Expression of oleosin and perilipins in yeast promotes formation of lipid droplets from the endoplasmic reticulum

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Summary

Most cells store neutral lipids in a dedicated compartment, the lipid droplet (LD). These LDs are structurally and functionally conserved across species. In higher eukaryotes, LDs are covered by abundant scaffolding proteins, such as the oleosins in plants and perilipins (PLINs) in animal cells. *Saccharomyces cerevisiae*, however, has no homologues of these scaffolding proteins. To analyze a possible function of these proteins in the biogenesis of LDs, oleosin and perilipin family members (PLIN1, ADRP/PLIN2 and TIP47/PLIN3) were expressed in yeast cells and their targeting to LDs, membrane association and function in neutral lipid homeostasis and LD biogenesis were analyzed. When expressed in wild-type cells, these proteins were properly targeted to LDs. However, when expressed in cells lacking LDs, oleosin was localized to the ER bilayer and was rapidly degraded. PLINs, on the other hand, did not localize to the ER membrane in the absence of LDs and lost their membrane association. Photobleaching experiments revealed that PLIN2 and PLIN3 rapidly exchanged their LD association, but PLINs did not move as quickly as integral membrane proteins, such as oleosin, over the LD surface. Interestingly, expression of these scaffolding LD proteins in mutant cells containing elevated levels of neutral lipids within the ER bilayer resulted in the formation of LDs. These results suggest that these LD scaffolding proteins promote the sequestration of neutral lipids from the ER bilayer and thereby induce LD formation. Consistent with this proposition, addition of a cell-permeable diacylglycerol (DAG) was sufficient to promote LD formation in cells expressing the LD scaffolding proteins but lacking the capacity to synthesize storage lipids.

Key words: Perilipin, Oleosin, Lipid droplets, Neutral lipids, Lipin, *Saccharomyces cerevisiae*

Introduction

Neutral lipids, particularly triacylglycerol (TAG) and steryl esters (STEs) are synthesized by enzymes that are located within the ER membrane. The neutral lipids themselves, however, are stored in a dedicated compartment, the lipid droplet (LD). LDs are generally believed to bud from the ER bilayer enclosing neutral lipids within a lipid monolayer containing a subset of ER proteins, many of which have functions in lipid metabolism (Fujimoto et al., 2008; Olofsson et al., 2009; Walther and Farese, 2012). In animal and plant cells, LDs are covered by abundant proteins – the perilipins (PLINs) and oleosins, respectively – which are thought to form a structural part of LDs. PLINs constitute a family of related proteins that share a common PAT domain, identified in the founding members of that family, perilipin/PLIN1, adipophilin/ADRP/PLIN2 and TIP47/PLIN3 (Kimmel et al., 2010; Miura et al., 2002). PLINs differ from one another in size, tissue expression and affinity for LDs, indicating that each of them has a distinct possibly cell-type-specific function, but all of them probably regulate the interface between LDs and the cellular environment (Bickel et al., 2009; Brasaemle and Wolins, 2012; Brasaemle, 2007; Londos et al., 1999). Perilipin/PLIN1 is translated on free polyribosomes and localizes to LDs post-translationally (Brasaemle et al., 1997a).

Oleosins, however, are abundant on plant oleosomes or oil bodies, particularly in seeds, where oleosins prevent coalescence of the LDs during desiccation and are important for lipid mobilization during seed germination. They are small integral membrane proteins with a hairpin topology and a central lipid-embedded domain containing a proline knot motif that is important for proper LD targeting of the protein (Hsieh and Huang, 2004). Even though oleosin lacks a classical N-terminal signal sequence, its translation in yeast depends on the signal recognition particle, indicating that the protein is cotranslationally inserted into the ER bilayer, from where it is then targeted to LDs (Beaudoin et al., 2000).

Yeast LDs are structurally and functionally related to those of animal and plant cells even though they have no homologues of either PLINs or oleosin and thus lack these abundant scaffolding components. The targeting of peripheral and integral membrane proteins to LDs, however, is conserved between yeast, plant and animal cells. Oleosin, for example, is properly targeted to LDs when expressed in yeast or mammalian cells (Beaudoin et al., 2000; Hope et al., 2002; Ting et al., 1997).

Synthesis of neutral lipids and thus the biogenesis, growth and abundance of LDs in yeast depends on the activity of four enzymes, two of which synthesize TAG and two generate STE

(Czabany et al., 2007). STE formation is catalyzed by two polytopic ER-localized acyl-CoA:sterol acyltransferases (ACATs), Are1 or Are2. TAG, however, can be synthesized either by Lro1 or Dga1. Lro1 encodes a lecithin cholesterol acyltransferase (LCAT)-related protein and synthesizes TAG through transesterification of a fatty acid from phospholipids to diacylglycerol (Dahlqvist et al., 2000; Oelkers et al., 2000). The second TAG synthesizing enzyme, Dga1, catalyzes the acyl-CoA-dependent synthesis of TAG from diacylglycerol (Oelkers et al., 2002; Sorger and Daum, 2002). Neutral lipid synthesis and storage are dispensable for the viability of *S. cerevisiae* because a quadruple mutant lacking all four biosynthetic enzymes is viable, makes no neutral lipids and lacks detectable LDs (Oelkers et al., 2002; Sandager et al., 2002).

We have previously analyzed the subcellular distribution of LD-localized proteins in cells lacking LDs as a result of deletion of all the genes required for the synthesis of neutral lipids. This study revealed that LD-localized membrane proteins such as Erg6, an enzyme of the ergosterol biosynthetic pathway, or the diacylglycerol acyltransferase Dga1, are localized in the ER bilayer in cells lacking LDs (Jacquier et al., 2011). Induction of neutral lipid synthesis in these cells, resulted in the *de novo* formation of LDs and a concomitant relocalization of marker proteins from the ER onto LDs, indicating that the ER bilayer is functionally connected to LDs (Jacquier et al., 2011).

In the present study, we analyzed whether the relocalization of otherwise LD-localized protein back to the ER bilayer in cells lacking LDs is a unique property of LD-localized yeast proteins or whether it is a more general feature of LD proteins and thus possibly conserved across species. Expression of oleosin and PLINs in cells containing or lacking LDs revealed that oleosin is localized to the ER bilayer and subject to rapid degradation if cells lack the capacity to synthesize neutral lipids. PLINs, by contrast, lose their membrane association in the absence of LDs, suggesting that they specifically associate with membrane domains enriched in neutral lipids. Consistent with this proposition, we observe that ectopic expression of either oleosin or PLINs results in LD formation from membranes enriched in neutral lipids, indicating that these proteins promote the formation of LDs, possibly by sequestering and condensing neutral lipids within the ER bilayer.

Results

To examine whether LD-localized proteins from plants and mammalian cells would also localize in LDs in wild-type yeast or in the ER bilayer when cells lack neutral lipids, we expressed GFP-tagged versions of oleosin 1 from *Arabidopsis thaliana*, and human perilipin/PLIN1, ADRP/PLIN2 and TIP47/PLIN3. Therefore, cDNAs of these genes were amplified and cloned into a centromeric vector that drives expression of N-terminally GFP-tagged versions of these proteins from a constitutively active alcohol dehydrogenase (ADH1) promoter. Correct size of the fusion proteins was controlled by western blot analysis. Expression of these fusion proteins in wild-type cells resulted in staining of punctate intracellular structures (Fig. 1A). These GFP-labeled structures were also marked by RFP-tagged Erg6, an enzyme of the late part of the ergosterol biosynthetic pathway and established marker protein of yeast LDs (Leber et al., 1994). Colocalization of the GFP-tagged LD scaffolding proteins with LDs was also observed when LDs were visualized by staining with the neutral lipid dye Nile Red (supplementary material Fig. S1). These results

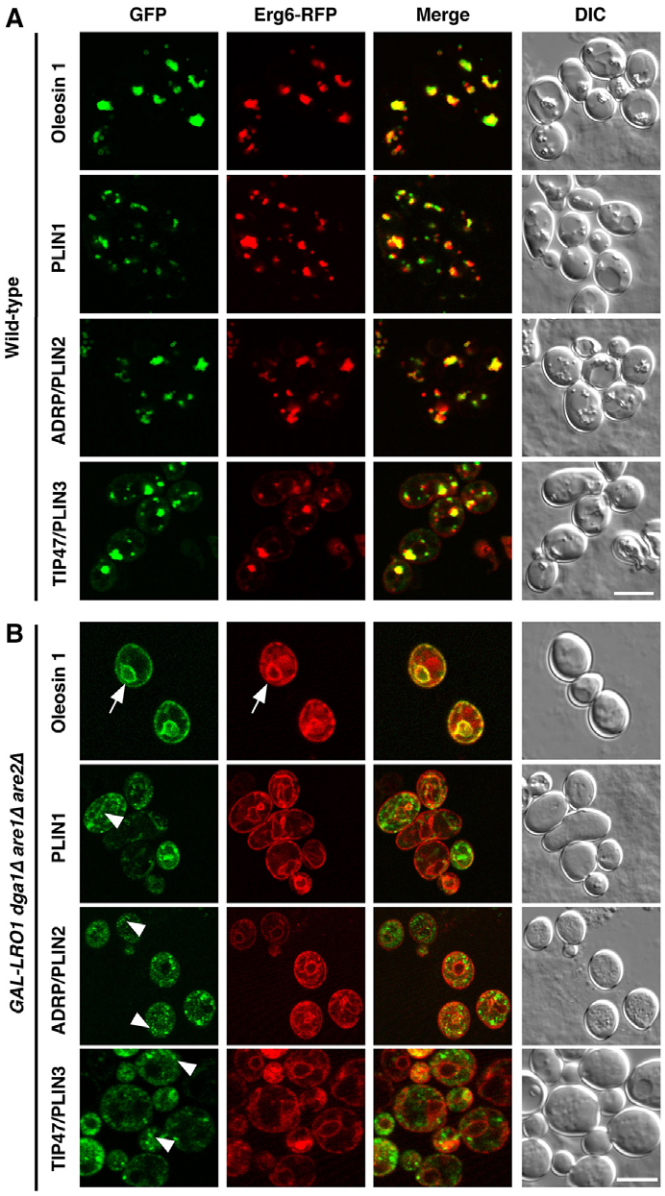

Fig. 1. Localization of foreign LD proteins in yeast cells containing or lacking LDs. (A) GFP-tagged versions of oleosin and perilipin family members are properly targeted to LDs in yeast. Colocalization of GFP-tagged versions of foreign LD proteins with the LD marker Erg6-RFP was analyzed by confocal microscopy. (B) Perilipin family members are mislocalized in the absence of LDs. The subcellular localization of the indicated fluorescently tagged proteins in triple mutant yeast cells lacking neutral lipids and LDs (*GAL-LRO1 dga1Δ are1Δ are2Δ*) was analyzed by confocal microscopy. ER localization of oleosin 1 and the endogenous LD marker Erg6-RFP is indicated by arrows. Granular localization of perilipin family members is indicated by arrowheads. Scale bars: 5 μm.

thus indicate that oleosin, PLIN1, PLIN2/ADRP and PLIN3/TIP47 are localized to LDs when expressed in yeast, consistent with earlier observations that oleosin is targeted to yeast LDs (Beaudoin et al., 2000; Ting et al., 1997). Although it is possible that the GFP tag attached to these proteins affects their physiological function, the observation that the fusion proteins are properly localized indicates that the targeting of the protein is not affected by the

presence of the tag. Moreover, expression of these foreign LD scaffolding proteins in yeast did not significantly appear to affect the number of LDs per cell, their morphology, or their close association with the ER bilayer. Wild-type cells displayed an average of 4.9 LDs per cell, whereas PLIN1 expressing cells had 5.6 LDs per cells (supplementary material Fig. S1). The average diameter of LDs in wild-type cells was 0.7 µm, whereas that in PLIN1-expressing cells was 0.6 µm. By contrast, cells lacking FLD1, the yeast homolog of the human lipodystrophy gene seipin, had significantly enlarged LDs (1.0 µm; supplementary material Fig. S2) (Fei et al., 2008; Szymanski et al., 2007). Colocalization experiments in cells expressing the ER marker Sec63 revealed close apposition of LDs with the ER bilayer in cells expressing GFP-tagged versions of oleosin or perilipins, as was observed in wild-type cells (supplementary material Fig. S3).

To examine the subcellular localization and fate of the foreign LD scaffolding proteins in cells lacking LDs, the GFP-tagged proteins were expressed in triple mutant cells (*GAL-LRO1 dga1Δ are1Δ are2Δ* strain) grown in the presence of glucose to repress the expression of LRO1 and therefore lacking the capacity to synthesize neutral lipids. These cells have no detectable LDs, STEs or TAGs when grown in glucose medium and thus essentially behave as a quadruple mutant lacking all four enzymes required for neutral lipid synthesis (supplementary material Fig. S4) (Jacquier et al., 2011; Sandager et al., 2002). In the absence of LDs, GFP-oleosin stained circular intracellular structures that correspond to the nuclear envelope or perinuclear ER and the peripheral ER (Fig. 1B). The structures stained by GFP-oleosin colocalized with Erg6-RFP, which is known to localize to the ER bilayer in the absence of LDs (Jacquier et al., 2011; Sorger et al., 2004). These results thus indicate that in the absence of LDs, relocalization to the ER bilayer is not only observed for endogenous yeast LD proteins, but also for a structural LD protein of plants, indicating that this property of LD-localized integral membrane proteins is conserved across species. Oleosin has a hairpin topology and is first integrated into the ER membrane before it is targeted to LDs (Beaudoin and Napier, 2002; Beaudoin et al., 2000; Ting et al., 1997). A similar relocalization from the ER bilayer to LDs is observed for the TAG biosynthetic enzyme Dga1 and the mammalian glycerol-3-phosphate acyltransferase 4 (GPAT4) (Jacquier et al., 2011; Wilfling et al., 2013).

Unlike oleosin, however, expression of the mammalian PLINs in cells lacking LDs, did not result in uniform labeling of the ER membrane, but instead revealed staining of small granular structures (Fig. 1B). These results indicate that PLINs have different properties to oleosin or yeast Erg6 and Dga1, in that they appear to lose their association with the ER bilayer in the absence of LDs. PLINs have no strongly predicted transmembrane domain or signal sequence and are synthesized on free polyribosomes before their post-translational targeting to LDs (Brasaemle et al., 1997a). The granular, possibly cytosolic localization of PLINs observed in the absence of LDs might thus be explained by the fact that PLINs are only peripherally associated with the LD surface, and suggests that these proteins may lose their membrane association in the absence of neutral lipids, as observed in animal cells (Bartholomew et al., 2012; Wolins et al., 2001; Wolins et al., 2005; Wolins et al., 2006).

Membrane association of PLINs depends on the presence of LDs

To confirm the LD-association of the foreign scaffolding proteins biochemically, spheroplasts were homogenized and LDs were enriched by flotation on a Ficoll gradient. Equal amounts of proteins were separated by SDS-PAGE and probed by antibodies against the LD marker proteins Erg6 and Ayr1 (Athenstaedt and Daum, 2000). This analysis revealed an enrichment of the scaffolding proteins in the floating LD fraction over the homogenate (Fig. 2A).

To examine whether the altered subcellular distribution of PLINs in cells lacking neutral lipids is due to differences in

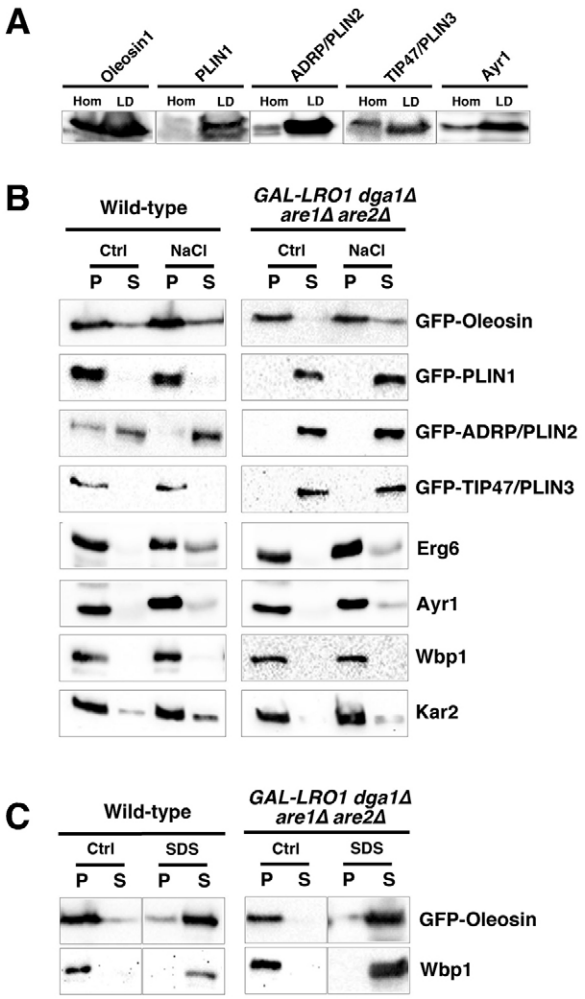

Fig. 2. Perilipin family members lose their membrane association in cells that lack neutral lipids and LDs. (A) Scaffolding proteins are enriched in a floating LD fraction. Cells were homogenized and LDs were floated on a Ficoll gradient. Equal amounts of proteins (1.5 µg) from the homogenate and the crude LD fraction were precipitated with TCA and analyzed by western blotting using Ayr1 as a marker protein for LDs. (B) Membrane association of the indicated protein was assessed by fractionating the cell homogenate of wild-type cells and cells lacking LDs (*GAL-LRO1 dga1Δ are1Δ are2Δ*) by centrifugation at 30,000 ***g***. Equal amounts of proteins (10 µg) from the pellet (P) and the supernatant fractions (S) were separated by SDS-PAGE, blotted and probed with antibodies against GFP, the LD markers Erg6 and Ayr1, and the ER markers Wbp1 and Kar2. To dissociate peripheral membrane proteins, the pellet fractions were incubated with 1 M NaCl or with buffer alone (Ctrl) for 30 minutes on ice, samples were centrifuged again and the levels of the respective protein in the soluble and insoluble fraction was monitored by western blotting. (C) Oleosin is solubilized upon detergent treatment. The 30,000 ***g*** pellet was incubated with 1% SDS or with buffer alone (Ctrl) for 30 minutes on ice, centrifuged and analyzed by western blotting.

membrane association of these proteins in the presence or absence of neutral lipids, we analyzed the biochemical properties of these proteins when expressed in wild-type cells and in cells lacking LDs (*GAL-LRO1 dga1Δ are1Δ are2Δ*). Cells were disrupted using glass beads, and microsomal membranes were prepared by centrifugation of the cell homogenate at 30,000 ***g***. Proteins from the pellet and the supernatant were separated by SDS-PAGE and the distribution of the GFP-tagged LD proteins in the two fractions was examined by western blotting. This analysis revealed that three of the LD proteins, oleosin, PLIN1 and TIP47/PLIN3 were limited to the pellet fraction (Fig. 2B). However, ADRP/PLIN2 was present both in the pellet and the supernatant. In yeast, PLIN1 thus behaves in a similar manner to that reported for animal cells, whereas ADR/PLIN2 and TIP47/PLIN3 appear to behave slightly differently: ADRP/PLIN2 does not show a soluble fraction in Leydig cells, but TIP47/PLIN3 displays a soluble pool in HeLa or Leydig cells in the presence of fatty acids (Brasaemle et al., 1997a; Brasaemle et al., 1997b; Wolins et al., 2001). The integral ER-membrane protein and subunit of the oligosaccharyl transferase complex, Wbp1, used as a control, was detected in the pellet fraction only (te Heesen et al., 1991). The soluble ER luminal chaperone Kar2, was also greatly enriched in the pellet, indicating that microsomal membranes remained sealed during the fractionation. The pellet fraction was also enriched in the LD marker proteins Ayr1 and Erg6, indicating that this fraction contains both LDs and ER-derived microsomes.

To examine whether membrane association of these LD proteins is disrupted by high salt concentrations, which disrupts ionic and polar interactions, but not hydrophobic interactions, and is thus indicative for peripheral association of proteins with membranes, the 30,000 ***g*** pellet fraction was washed with 1 M NaCl and proteins were again separated into soluble and insoluble fractions. This salt treatment resulted in the complete dissociation of ADRP/PLIN2 from membranes, but did not affect membrane association of the other LD proteins, oleosin, PLIN1 and TIP47/PLIN3. These results thus indicate that ADRP/PLIN2 displays the weakest membrane association of the four LD proteins tested.

Next, we examined membrane association of these proteins in cells lacking LDs. This analysis revealed that oleosin remained associated with the membrane irrespective of whether it was expressed in cells containing or lacking LDs, which is consistent with its localization in the ER bilayer in cells lacking LDs. PLIN1, ADRP/PLIN2 and TIP47/PLIN3, however, were present in the soluble fraction, indicating that these proteins lost their membrane association when cells had no LDs. These fractionation properties of the perilipin family members are consistent with the observed subcellular redistribution of these proteins in cells lacking LDs (Fig. 1) and suggests that these proteins associate specifically with the surface membrane of LDs but not with other cellular membranes. Consistent with the presence of oleosin within the ER bilayer in cells lacking LDs, oleosin was solubilized upon treatment of the pellet fraction with detergent (Fig. 2C).

The presence of LDs affects the stability of LD-localized proteins

To examine whether the presence or absence of LDs would affect the stability of these LD-targeted proteins, translation was blocked using cycloheximide, and turnover of these proteins was assessed by Western blotting. These experiments revealed that oleosin was stable and had a half-life of about 4 hours in wild-type cells, but in cells lacking LDs, oleosin was rapidly degraded, with a half-life of less than 1 hour (Fig. 3). Oleosin thus appears to be unstable when localized in the ER bilayer and thus requires association with LDs for its stability. Phosphoglycerate kinase, Pgk1, a soluble cytosolic protein used as a control in these experiments, did not display differences in its stability in cells lacking or containing LDs. PLIN1, however, was unstable both in wild-type cells and in cells lacking LDs with a half-life of less than 1 hour (Fig. 3). The stability of ADRP/PLIN2 and that of TIP47/PLIN3, by contrast, were not affected by the presence or absence of LDs (Fig. 3). Membrane association of ADRP/PLIN2 and TIP47/PLIN3 is thus not required for the stability of these proteins. These observations are consistent with the fact that PLIN1 truncations that do not target to LDs are rapidly degraded in adrenal cortical cells, and that PLIN1 is stable only in cells with large LDs (Brasaemle et al., 1997a).

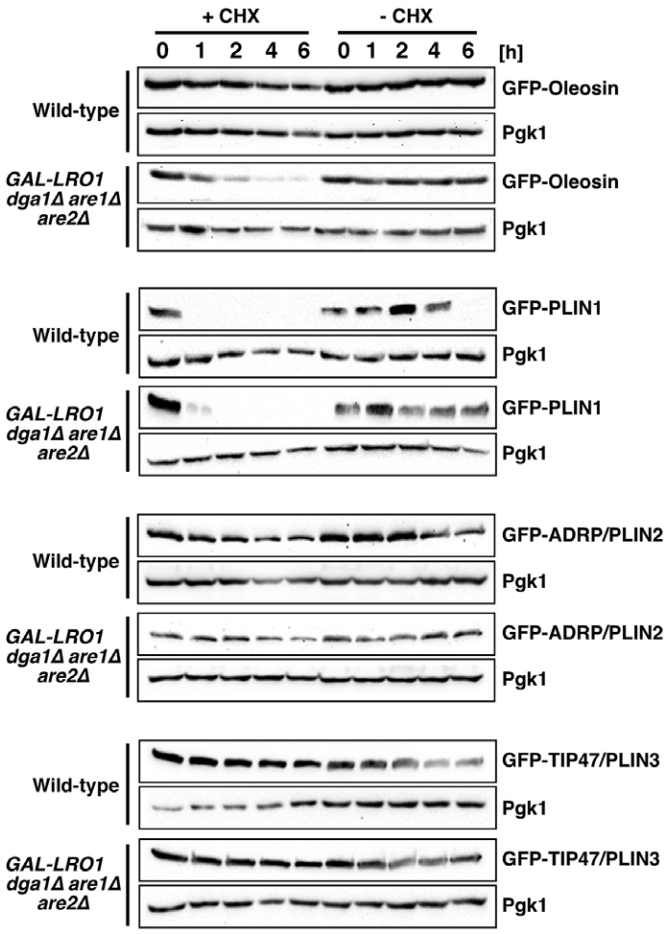

Fig. 3. Oleosin is unstable in cells lacking LDs. The stability of the indicated protein in cells containing (wild-type) or lacking LDs (*GAL-LRO1 dga1Δ are1Δ are2Δ*) was examined by cycloheximide chase. Cells expressing the indicated protein were diluted in fresh medium with or without cycloheximide (CHX, 50 μg/ml) and samples were removed at the time points indicated (0–6 hours). The relative abundance of the indicated protein was assessed by western blotting. Phosphoglycerate kinase 1 (Pgk1) was used as a loading control.

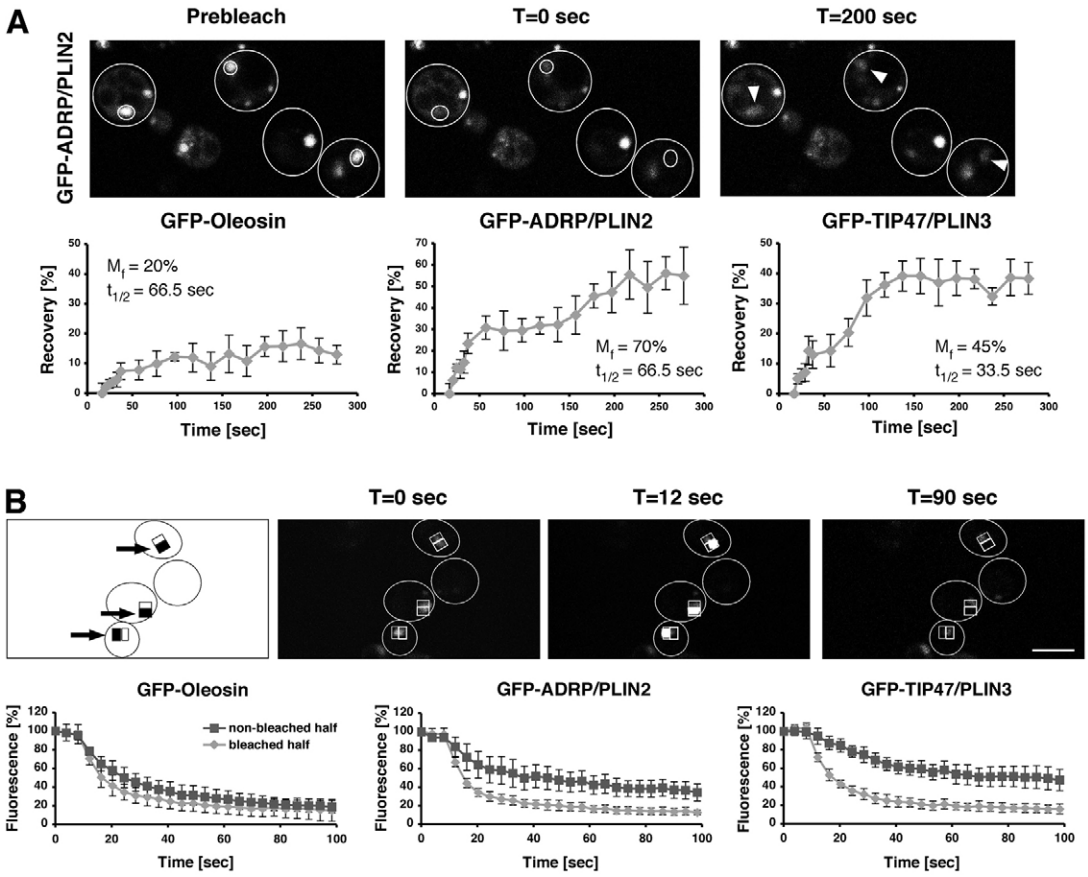

Fig. 4. Perilipin family members dynamically exchange their LD association and display a reduced mobility over the LD surface. (A) The exchange of LD-associated oleosin, ADRP and TIP47 was examined by photobleaching. Individual LDs were photobleached, and time-dependent recovery of fluorescence was recorded (n=9). The perimeter of the cells is indicated, LDs that were photobleached are encircled and fluorescence recovery over the bleached area 200 seconds after photobleaching is indicated by arrowheads. (B) Individual LDs were divided into two parts, one was constantly photobleached (filled square) and fluorescence loss (FLIP) over the non-bleached part of the LD (open square) was monitored over time.

LD-localized perilipin family members rapidly exchange their LD association

We have previously shown that yeast LD proteins can move between the ER bilayer and LDs and that this protein transport route operates independent of temperature and energy, suggesting that it might occur by a diffusion-driven mechanism (Jacquier et al., 2011). To examine whether the heterologously expressed LD-localized proteins can dynamically exchange between different LDs, individual LDs from cells expressing GFP-tagged versions of oleosin, and the three perilipin family members were subjected to photobleaching and recovery of fluorescence was monitored over time. These FRAP experiments (fluorescence recovery after photobleaching), revealed that oleosin recovered only to 20% of the initial fluorescence level (mobile fraction M_f=20%), with a half-life of $t_{½}$ =66.5 seconds, indicating that the protein is relatively immobile and that there is little exchange of oleosin between different, possibly interconnected LDs (Fig. 4A). As a comparison, similar experiments performed with the TAG synthase Dga1, which is an integral membrane protein, revealed an M_f of 85% and a $t_{½}$ for the exchange of 83 seconds (Jacquier et al., 2011).

The two perilipin family members, ADRP/PLIN2 (M_f=70%, $t_{½}$=66.5 seconds) and TIP47/PLIN3 (M_f=45%, $t_{½}$=33.5 seconds), showed much higher rates of fluorescence recovery after photobleaching, compared with oleosin (Fig. 4A). These results indicate that the bleached pool of LD-associated ADRP/PLIN2 and TIP47/PLIN3 is in constant exchange with a non-bleached pool of these proteins. A large fraction of the exchangeable pool of these proteins is likely to be associated with LDs because these are the only structures that are labeled under steady-state conditions (Fig. 1), and because these proteins co-sediment either entirely (TIP47/PLIN3) or partially (ADRP/PLIN) with membranes and LDs upon fractionation (Fig. 2). Recovery for PLIN1 was not examined in these experiments because of the high instability of this protein.

ADRP/PLIN2 and TIP47/PLIN3 display reduced lateral mobility on the surface of LDs

We have previously shown that integral membrane proteins such as Dga1 and Erg6 display rapid exchange at the surface of LDs, which is likely to reflect movement of the protein over the LD surface (Jacquier et al., 2011). To analyze whether the integral membrane protein oleosin, and the peripheral membrane-associated ADRP/PLIN2 and TIP47/PLIN3 would also move over the LD surface, we performed fluorescence loss in photobleaching (FLIP) experiments. Therefore, one half of a LD was constantly bleached and fluorescence of the non-bleached half of the LD was recorded. Rapid movement of proteins around the LD would lead to a rapid fluorescence loss in the non-bleached

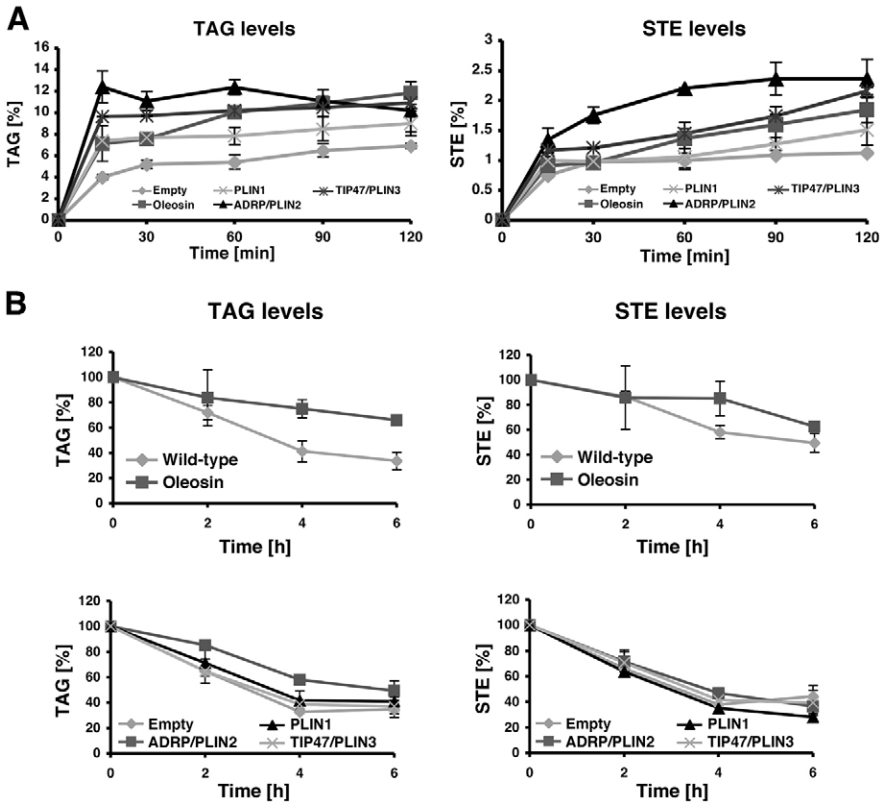

Fig. 5. Expression of LD scaffolding proteins results in increased synthesis of TAG and STE, whereas expression of oleosin results in slower turnover of TAG. (A) Wild-type cells expressing the indicated LD protein were incubated with [^{3}H]palmitic acid (10 μCi/ml) and samples were removed at the indicated time points, lipids were extracted, separated by TLC and quantified by radioscanning. (B) Wild-type cells expressing the indicated LD protein were radiolabeled with [^{3}H]palmitic acid (10 μCi/ml) overnight, cells were diluted into fresh medium containing cerulenin and terbinafine, and samples were removed at the time points indicated, lipids were extracted, separated by TLC and quantified by radioscanning. Values are means ± s.d. of two independent experiments.

half of the LD. By contrast, slow movement of the protein over the LD surface would result in a slow loss of fluorescence in the non-bleached half of the LD. Oleosin displayed a rapid loss of fluorescence in the non-bleached part of the LD, indicating that this protein is able to rapidly move over the LD surface (Fig. 4B). Compared with oleosin, ADRP/PLIN2 and TIP47/PLIN3 showed a slower loss of fluorescence, indicating that their mobility on the LD surface is restricted (Fig. 4B). Restricted mobility was particularly evident for TIP47/PLIN3, suggesting that once the protein has associated with an LD, it rather adheres to the exact spot where it has bound and thus exchanges its lateral position with slower kinetics than does an integral membrane protein, such as oleosin or Dga1 (Jacquier et al., 2011). This partial local immobilization of the perilipin family members might be explained by their proposed scaffolding function, by possible interactions between themselves, by their interactions with other relatively immobile surface components of LDs, or by their binding to other relatively immobile components, such as the cytoskeleton. These data on the lateral mobility of LD proteins are in agreement with FRAP data from animal cells, which revealed that PLIN1 and ADRP/PLIN2 are both able to slowly move over the LD surface (Wang et al., 2009).

Peripheral LD proteins affect neutral lipid synthesis

To examine whether the overexpression of LD proteins would affect the synthesis or turnover of neutral lipids in yeast, cells were labeled with [^{3}H]palmitic acid and the time-dependent incorporation of the labeled fatty acid into neutral lipids was examined (Jacquier et al., 2011). This analysis revealed that incorporation of palmitic acid into TAG and STE was rapid for the first 15 minutes and then either plateaued or increased only slowly for up to 120 minutes (Fig. 5A). Levels of newly synthesized TAG, however, were increased in cells expressing the heterologous LD proteins compared with control cells. This increase in newly synthesized TAG levels was most pronounced for cells expressing ADRP/PLIN2 and resulted in a twofold higher level of TAG compared with control cells (Fig. 5A). The synthesis of STE was also elevated in cells expressing the heterologous LD proteins and was again most pronounced for cells expressing ADRP/PLIN2 (Fig. 5A).

To examine turnover of neutral lipids, cells were labeled with [^{3}H]palmitic acid overnight and then diluted into fresh medium containing cerulenin and terbinafine, drugs that block fatty acid and sterol synthesis and hence force the cells to hydrolyze their neutral lipid pool. Samples were removed after different periods of growth, lipids were extracted, separated by TLC and quantified by radioscanning. This analysis revealed that heterologous expression of oleosin reduced the rate of TAG turnover significantly, but did not affect mobilization of STE (Fig. 5B). Expression of PLIN1, ADRP/PLIN2 and TIP47/PLIN3, by contrast, did not affect the rate of turnover of TAG and STE. These results thus indicate that oleosin protects the TAG pool from hydrolysis by lipases but that the PLINs do not affect access of the endogenous lipases to the stored neutral lipid pool, as is the case in animal cells (Bickel et al., 2009; Brasaemle et al., 2000; Brasaemle, 2007).

LD scaffolding proteins induce the formation of LDs

The increased steady-state levels of TAG that were observed upon expression of any of the heterologous LD structural components is striking and appears not to be due to reduced activity of the respective lipases because the TAG mobilization assays did not reveal any strong difference between cells expressing the PLINs and cells bearing an empty vector control

(Fig. 5B). We have previously shown that LDs are functionally connected to the ER bilayer and proposed that LDs could act as a metabolic sink to sequester neutral lipids that are formed by ER-localized enzymes (Jacquier et al., 2011). Thus, the increased synthesis of TAG and STE observed in cells that express these structural components of LDs from plants and animal cells could be explained by a more-efficient sequestration of neutral lipids within the ER membrane and their efficient packaging within LDs, releasing product inhibition of the respective enzyme and thus increasing the flux towards neutral lipids formation.

To test whether expression of these LD proteins reduces the levels of free neutral lipids within the ER bilayer and promotes their packaging into LDs, we examined LD formation in cells with elevated levels of neutral lipids, particularly TAG and STE, within the ER bilayer. The rate-limiting step for TAG formation is the dephosphorylation of phosphatidic acid to DAG, which is catalyzed by Pah1, the yeast homologue of human lipin, mutations in which result in lipodystrophy (Csaki and Reue, 2010; Pascual and Carman, 2013).

Cells lacking PAH1 have a reduced number of LDs but they contain as many neutral lipids as wild-type cells and they accumulate these neutral lipids, particularly STE, within the ER bilayer, indicating that DAG produced by Pah1 is important for the biogenesis of LDs (Adeyo et al., 2011). Accumulation of neutral lipids in the ER bilayer of *pah1Δ* mutant cells can be visualized by staining with the neutral lipid dye BODIPY 493/503, Nile Red or Oil Red O, and it also appears as electron-translucent inclusions in an aberrant ER when cells are analyzed by transmission electron microscopy (TEM) (Adeyo et al., 2011). In wild-type cells, Nile Red efficiently and prominently stains LDs and shows very low levels of ER staining. To test whether the accumulation of neutral lipids in the ER bilayer and the defect in LD formation in the *pah1Δ* mutant could be overcome by expression of heterologous LD scaffolding proteins, we expressed GFP-tagged version of the LD proteins in the *pah1Δ* mutant background and analyzed their localization in cells that were co-stained with Nile Red. These experiments revealed that the *pah1Δ* mutant alone showed strong Nile Red staining of the ER bilayer, with no detectable punctate staining as would be indicative for the presence of LDs, consistent with an elevated level of neutral lipids within the ER bilayer and a defect in LD formation (Adeyo et al., 2011) (Fig. 6A). Expression of GFP-oleosin, however, resulted in the appearance of punctate structures that were stained with Nile Red. At the same time, Nile Red

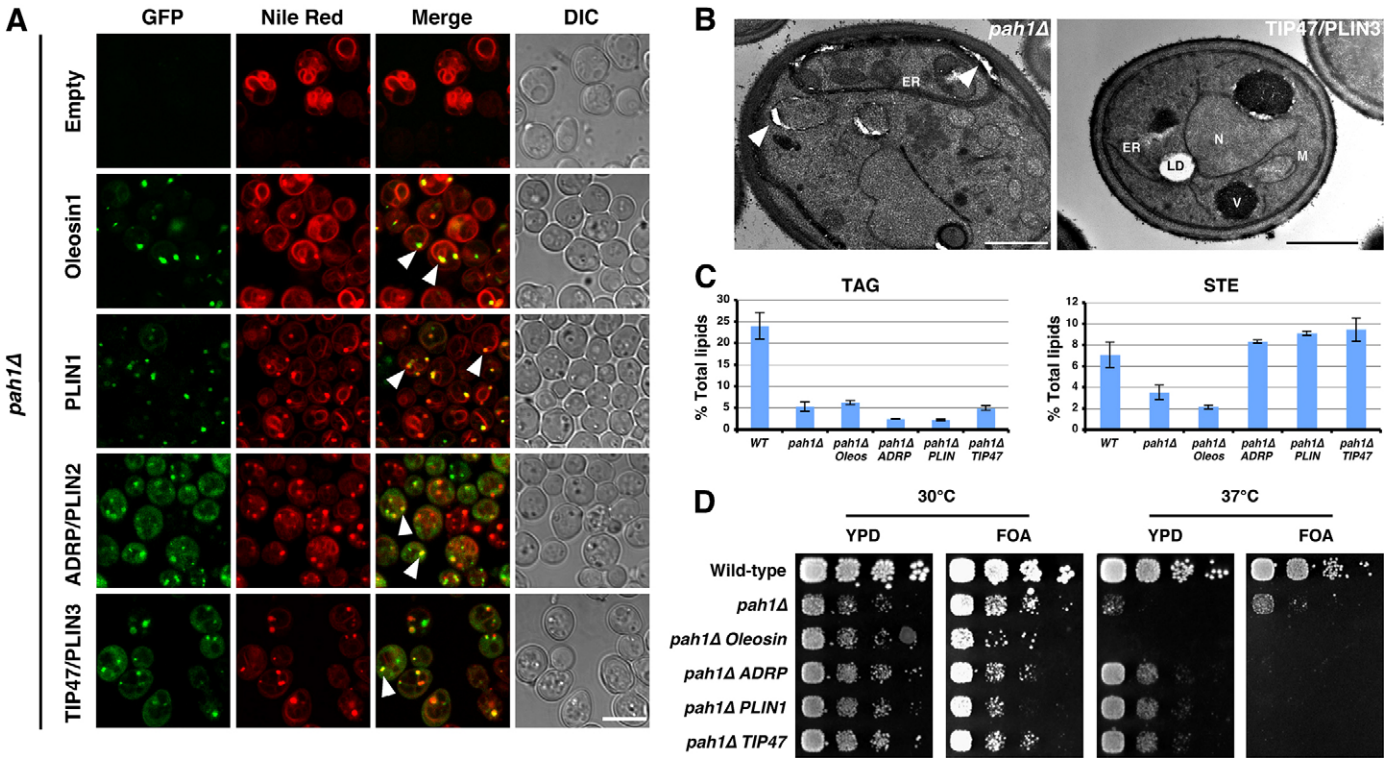

Fig. 6. Expression of LD proteins in a *pah1Δ* mutant results in LD formation, increased levels of STE and rescues the temperature-sensitive growth phenotype. (A) The subcellular localization of GFP-tagged LD scaffolding proteins in the *pah1Δ* mutant was analyzed by confocal microscopy. Neutral lipids in the ER and LDs were stained with Nile Red and the colocalization of LD proteins with Nile-Red-stained material within punctate structures is indicated by arrowheads. Scale bar: 5 μm. (B) Expression of TIP47/PLIN3 rescues the aberrant ER morphology of the *pah1Δ* mutant strain and results in the formation of LDs. The *pah1Δ* mutant strain or a *pah1Δ* mutant expressing TIP47/PLIN3 was cultivated in minimal media, fixed with glutaraldehyde, embedded in Spurr's resin and processed for ultrastructural analysis by TEM. The membrane of the ER is indicated and arrowheads indicate aberrant accumulation of neutral lipids. N, nucleus; V, vacuole; M, mitochondria; LD, lipid droplet. Scale bars: 1 μm. (C) Expression of perilipins results in increased levels of STE in the *pah1Δ* mutant. Wild-type cells and the *pah1Δ* mutant expressing the indicated LD protein were radiolabeled with palmitic acid overnight, lipids were extracted, separated by TLC and quantified by radioscanning. Values are means ± s.d. of two independent experiments. (D) Expression of perilipins rescues the growth defect of *pah1Δ* at 37℃. Cells of the indicated genotype were cultivated overnight, serially diluted 10-fold and dilutions were spotted on YPD plates and on plates containing FOA to counterselect for the presence of the plasmid-borne copy of the respective LD-localized gene product. Plates were incubated at either 30℃ or 37℃ for 3 days.

staining of the ER bilayer appeared to be reduced, indicating that expression of oleosin in a *pah1Δ* mutant background promoted the formation of LDs, possibly by sequestering neutral lipids from the ER bilayer and promoting their packaging into LDs. Expression of the three perilipin family members, PLIN1, ADRP/PLIN2 and TIP47/PLIN3, resulted in a similar if not stronger induction of LD formation compared with that observed by expression of oleosin (Fig. 6A). The fact that expression of all four proteins promoted the formation of LDs in this sensitized genetic background, suggests that LD formation, possibly by sequestering neutral lipids from the ER bilayer, might be a common mode of action of these scaffolding proteins. This is consistent with a morphological analysis of the *pah1Δ* mutant expressing TIP47/PLIN3 by TEM. The *pah1Δ* mutant displays electron-translucent material in an aberrant ER membrane and no, or only a few, LDs are detectable (Adeyo et al., 2011). Mutant cells expressing TIP47/PLIN3, however, show a normal ER morphology and display morphologically normal-looking LDs (Fig. 6B).

To determine whether expression of these LD proteins in the *pah1Δ* mutant resulted in increased levels of TAG or STE, as would be predicted if the presence of these scaffolding proteins shifted the biosynthetic equilibrium towards storage of neutral lipids within metabolically more inert LDs, we labeled cells with palmitic acid overnight and quantified the levels of neutral lipids. This analysis, revealed that the reduced level of TAG in the *pah1Δ* mutant was not increased by the expression of the LD scaffolding proteins, which is consistent with the fact that Pah1 activity is rate-limiting for TAG formation, even in the presence of proteins that promote LD formation (Fig. 6C). Expression of the perilipins, however, greatly increased the levels of STE, suggesting that the presence of these proteins releases a metabolic block in STE formation that is present in the *pah1Δ* mutant. We also note that expression of oleosin does not result in increased levels of STE formation.

The *pah1Δ* mutant is temperature-sensitive for growth at 37℃ (Santos-Rosa et al., 2005). Remarkably, this growth defect is ameliorated by expression of the perilipins, but not by that of oleosin (Fig. 6D). These data indicate that temperature-sensitive growth defect of the *pah1Δ* mutant correlates with the reduced levels of STEs observed in the mutant, and suggest that sequestration of STEs into LDs might become important if cells are temperature stressed, possibly because they accumulate toxic levels of free sterols and/or STEs within the ER bilayer, if these free sterols cannot efficiently be esterified and packaged into LDs (Adeyo et al., 2011; Bacia et al., 2005).

Diacylglycerol is sufficient to induce LDs formation by the scaffolding proteins

To examine whether increased levels of DAG would be sufficient to promote LD formation by the scaffolding proteins, we examined the subcellular localization of these proteins in cells lacking the capacity to form neutral lipids and bearing a deletion of the DAG kinase *DGK1*. *DGK1* encodes the major DAG kinase in yeast, and cells lacking this activity have elevated levels of endogenous DAG (Fakas et al., 2011; Han et al., 2008). Fluorescence microscopy of cells expressing PLIN1 or of those expressing TIP47/PLIN3 revealed the presence of punctate structures that colocalized with Nile-Red-stained LDs in cells lacking DGK1, indicating that elevated endogenous levels of DAG is sufficient to promote LD formation from membranes enriched with DAG (Fig. 7). To examine whether exogenously

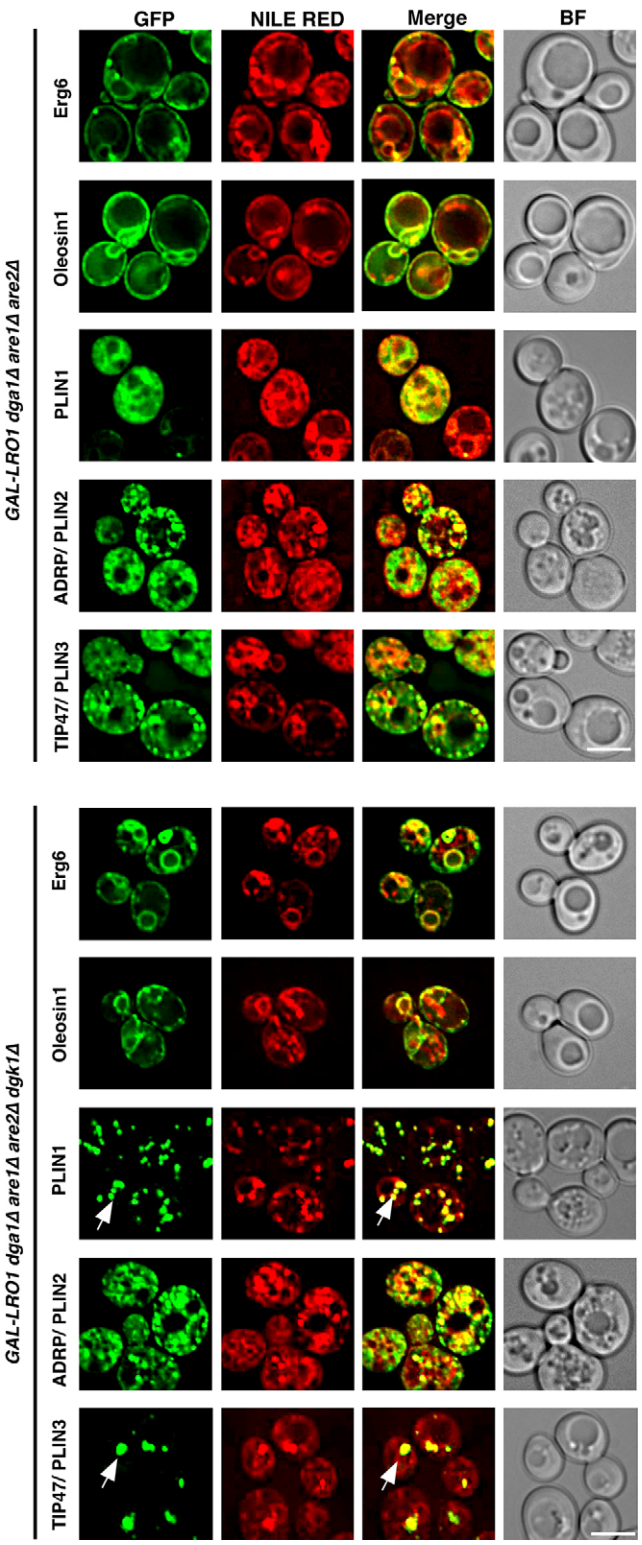

Fig. 7. Increase of endogenous DAG levels is sufficient to promote LD formation in cells expressing PLIN1 or TIP47/PLIN3. The colocalization of GFP-tagged LD scaffolding proteins with Nile-Red-stained LDs in cells lacking the capacity to form neutral lipids (*GAL-LRO1 dga1Δ are1Δ are2Δ*) and containing or lacking a functional copy of the DAG kinase *DGK1*, was analyzed by fluorescence microscopy. The colocalization of LD proteins with Nile-Red-stained material within punctate structures is indicated by arrowheads. Scale bars: 5 μm.

added cell-permeable DAG (C8-DAG) could also induce the formation of LDs in cells lacking the neutral lipid biosynthetic enzymes (*GAL-LRO1 dga1Δ are1Δ are2Δ*), but expressing the scaffolding proteins, cells were incubated with C8-DAG or with the solvent alone for 45 minutes and LD formation was analyzed by fluorescence microscopy. These experiments revealed that DAG addition promoted LD formation in cells expressing the scaffolding proteins, as indicated by the co-staining of punctate structures by the GFP-tagged scaffolding proteins and Nile Red. No comparable LD formation was observed if cells were incubated with the solvent alone, or if they did not express any of the scaffolding proteins (Fig. 8). A similar induction of LDs

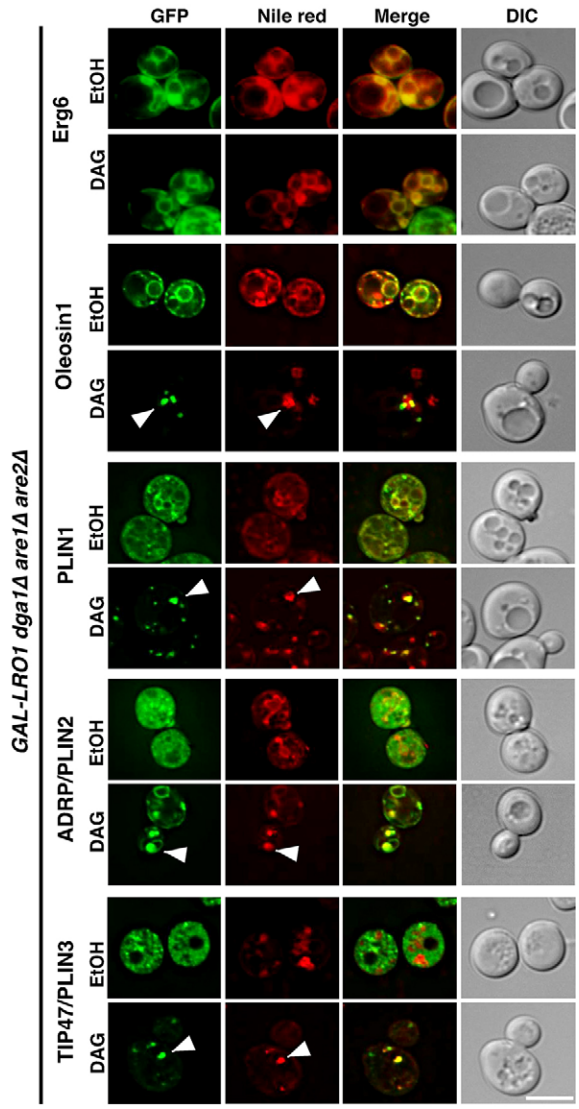

Fig. 8. Cell-permeable DAG induces LD formation in cells expressing the LD scaffolding proteins and promotes membrane association of TIP47/PLIN3. The subcellular localization of GFP-tagged LD scaffolding proteins in cells lacking the capacity to form neutral lipids (*GAL-LRO1 dga1Δ are1Δ are2Δ*) was analyzed by fluorescence microscopy. Cells were incubated with the cell-permeable DAG or with ethanol alone for 45 minutes at room temperature, stained with Nile Red and analyzed by fluorescence microscopy. The colocalization of LD proteins with Nile-Red-stained material within punctate structures is indicated by arrowheads. Scale bar: 5 μm.

was also observed in quadruple mutant cells lacking all four enzymes required for storage lipid synthesis (supplementary material Fig. S5). Analysis of uptake of the cell-permeable DAG in cells labeled with [^{3}H]palmitic acid indicated that the short-chain DAG is efficiently taken up by the cells and that it can be metabolically converted to TAG if Lro1 expression is induced by galactose (supplementary material Fig. S6).

To examine whether the LDs that were formed by the addition of DAG to cells expressing the scaffolding proteins are morphologically comparable to LDs of wild-type cells, cells were analyzed by TEM. These experiments revealed the presence of characteristic electron-translucent globular structures that were limited by an electron-dense structure in DAG-treated cells. These structures were morphologically indistinguishable from LDs present in wild-type cells (supplementary material Fig. S7).

Quantification of the number of LDs formed upon DAG treatment indicates that the addition of DAG to cells expressing TIP47/PLIN3 resulted in an average of 2.8 LDs per cell, whereas 0.8 LDs were observed in cells treated with the solvent alone. Wild-type cells, however, contained an average of 3.6 LDs per cell section (supplementary material Fig. S7). These results indicate that TIP47/PLIN3-dependent formation of LDs upon addition of DAG is efficient. On the basis of these results, we conclude that addition of the cell-permeable DAG is sufficient to promote membrane association of TIP47/PLIN3 and to induce the formation of LDs that are morphologically indistinguishable from LDs in wild-type cells.

Discussion

Here, we show that heterologous expression of LD scaffolding proteins from plants and animal cells in yeast promotes the formation of LDs from an ER bilayer enriched in neutral lipids, as is the case in *pah1Δ* mutant cells, or in cells lacking the capacity to form neutral lipids but having elevated levels of DAG as a result of the deletion of the DAG kinase, or upon treatment with a cell-permeable DAG. These data indicate that expression of these LD scaffolding components is sufficient to induce LD formation from a sensitized membrane – one containing elevated levels of neutral lipids such as TAG, STE or DAG – suggesting that these proteins sequester neutral lipids within a bilayer membrane to induce droplet formation. The fact that expression of any of these components is sufficient to induce droplet formation in yeast indicates that any additional factors required for LD formation are either conserved or directly promote LD formation. The fact that the two classes of scaffolding proteins analyzed here, oleosin and PLINs are structurally very different (oleosin is an integral membrane protein whereas PLINs are soluble in the absence of LDs) indicates that the function of these proteins in LD formation is more direct.

Cells lacking the phosphatidate phosphatase Pah1 have previously been shown to exhibit a defect in LD formation and accumulate elevated levels of neutral lipids, particularly STE, in the ER bilayer (Adeyo et al., 2011). This defect in LD biogenesis of *pah1Δ* mutant cells can be bypassed by deletion of the DAG kinase DGK1, and thus has been attributed to reduced levels of DAG, which possibly needs to be formed in the proximity of the growing droplets (Adeyo et al., 2011).

The rescue of the defect in LD biogenesis upon expression of oleosin and PLINs observed in this study could thus be explained by an increase in DAG levels. Although we cannot formally rule out this possibility, we consider it unlikely because both oleosins

and PLINs are abundant proteins and their abundance correlates with the amount of storage lipids present in a given cell, which is indicative of a structural rather than catalytic role of these proteins in droplet formation. Even if these proteins had no catalytic function in DAG formation, their expression could result in activation of other pathways that result in increased DAG production, such as an increased synthesis or turnover of sphingolipids, however, we also consider this possibility unlikely because there is no structural conservation between oleosin and PLINs that could explain a shared function of these proteins in activating such an alternative pathway to bypass Pah1 function.

The fact that expression of the LD scaffolding proteins results in an increased incorporation of palmitic acid into neutral lipids might be explained by (1) an elevated activity of the respective biosynthetic enzymes, such as Lro1 and Dga1 in TAG formation, and Are1 or Are2 in STE synthesis; (2) more-efficient packaging of the neutral lipids into LDs, thus releasing any possible negative-feedback inhibition of the respective biosynthetic enzymes through product accumulation in the ER bilayer; or (3) reduced activity and/or access of the respective lipases to the neutral lipid core of LDs and thus a reduced basal turnover of these neutral lipids.

The observation that oleosin is in the ER bilayer in cells having no LDs and that the protein is rapidly degraded is interesting and suggests that oleosin is unstable when present in a bilayer membrane and that the protein becomes stabilized only when present on LDs. When translated in a reticulocyte lysate in the presence of an artificial oil emulsion composed of TAG and phospholipids, oleosin is targeted to the oil emulsion (Chen and Tzen, 2001). This *in vitro* targeting of oleosin to oil droplets, suggests that targeting of the protein to LDs is an intrinsic property of the protein itself that is possibly due to a high affinity of the protein towards membranes containing elevated levels of neutral lipids and/or having an unusual structure, such as the monolayer surrounding LDs. High affinity of oleosin to neutral lipids could also account for the low degree of exchange of GFP-oleosin between different LDs, as observed in the photobleaching experiments, which indicate that the rate at which oleosin dissociates from LDs is low, compared with that of other integral membrane proteins, such as Dga1 (Jacquier et al., 2011). In addition, the rate of exchange of oleosin between different LDs and between the ER bilayer and LDs might be further reduced by the high rate of turnover of the protein within the ER bilayer, which it might have to cross to move from one LD to the next.

Photobleaching experiments with ADRP/PLIN2 and TIP47/PLIN3, by contrast, revealed rapid exchange of these soluble proteins between different LDs. In animal cells, PLIN1 and ADRP/PLIN2 are present in cells containing neutral lipids where they localize to LDs and both are rapidly degraded in the absence of neutral lipids (Brasaemle et al., 1997a; Wolins et al., 2006; Xu et al., 2005). In our study, PLIN1 was rapidly turned over, both in the presence and absence of LDs. Even though it still decorated LDs under steady-state conditions, PLIN1 might not be able to adopt a stable fold when bound to yeast LDs. This is in contrast to ADRP/PLIN2, which was stable both in the presence and absence of LDs, even though it was the most soluble of the three PLINs tested here because it partitioned with the soluble fraction even in cells containing LDs.

TIP47/PLIN3, however, is stable even in the absence of LDs (Wolins et al., 2001; Wolins et al., 2005; Wolins et al., 2006). Consistent with its stability in animal cells lacking LDs, TIP47/PLIN3 was also stable in yeast having no LDs and it rapidly exchanged between different LDs as revealed by the photobleaching experiments. These results are thus in agreement with FRAP experiments performed in Chinese hamster ovary cells, which revealed that ADRP/PLIN2 rapidly exchanged between an LD-associated and a cytoplasmic pool, whereas PLIN1 did not (Wang et al., 2009). Both proteins, however, can slowly move over the surface of the LD and PLIN1 has recently been shown to move between LDs and the ER (Skinner et al., 2013; Wang et al., 2009).

How do these proteins recognize the LDs and tightly bind to an apparently unique feature on the peripheral membrane monolayer of LDs? The observation that PLIN1 and TIP47/PLIN3 sediment with microsomes and LDs and that they even resist solubilization in 1 M NaCl, indicates that these proteins are very tightly anchored within the periphery of LDs. Our observation that expression of PLINs in cells having elevated levels of neutral lipids in the ER membrane, i.e. in the *pah1Δ* or *dgk1Δ* mutant, promotes the formation of LDs suggests that these proteins directly act in sequestering neutral lipids within the ER bilayer and possibly induces formation of LDs from the ER membrane. PLINs might thus assemble on membranes containing neutral lipids and in doing so induce a lateral concentration of neutral lipids within the membrane. In mammalian cells, PLINs have previously been shown to promote LD formation from cellular membranes loaded with cell-permeable DAG (Skinner et al., 2009). It has been proposed that TIP47/PLIN3 recognizes membranes enriched in neutral lipids by their increased spacing of phospholipid head groups, which is induced to accommodate neutral lipids within the bilayer (Bulankina et al., 2009). PLINs might thus function in a similar manner to lipoproteins in that they promote package of a membrane-enclosed particle enriched in neutral lipids (Bulankina et al., 2009). The recent observation that different PLINs sequester to LDs that contain either TAG or STE could indicate that different PLINs have distinct affinities for membranes enriched in either TAG or STE (Hsieh et al., 2012).

Consistent with such a lipid-sequestering function of PLINs, the C-terminal domain of TIP47/PLIN3 has structural similarities to apolipoprotein E and the protein can reorganize liposomes into small lipid discs *in vitro*, as does ApoE (Bulankina et al., 2009; Hickenbottom et al., 2004). The conserved N-terminal domain of TIP47/PLIN3, however, is composed of 11-mer repeats, which have been postulated to form an unusual right-handed helix with three turns per 11-mer repeat, possibly forming a TAG-miscible and a water-exposed surface (Bussell and Eliezer, 2003). How these two domains within TIP47/PLIN3 cooperate in lipid binding and formation of nascent LDs remains to be determined.

Taken together, the observation that expression of oleosin and PLINs in yeast cells, which have elevated levels of neutral lipids within the ER membrane, is sufficient to promote LD formation, suggests that steady-state levels of neutral lipids within a biosynthetically active ER bilayer might be high enough to sustain a protein-assisted sequestration into domains and ultimately the formation of a particle filled with neutral lipids. Although yeast does not require oleosin or PLINs for LD formation if DAG levels within the ER are sufficiently high, it might be that the small size of LDs formed in yeast (~0.3 μm) compared with those of plant and animal cells allows these cells to form these lipid deposits without the help of such scaffolding components. Alternatively, the function of oleosin and PLINs in sequestering neutral lipids within a bilayer and promotion of LD

formation could be taken over by other lipid-binding proteins, such as the Dga1 and/or other lipid-modifying enzymes that reside in high abundance on LDs in yeast.

Materials and Methods

Yeast strains and growth conditions

Yeast strains and their genotypes are listed in supplementary material Table S1. Strains were cultivated in YP-rich medium (1% Bacto yeast extract, 2% Bacto peptone (USBiological Swampscott, MA) or minimal medium [0.67% yeast nitrogen base without amino acids (USBiological), 0.73 g/l amino acids] containing 2% glucose.

GFP tagging, subcellular fractionation and western blot analysis

Genes encoding oleosin 1 from *Arabidopsis* (ATol1) and the perilipin family members were amplified by PCR from the respective cDNAs (*Arabidopsis* Biological Resource Center, Ohio State University, Columbus, OH; Source BioScience, Nottingham, UK) and cloned as N-terminal fusions with GFP into a pGREG576-based plasmid, in which the GAL promoter was replaced by an ADH promoter (Jansen et al., 2005). Differential fractionation, salt and detergent extractions were performed as previously described (Köffel et al., 2005). Crude LDs were isolated by flotation on a 12% Ficoll 400 gradient as described (Leber et al., 1994). Protein concentration was determined using the Folin reagent and BSA as standard.

Lipid labeling

Neutral lipids synthesis and accumulation was monitored by labeling cells that were grown with 10 µCi/ml [9,10-^{3}H]palmitic acid (10 mCi/ml; American Radiolabeled Chemicals, St Louis) for the indicated period of time as previously described (Jacquier and Schneiter, 2010). TAG mobilization was monitored in cells that were labeled overnight with 10 µCi/ml [^{3}H]palmitic acid and then diluted in fresh medium supplemented with cerulenin (10 µg/ml) and terbinafine (30 µg/ml) as described (Köffel et al., 2005).

Fluorescence microscopy

Localization of GFP- and RFP-tagged proteins was performed by fluorescence microscopy of living cells using a Leica TCS SP5 confocal microscope with LAS AF software, or a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA). Confocal images were recorded with a 63× /1.20 HCX PL APO objective with a zoom of 6. Cells were stained with Nile Red (10 µg/ml, Sigma-Aldrich, St Louis, MO) for 1 minute at room temperature and washed twice with PBS. The cell-permeable DAG analog (1,2-dioctanoyl-sn-glycerol; Avanti Polar Lipids, Alabaster, AL) was added at 1 mg/ml to cells and cells were incubated for 45 minutes at room temperature before live-cell imaging or subcellular fractionation. Double labeling and photobleaching experiments were recorded as previously described (Jacquier et al., 2011).

Electron microscopy

10 OD_{600} units of exponentially growing wild-type and mutant cells were harvested and treated with the cell-permeable DAG analog (1 mg/ml) or the solvent alone for 45 minutes at room temperature, and fixed in 1 ml of fixative (1% glutaraldehyde, 0.2% paraformaldehyde and 40 mM potassium phosphate, pH 7.0) for 5 minutes at room temperature. Cells were washed, dehydrated and embedded in Spurr's low-viscosity resin (EMS, Hatfield, PA) as previously described (Jacquier et al., 2011). Semi- and ultra-thin sections were produced, collected on 200 mesh grids, post stained with uranyl acetate and lead citrate, and visualized with a Philips CM-100 TEM, operating at 80 kV. Pictures were recorded on a side-mounted digital TEM camera (Morada Soft Imaging System, Olympus) and images were processed with iTEM software.

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Author contributions

N.J., S.M., V.C. and R.S. designed the experiments; N.J., S.M. and V.C. performed the experiments; N.J., S.M., V.C. and R.S. analyzed the data; N.J. and R.S. wrote the manuscript.

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