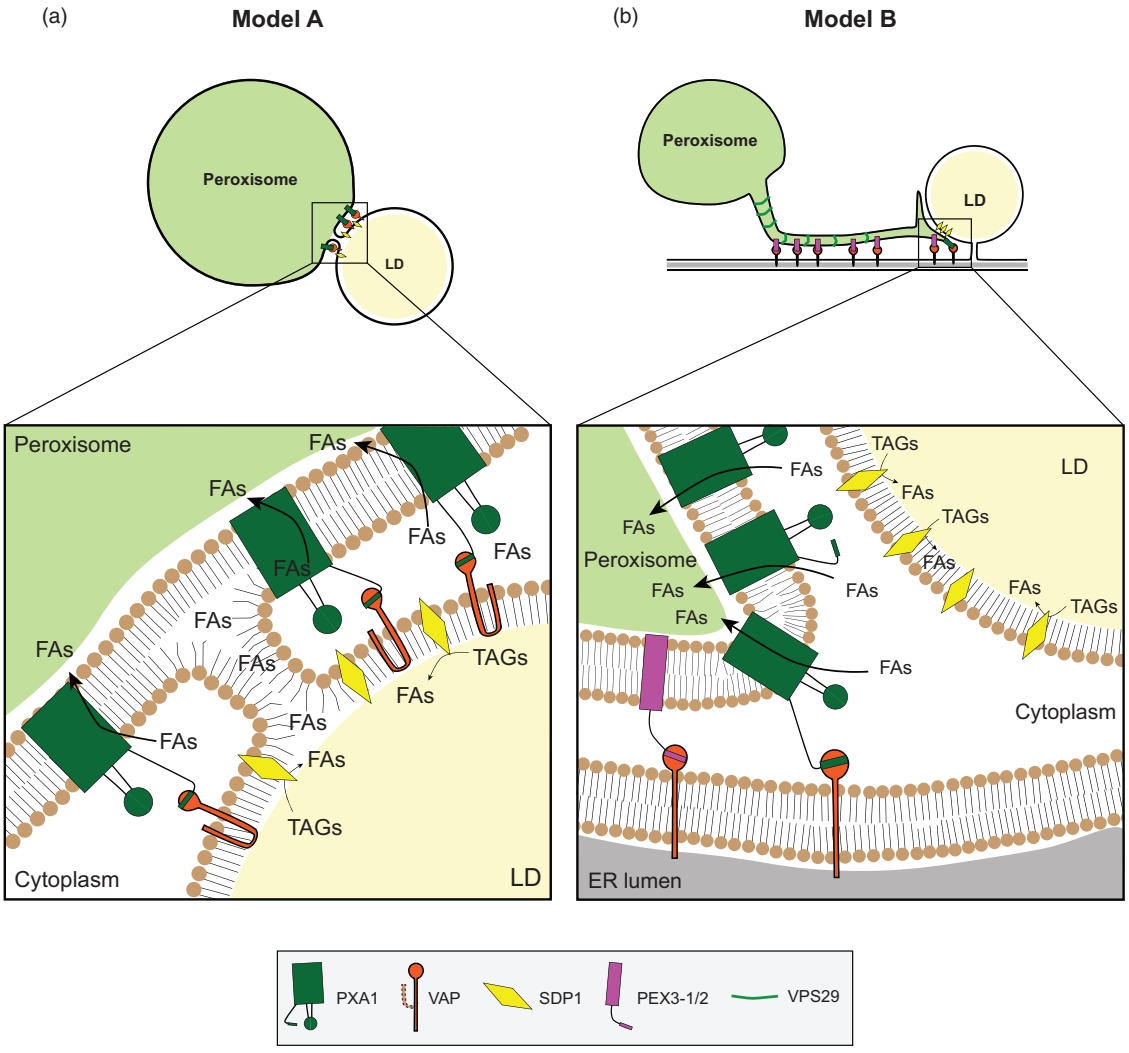

Figure 3. Hypothetical Models for a Membrane Connection Between LDs and Peroxisomes in Plants. Panel A: A scheme for interaction between FFAT-like motif-containing PXA1 and LD-localized VAPs, which could help stabilize an LD–peroxisome MCS to facilitate the transfer of FAs from the LDs to peroxisomes during seedling establishment (Model A). In this model, the peroxisomal membrane might form a hemifusion with the LD monolayer to help deliver SDP1 from peroxisomes (via peroxules) to the surface of the LDs for TAG breakdown. In addition, a connection could be stabilized by the interaction of the putative FFAT-like motif of PXA1 with the major sperm domain of the LD-localized VAP. LD localization of VAPs would likely require a change in conformation of the VAP from its typical tail-anchored form in the ER bilayer to a hairpin-like configuration such that the few polar residues at the extreme C-terminus would extend to the LD surface. A resulting connection between peroxisomes and LDs would facilitate the large flux of FAs from LDs to the matrix of peroxisomes by diffusion via a lipid bridge or by direct translocation by the action of the PXA1 transporter. Panel B: Alternatively, a scheme that involves three compartments—the ER, peroxisome, and LDs—could be envisioned (Model B), inspired by the multiple membrane interactions mediated by the snazarus protein in *Drosophila* (Ugrankar et al., 2019). In this model, peroxules are guided by the ER via interaction of FFAT-like motif-containing peroxisomal proteins, such as PEX3-1 and/or PEX3-2 with ER-localized VAPs. The formation of these extensions is also dependent on the activity of the retromer complex. Then, at the ER–peroxule–LD junction, the peroxisomal PXA1 protein, through its cytoplasm-oriented FFAT-like motif, might interact with ER-localized VAPs to stabilize a connection for the transfer of SDP1 from the peroxule to the LD surface and the efficient translocation of FAs released from the LD by SDP1 activity. In this model, it is unclear what mediates the direct tether between LDs and peroxisomes. LD = lipid droplet; FAs = free fatty acids or acyl-CoA; TAGs = triacylglycerols; SDP1 = sugar-dependent protein 1; PXA1 = peroxisomal ABC transporter 1 protein; VAP = vesicle-associated membrane protein-associated protein; ER = endoplasmic reticulum; PEX3-1/2 = peroxin 3-1/3-2.

bilayer and mediate (via their cytoplasmic major sperm domain) interaction with peroxisome proteins, PXA1 and/or PEX3-1/2, through their FFAT-like motifs. Here, the VAPs would be oriented in their usual manner (i.e., $N_{cytoplasm}$–$C_{ER\ lumen}$), and the ER would provide a scaffold for the expansion of peroxules and the close connection between LDs and peroxisomes. Although this latter model accounts for the more

commonly accepted localization of VAPs in the ER bilayer, the direct connection between LDs and peroxisomes remains unclear. It is possible that there are features of both models that are important for LD–peroxisome connections. In any case, further experimental evidence will be required to provide support for these or other models that describe the close cooperation between LDs and peroxisomes in plants.

Based on FFAT and FFAT-like motifs described by Mikitova and Levine (2012), such motifs can also be found in four plant peroxisomal membrane-associated proteins, including PXA1, PEX3-1, PEX3-2, and CGI-58 (Figure 4, based on calculations according to Murphy and Levine, 2016). As described previously, PXA1 is the primary peroxisomal membrane transporter involved in the uptake of FAs into the peroxisome matrix (Baker et al., 2015). PXA1 was shown to be important for the interaction of peroxisomes and LDs in plant cells (Cui et al., 2016). The FFAT-like motifs in PXA1 are located in the C-terminal region of the protein (Figure 4), which is predicted to face the cytoplasm (Dietrich et al., 2009; Park et al., 2013). PEX3-1 and PEX3-2, on the other hand, are peroxisome biogenesis factors known to be important for maintaining peroxisome morphology (Nito et al., 2007). Both of these PEX3 homologs possess two TMDs (Hunt and Trelease, 2004), and, similar to PXA1, both proteins contain an FFAT-like motif predicted to face the cytoplasm (Figure 4). Furthermore, CGI-58, which is localized to peroxisomes in plant cells through its interaction with PXA1 (Park et al., 2013), also contains an FFAT-like motif (Figure 4).

A confidence score can be assigned to each FFAT-like motif depending on its amino acid composition. The FFAT-like motifs in PXA1 and CGI-58 have "weaker" scores ($\geq$3), whereas the FFAT-like motifs in PEX3-1 and PEX3-2 have "stronger" scores (between 0 and 3; Figure 4; Murphy and Levine, 2016). This "score" indicates the probability of the FFAT-like motif to function as an FFAT motif, and more than one FFAT-like motif in the protein increases the interaction of the protein with VAPs. By way of example, the human ACBD5 peroxisomal protein, which interacts with VAP-A and VAP-B in the ER (Costello et al., 2017; Hua et al., 2017), contains a single FFAT-like motif with a score of 3.5. Thus, in the case of plant peroxisomes, several FFAT-like-motif-containing proteins are located on the peroxisome surface and may participate in MCS formation via VAPs. In particular, PXA1 appears to be a good candidate for participating in VAP-mediated tethering during seed germination, since PXA1 is directly involved in the process of FA transfer from LDs to peroxisomes (Park et al., 2013). Furthermore, PXA1 has two FFAT-like motifs and PXA1 mutants, *ped3-1* and *ped3-3*, that lack these motifs display more severe phenotypes compared with other alleles (Hayashi et al., 2002). Consequently, the transport process for FAs from LDs to peroxisomes might be enhanced by the binding of PXA1 to an LD-localized VAP(s), which would bring the two membranes into closer proximity (Figure 3a).

As mentioned earlier, VAP homologs were identified in the proteomes from isolated LDs, including from seeds, seedlings, and senescing leaves (Brocard et al., 2017; Zhi et al., 2017; Kretzschmar et al., 2018). Presumably, VAPs in the LDs would have become associated during the biogenesis of LDs in the ER at the time of seed development. The relocalization of transmembrane proteins to LDs has been described for several proteins including certain glycerol-3-phosphate

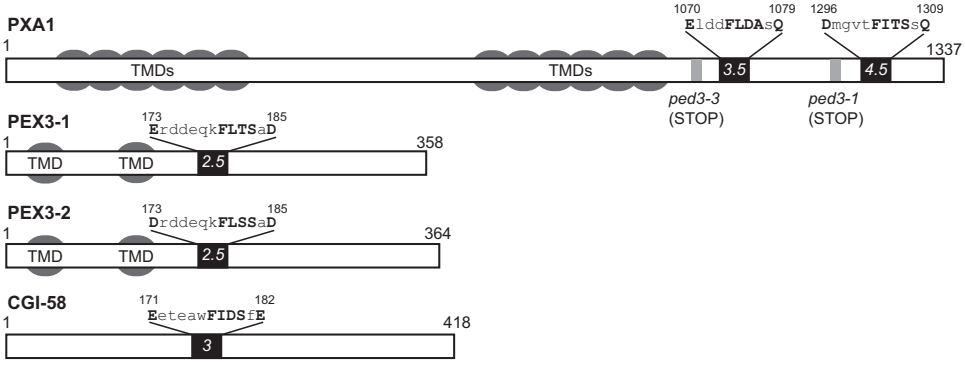

Figure 4. Putative FFAT-Like Motifs in the Plant Peroxisomal Proteins PXA1, PEX3-1, PEX3-2, and CGI-58. FFAT-like motifs, as described in Mikitova and Levine (2012), were identified using the ScanProsite program available at www.prosite.expasy.org and the following motif [DE]-X(0,5)-X-[FY]-[FYCILMVWH]-[DEST]-[ACST]-X-[DESTGNQ]. All motifs are located in regions of the proteins predicted to be localized on the cytoplasmic side of the peroxisomal membrane. FFAT scores (numbers in black boxes) are calculated according to Murphy and Levine (2016). The *pxa1* mutant alleles, *ped 3-1* and *ped 3-3* (Hayashi et al., 2002), introduce premature stop codons that remove one or both of the FFAT-like motif(s) in PXA1 and could thereby disrupt the functional association of PXA1 with VAPs. Also depicted for PXA1, PEX3-1 and PEX3-2 are the putative TMDs (based on Hunt and Trelease, 2004; Dietrich et al., 2009; Park et al., 2013). TMD = transmembrane domain.

acyltransferases and DGATs (reviewed in Kory et al., 2016). Furthermore, the abundant seed LD proteins, the oleosins, are cotranslationally inserted into the ER before reorienting into the hairpin configuration in the LD monolayer (Abell et al., 2002). Of course, any candidate LD-localized VAPs will need to be verified experimentally and also tested for their ability to interact with any peroxisomal proteins. Nevertheless, we speculate that LD-localized VAPs might provide an anchor that tethers the peroxisomal membrane to the LD surface through interaction with PXA1 and/or CGI-58, to help improve the efficient delivery of FAs from LDs to the peroxisomal matrix (Figure 3a).

An additional feature of the LD–peroxisome connection in plants that needs to be resolved is how SDP1 traffics from peroxisomes to the LD surface during postgerminative seedling growth; here, VAPs might also play an indirect role. As described earlier, core retromer proteins are required for the formation of peroxisomal tubules (peroxules) that are thought to deliver SDP1 from the peroxisomal membrane to the LD surface (Thazar-Poulot et al., 2015). One plausible hypothesis to help facilitate protein transfer is hemifusion between the outer leaflet of the peroxisomal membrane and the LD monolayer (Figure 3a), similar to the pexopodia–LD hemifusion event hypothesized for yeast cells (Binns et al., 2006). This hemifusion would allow for the passive diffusion of SDP1 from peroxisomal membranes to the LD monolayer, thereby requiring less energy than a direct protein transfer. Similar diffusion-based transfer events are known to exist for animal acyltransferases, DGAT2 and glycerol-3-phosphate acyltransferase-4, which traffic from the ER bilayer to the LD monolayer via a continuous lipid bridge during lipid-loading conditions (Wilfling et al., 2013).

Concluding Comments

Although few specific details exist regarding the molecular players that facilitate the interaction between peroxisomes and LDs in plant cells, the literature is replete with circumstantial evidence for an intimate connection between these two organelles that is important for the rapid metabolic mobilization of storage lipids during seedling establishment (Figure 1). We propose that MCSs, possibly involving VAPs on LDs and FFAT-like-motif-containing proteins on the peroxisomal surface, and/or hemifusion of peroxisome and LD membranes, help to mediate the retromer-dependent delivery of SDP1 from peroxisomes (peroxules) to LDs to activate lipid hydrolysis. These MCSs would further help facilitate the transfer of FAs into the peroxisomal matrix for β-oxidation. Two hypothetical models are provided and are distinguished from each other by the proposed involvement of the ER (Figure 3). Both models are consistent with retromer-component mutant phenotypes (Thazar-Poulot et al., 2015; Huang et al., 2019) and putative FFAT-like motifs within cytoplasmic regions of key peroxisomal proteins, including PXA1, PEX3-1, PEX3-2, and CGI-58 (Figure 4). Support for a model describing a direct connection between LDs and peroxisomes includes the presence of VAP family proteins in plant LD proteomes (Brocard et al., 2017; Kretzschmar et al., 2018). In addition, PXA1 is required for proper peroxisome–LD connections and the mobilization of seed storage lipids (Hayashi et al., 2002; Cui et al., 2016). Undoubtedly, a major breakthrough in understanding connections between peroxisomes and LDs will come from the isolation of protein tethering complexes, which might be markedly altered in peroxisome-deficient mutants like *ped1* (Hayashi et al., 2001), *pex6*/*26* (Gonzalez et al., 2017), or *pex10* (Schumann et al., 2003), where, in the latter, the formation of both LDs and peroxisomes is disrupted. Likewise, additional understanding of the LD–peroxisome connection in plants might be gained by studies involving femtosecond laser technology, such as that used to investigate the connections between peroxisomes and chloroplasts in plants (Oikawa et al., 2015; reviewed in Oikawa et al., 2019).

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