Identification of seipin-linked factors that act as determinants of a lipid droplet subpopulation

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Functional heterogeneity within the lipid droplet (LD) pool of a single cell has been observed, yet the underlying mechanisms remain enigmatic. Here, we report on identification of a specialized LD subpopulation characterized by a unique proteome and a defined geographical location at the nucleus–vacuole junction contact site. In search for factors determining identity of these LDs, we screened ~6,000 yeast mutants for loss of targeting of the subpopulation marker Pdr16 and identified Ldo45 (LD organization protein of 45 kD) as a crucial targeting determinant. Ldo45 is the product of a unique splicing event connecting two adjacent genes (YMR147W and YMR148W/OSW5/LDO16). We show that Ldo proteins cooperate with the LD biogenesis component seipin and establish LD identity by defining positioning and surface-protein composition. Our studies suggest a mechanism to establish functional differentiation of organelles, opening the door to better understanding of metabolic decisions in cells.

Introduction

Lipid droplets (LDs) are evolutionarily conserved lipid storage organelles (Gross and Silver, 2014). As such, they need to dynamically balance deposition and mobilization of diverse lipid species to sustain crucial cellular functions, including metabolic homeostasis and biosynthesis of membrane lipids (Farese and Walther, 2009; Welte, 2015; Schuldiner and Bohnert, 2017). Accordingly, LD dysfunction is connected to various pathologic conditions, including obesity, diabetes, steatohepatitis, and lipodystrophy (Greenberg et al., 2011; Walther and Farese, 2012). LDs consist of a central core of neutral storage lipids (mainly triacylglycerols and sterol esters) shielded from the aqueous cytosol by a phospholipid monolayer that accommodates numerous surface proteins (Thiam et al., 2013). Most LD surface proteins are enzymes involved in lipid metabolism, but the function of several LD proteins is still unknown (Athenstaedt et al., 1999; Binns et al., 2006; Grillitsch et al., 2011; Currie et al., 2014; Ohsaki et al., 2014). Intriguingly, in recent years, several cases have been reported in which specific LD surface proteins were found enriched or even exclusively localized on a fraction of LDs (Wolins et al., 2005, 2006; Krahmer et al., 2011; Hsieh et al., 2012; Wülfing et al., 2013; Ren et al., 2014; Moldavski et al., 2015; Zhang et al., 2016; Thiam and Bélanger, 2017). Similarly, various lipids have been found distributed unevenly among LDs (Rinia et al., 2008; Hsieh et al., 2012). These findings demonstrate that the LD pool within a single cell consists of distinct LD subpopulations and suggest that a functional differentiation of LDs might contribute to cellular lipid homeostasis. However, the molecular mechanisms that establish and maintain LD heterogeneity are currently unknown.

In Saccharomyces cerevisiae (from here on termed “yeast”), one protein that localizes preferentially to a subpopulation of LDs is the phosphatidylinositol transfer protein Pdr16 (Ren et al., 2014; Moldavski et al., 2015). In this study, we used systematic screening approaches to characterize the Pdr16-rich LD subpopulation and identified six additional proteins enriched on the same LDs. We show that two of those subpopulation residents, which we term Ldo45 (Ymr147w + Ymr148w) and Ldo16 (Ymr148w/OSW5/LDO16), are the product of a unique splicing event of two overlapping genes and act as key determinants of LD identity. Ldo45 is crucial for targeting of Pdr16 to the LD subpopulation, and Ldo16 mediates accumulation of LDs in a unique niche in the cell, the nucleus–vacuole junction (NVJ) contact site, under conditions of nutrient deprivation. Ldo45 and Ldo16 interact with the seipin complex that controls LD composition. Indeed, overexpression of Ldo45 results in a generalized loss of LD identity similar to loss of function seipin mutants. Our results suggest that through localized modulation of seipin, Ldo proteins mediate LD differentiation.

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Results

A unique LD subpopulation resides proximal to the NVJ

We have previously shown that the LD protein Pdr16 is strongly enriched on just a fraction of cellular LDs in exponentially growing yeast cells (Moldavski et al., 2015). Typically, Pdr16 can be found on one LD per cell or alternatively on few LDs that are often close to each other (Fig. 1 A). Pdr16 is part of the family of Sec14-like phosphatidyl inositol transfer proteins (Li et al., 2000; Schnabl et al., 2003; Ren et al., 2014). This class of proteins has previously been suggested to preferentially localize to organellar contact sites (Šimová et al., 2013; Moldavski et al., 2015; Selitrennik and Lev, 2016), which are specific cellular subdomains where the surfaces of two organelles are actively positioned directly adjacent to each other by tether proteins (Eisenberg-Bord et al., 2016). We therefore sought to determine whether the Pdr16-rich LDs are in close proximity to any other cellular membrane. To this end, we expressed GFP-labeled Pdr16 (Pdr16-GFP) in cells with the different cellular membranes labeled with RFP or Cherry (Fig. S1 A). Although we could not detect any specific spatial relationship between Pdr16-rich LDs and the plasma membrane, peroxisomes or mitochondria, we found that LDs marked by Pdr16 were closely associated with both vacuolar and ER/perinuclear membranes.

To visualize both organelles at the same time, we labeled vacuoles with the blue vacuole luminal dye 7-amino-4-chloromethylcoumarin (CMAC) in cells expressing Pdr16-GFP and the ER marker Sec63-RFP (Fig. 1 B). We found that indeed, Pdr16-rich LDs were often found adjacent to the area where the nucleus and the vacuole were in close proximity to each other, a contact site termed the NVJ (Pan et al., 2000). To test whether Pdr16-rich LDs have a defined spatial relationship to this structure, we genomically tagged the NVJ marker protein Nvj1 with Cherry and found that Pdr16-rich LDs were preferentially located adjacent to the NVJ, whereas Pdr16-poor LDs (labeled by the neutral lipid dye monodansylpentane [MDH]) were dispersed throughout the cell (Fig. 1 C and Fig. S1 B). We conclude that Pdr16-rich LDs are spatially confined to a specific cellular location next to the NVJ.

A high-content screen uncovers modulators of Pdr16 localization

An LD subpopulation that has both a defined local and a unique surface protein must have a molecular mechanism in place to determine its identity. To identify molecular determinants of this LD subpopulation, we used an unbiased systematic screen for factors involved in Pdr16 localization. We generated a genome-wide collection of ∼6,000 yeast strains expressing Pdr16-Cherry in the background of loss of function mutations (deletions for nonessential genes and decreased abundance by mRNA perturbation alleles for essential genes; Tong et al., 2001; Giaever et al., 2002; Tong and Boone, 2006; Breslow et al., 2008; Cohen and Schuldiner, 2011). All strains were imaged by automated microscopy, followed by manual inspection (Breker et al., 2013) to identify mutants with altered Pdr16 localization (Fig. 2 A). We identified 22 mutants that failed to target Pdr16 efficiently to LDs (Fig. 2 B) as well as 30 mutants with an increased number of Pdr16-positive foci compared with the control (Table S1).

Any one of the mutants that failed to properly target Pdr16 could be a direct determinant of LD identity or an indirect effector. To uncover the direct effectors, we performed two follow-up screens. First, we reasoned that a component involved in Pdr16 targeting should be found in proximity to Pdr16 and, thus, used a whole-genome, split dihydrofolate reductase (DHFR) complementation screen (Tarassov et al., 2008). In that screen, Pdr16 was tagged with one half of the DHFR enzyme and assayed for complementation with all other yeast proteins tagged with the other half, to search for components that are localized in the vicinity of Pdr16 (Fig. 2 C). We identified 39 hits in the split DHFR screen (Table S1). Second, we reasoned...
that factors required for Pdr16 targeting should be enriched on the Pdr16-rich subpopulation. To address that point, we assembled a collection of strains expressing all known LD proteins fused to either a C-terminal GFP moiety from the genome-wide C-terminal GFP library (Huh et al., 2003), or an N-terminal GFP tag under the control of a NOP1 promoter from the SWAT
GFP library (Yofe et al., 2016). We used an automated mating approach to cross each GFP-tagged strain with either a strain expressing Erg6-Cherry, which marks all LDs in the cell, or Pdr16-Cherry as a marker of our LD subpopulation. All strains were then imaged by automated microscopy and inspected manually. We identified five additional proteins that were enriched on the Pdr16-rich LD subpopulation (Fig. 2 D, Fig. S1 C, and Table S1): the lipid metabolism enzymes Erg2, Tgl4, and Srt1; and Ymr148w/Osw5 and Bsc2, two proteins of unknown function. We concluded that the Pdr16-rich LD subpopulation has both a unique cellular distribution and is equipped with a special set of proteins.

When cross-comparing the hits from all three screens (the primary Pdr16 targeting screen, the split DHFR screen, and the colocalization screen), we found that only one gene, YMR148W/OSW5, encoding a protein of unknown function, was identified by all three approaches. Ymr148w is thus enriched on Pdr16-rich LDs, can be found in close proximity to the Pdr16 protein, and in its absence, Pdr16 is no longer targeted to LDs. We, therefore, considered Ymr148w a highly promising candidate for a direct determinant of Pdr16 targeting.

Interestingly, YMR147W, the gene just upstream of YMR148W, was also a hit in the Pdr16-targeting screen. Manual recreation of the strains Pdr16-Cherry Δymr148w and Pdr16-Cherry Δymr147w showed that, although LDs were still present in both mutants, as confirmed by labeling with the neutral lipid dye boron-dipyrromethene (BODIPY) 493/503, both deletion of YMR148W and YMR147W led to a complete loss of Pdr16 targeting to LDs (Fig. 2 E).

A unique splicing isoform is required for Pdr16 targeting to LDs

YMR148W was our main candidate for a factor involved in Pdr16 targeting, and we wondered whether the genomic manipulation of the neighboring gene YMR147W was, in fact, affecting the expression of YMR148W. To determine which of the two genes was responsible for the Pdr16-targeting phenotype, we created a Pdr16-Cherry strain expressing YMR148W under the control of the inducible/repressible GALI promoter (GALIp), as well as a strain expressing YMR147W under that same promoter. In the presence of glucose (i.e., GALip repression), we observed complete loss of Pdr16 targeting to LDs in both strains (Fig. 3 A, left). Surprisingly, induction of YMR148W expression by addition of galactose failed to restore Pdr16 localization (Fig. 3 A, right), whereas Pdr16 was targeted efficiently to LDs in the GALip-YMR147W strain under the same conditions, pointing toward a direct role for YMR147W in Pdr16 targeting. Of note, we observed Pdr16 targeting to all LDs instead of a subpopulation under this condition, likely because the GALI promoter is strongly induced in the presence of galactose (Fig. 3 A, right, bottom). Additionally, we observed alterations in LD morphology upon overexpression of both proteins (Fig. 3 A, right; see section Overexpression of Ldo proteins results in LD clustering at the NVJ).

Our rescue experiments were confusing because, on one hand, Ymr148w came up in our split DHFR screen and was enriched in the LD subpopulation, but, on the other hand, it appeared that it was, in fact, Ymr147w that was required for Pdr16 targeting. In databases, YMR147W was originally annotated as a gene encoding a putative protein of unknown function. Intriguingly, several transcriptome studies identified a YMR147W transcript that does not correspond to the annotated gene (Miura et al., 2006; Reiner-Benaim et al., 2014; Schreiber et al., 2015). Strikingly, in this transcript, YMR147W is spliced to its downstream gene, YMR148W. Reverse transcription experiments templated with a total polyA+ RNA fraction purified from our WT strain, coupled to DNA sequence analysis of the amplified cDNA product, confirmed expression of this spliced RNA (unpublished data). The spliced transcript encodes a putative protein corresponding to amino acids 1–194 of the annotated gene YMR147W, 70 amino acids corresponding to part of the annotated promoter of YMR148W, as well as the complete YMR148W sequence (148 amino acids; Fig. 3 B).

To test whether such a fusion protein of Ymr147w and Ymr148w is expressed in vivo, we analyzed a strain with a C-terminal GFP tag on Ymr148w by Western blot using anti–GFP antibodies (Fig. 3 C, lane 2). Indeed, we detected two bands: the upper band corresponded to the expected size of the putative Ymr147w–Ymr148w fusion protein carrying a GFP tag (72 kD) whereas the lower band corresponded to the expected size of regular Ymr148w–GFP (43 kD). Anti-GFP Western blot of a strain encoding an N-terminal GFP tag on YMR147W resulted in just the 72-kD band corresponding to a tagged version of the putative fusion protein, indicating that this large protein was the major Ymr147w variant, whereas a protein corresponding to just the YMR147W gene is not expressed in detectable amounts (Fig. 3 C, lane 3). N-terminal GFP tagging of YMR148W resulted in the expected 43 kD band (Fig. 3 C, lane 4). Finally, we N-terminally tagged YMR147W with Cherry in a Ymr148w–GFP strain and subjected the cells to anti–GFP Western blot. We detected a band migrating ~30 kD higher than the putative GFP-Ymr147w–Ymr148w band (Fig. 3 C, lanes 6 and 7), a shift that corresponds to the size of Cherry. Hence, the detected protein must contain both the GFP tag (used for detection) and the Cherry tag (causing the altered migration), further corroborating that the fusion protein from this unique intergenic splicing reaction was indeed expressed in vivo. We, thus, decided to name Ymr147w Ldo45 for LD organization protein of 45 kD, according to the molecular weight of the spliced product. Accordingly, we suggest renaming Ymr148w/Osw5 as Ldo16 (according to the molecular weight of this protein; also see Teixeira et al. in this issue).

In light of these findings, it is apparent that Ldo45, not Ldo16, is crucial for Pdr16 targeting. Introduction of the GALIp cassette 5′ to the LDO16 gene leads to disruption of expression of the spliced form of LDO45 (Fig. 3 B), resulting in loss of Pdr16-Cherry targeting, both under induction and repression conditions (Fig. 3 A). In contrast, galactose induction of the GALIp-YMR147W strain, which leads to synthesis of Ldo45 (Fig. 3 B), resulted in rescue of Pdr16-Cherry targeting (Fig. 3 A).

We asked whether Ldo45 was dispensable for Pdr16 targeting or whether both Ldo16/45 splice variants were required. Because deletion of the YMR148W gene resulted in the absence of both Ldo16 and Ldo45, we constructed a cDNA plasmid encoding only GFP-Ldo45 under control of a constitutive TEF2 promoter that would allow for individual expression of Ldo45. Importantly, the LDO45 sequence included both the complete LDO16 coding sequence and part of the LDO16 promoter (Fig. 3 B). Therefore, we deleted the three bases corresponding to the start codon of LDO16 to abolish expression of LDO16 from the plasmid, resulting in a plasmid that expressed only LDO45 (pGFP-LDO45, Fig. 3 B). As a control, we constructed an analogous plasmid pGFP-LDO16. When we
Figure 3. Ldo45, the product of an intergenic splicing event, is required for Pdr16 targeting to LDs. (A) A GAL1 promoter was genomically integrated 5′ to YMR147W or YMR148W and targeting of Pdr16-Cherry to LDs, visualized with BODIPY, was assessed by fluorescence microscopy. Pdr16-Cherry targeting was abolished in the presence of glucose (repression) in both strains but rescued by incubation with galactose (induction) for 4 h only in GAL1p-YMR147W cells. Bar, 5 µm. (B) Schematic representation of the YMR147W (blue) and YMR148W (green) loci. A splicing reaction gives rise to a YMR147W-YMR148W fusion transcript (Miura et al., 2006) encoding Ldo45. That transcript corresponds to most of the YMR147W sequence (dark blue) excluding the last 90 nucleotides (light blue), 210 nucleotides of the annotated YMR148W promoter (dark gray), and the full YMR148W sequence (green). Ldo16 is the translation product of YMR148W. (C) Proteins from indicated cells were extracted and subjected to SDS-PAGE and Western blotting using anti-GFP antibodies. C-terminal GFP-tagging of YMR148W resulted in two bands corresponding to Ldo16-GFP and Ldo45-GFP. N-terminal tagging of YMR148W or YMR147W gives rise to only one tagged protein (GFP-Ldo16 or GFP-Ldo45, respectively). Simultaneous tagging of YMR147W with Cherry and YMR148W with GFP gives rise to a Cherry-Ldo45-GFP protein migrating ~30 kD higher than GFP-Ldo45. Promoters used included an endogenous promoter, lanes 2 and 3; NOP1p, lane 4; and TEF2p, lanes 6 and 7. (D) cDNA plasmids encoding GFP-Ldo16 or GFP-Ldo45* (asterisk marking deletion of the YMR148W start codon) under control of a strong constitutive TEF2 promoter or empty vector (e.v.) were transformed into cells with a genomic ymr148w deletion (lacking both Ldo16 and Ldo45) and targeting of Pdr16-Cherry to LDs, visualized with MDH, was assessed by fluorescence microscopy. Ldo16 is dispensable for Pdr16 localization, but overexpression of both Ldo isoforms induces alterations of LD morphology. Bar, 5 µm.
transformed these plasmids into Δymr148 cells, we found that pGFP-LDO45*, but not pGFP-LDO16, fully rescued the targeting of Pdr16-Cherry, showing that Ldo45 is essential for recruiting Pdr16 to LDs, whereas Ldo16 is dispensable (Fig. 3D; Teixeira et al., 2018).

Overexpression of Ldo proteins results in LD clustering at the NVJ

Knowing that Ldo45, but not Ldo16, is required for targeting of Pdr16 to the LD subpopulation, we were curious to determine the function of Ldo16. Intriguingly, we consistently observed that overexpression of either Ldo variant led to alterations in LD morphology, with numerous cells displaying a large, multilobed, neutral lipid-dye–positive structure located in the center of the cell (Fig. 3A and D). We, therefore, asked whether, next to its function in Pdr16 targeting, Ldo16/45 could have an additional role in determining the specific subcellular localization of the Pdr16-rich LD subpopulation, close to the NVJ.

To assess a possible effect of Ldo16 or Ldo45 overexpression on the association of LDs with the NVJ, we integrated a TEF2 promoter for constitutive overexpression of either GFP-Ldo16 or GFP-Ldo45 into an Nvj1-Cherry strain and stained the cells with the LD dye MDH. Similar to the phenotype observed upon acute induction of GAL1p-YMR148W and GAL1p-YMR147W (Fig. 3A), constitutive overexpression of either Ldo isoform resulted in dramatic alterations of LD morphology with frequent appearance of a single, large, multilobed MDH-positive structure per cell, as opposed to approximately five small LDs per focal plane in control cells with unaltered Ldo16/45 levels (Fig. 4A). Approximately 75% of the large MDH-positive structures in cells overexpressing Ldo16 or Ldo45 overlapped with the Nvj1-Cherry signal, as opposed to just 45% of control LDs (Fig. 4A). These results prompted us to turn to immunoelectron microscopy to obtain high-resolution information on the exact nature of the multilobed structure as well as the spatial relationship between the NVJ and the altered LDs upon Ldo16/45 overexpression. Immunoelectron microscopy analysis revealed that the large MDH and BODIPY-positive structures in cells overexpressing Ldo proteins are clusters of several, tightly packed LDs, with Ldo45 overexpression resulting in larger LD clusters than Ldo16.

Figure 4. Role of Ldo16 and Ldo45 in LD accumulation at the NVJ. (A) Overexpression of GFP-Ldo45 and GFP-Ldo16 leads to accumulation of LDs next to the NVJ, marked by Nvj1-Cherry (arrows; top). This effect was quantified by counting the number of LDs labeled by MDH that were close to the NVJ (black) and LDs that were far from the NVJ (gray; bottom). n = 200 LDs. Bar, 5 µm. (B) Overexpression of GFP-Ldo45 or GFP-Ldo16 results in the formation of LD clusters adjacent to vacuolar and nuclear membranes, compared with Ymr148w-GFP control cells expressing Ldo proteins from their endogenous promoters, as determined by immunoelectron microscopy using anti–GFP antibodies. Asterisks mark LDs. N, nucleus; V, vacuole. Bar, 250 nm.
produced. These LD clusters frequently appeared embraced from all sides by the nuclear and vacuolar membranes (Fig. 4 B). These results indicate synthetic overexpression of either Ldo protein is sufficient to relocate LDs to the NVJ.

**Ldo16 is a critical determinant for LD distribution during entry into stationary phase**

We went ahead and searched for physiologic conditions under which LDs accumulate at the NVJ. In our standard experimental condition of exponentially growing culture, we usually find LDs dispersed throughout the cytosol; however, LDs have previously been found to accumulate at the NVJ once cells enter the stationary growth phase (Wang et al., 2014b; Barbosa et al., 2015; Fig. 5, A and B). Strikingly, we found that although 87% of WT LDs are in close proximity to the NVJ in stationary phase, only 43% of LDs in cells with a genomic deletion of \( \text{LDO16} \) were next to the NVJ under the same conditions (Fig. 5 A). This is virtually identical to the basal LD-NVJ colocalization determined in control cells in the exponential phase (45%; Fig. 4 A), indicating that, in \( \Delta \text{ldo16} \) cells, LD accumulation at the NVJ during the diauxic shift was completely abolished. We found that deletion of \( \text{YMR147W} \), which results in the absence of Ldo45, but normal expression of Ldo16, did not negatively affect LD accumulation at the NVJ (Fig. 5 B), indicating that, under physiologic conditions, Ldo16 is required for correct LD distribution, a function that can be fulfilled in the absence of Ldo45.

**A link between Ldo proteins and the LD biogenesis factor seipin**

We asked by which means Ldo16 and Ldo45 proteins exert their roles in defining LD identity. Because we could not detect significant alterations of the phospholipid composition between LD-enriched fractions from control, \( \Delta \text{ldo16/45} \), and Ldo16/45-overexpressing cells (Fig. S2 A), we reasoned that the Ldos might work through a proteinaceous machinery and thus searched for Ldo partner proteins. In a first approach, we performed a high-content screen for components required for the phenotype observed upon overexpression of Ldo45. We introduced a \( p\text{TEF2-GFP-LDO45} \) allele into the genome-wide deletion/decreased abundance by mRNA perturbation library and analyzed all strains by automated microscopy (Fig. 6 A). We identified several phenotypic classes (Fig. 6 B). (1) Strains that lost the LD clustering phenotype typical for Ldo45-overexpressing cells, resulting in reversion to a WT phenotype with LDs being dispersed throughout the cytosol; among these were components associated with splicing, the cytoskeleton, and nuclear and, especially, vacuolar membrane structure and dynamics; we speculate that in the splicing mutants, Ldo45 is not formed in sufficient amounts. (2) Several strains that had supersized LDs, all carrying mutations previously reported to induce supersized LDs by affecting phospholipid biosynthesis (Guo et al., 2008; Fei et al., 2011b). (3) Mutants with enhanced GFP signal, including genes involved in gene expression control. (4) Mutants displaying a weaker GFP-Ldo45 signal, comprising components required for splicing as well as the seipin component \( \text{SEI1} \) (Fig. 6 B).

In a second approach, we performed coimmunoprecipitation of a Ymr148w-GFP strain (expressing both Ldo16-GFP and Ldo45-GFP; Fig. 3 B), followed by mass spectrometry (MS; Fig. 6 C). The top Ldo interactor was Ldb16, an ER protein that, alongside its partner protein Sei1, forms the seipin complex. In contrast, Ldb16 was not copurified from a strain expressing the highly abundant LD protein Erg6-GFP.
In support of a link of seipin and LDO machineries, Ymr147w was previously identified by MS in coimmunoprecipitations using a GFP-tagged variant of Sei1, the second seipin component, as bait (Pagac et al., 2016). Furthermore, SEI1 was also a hit in our visual screen for Ldo partner proteins, with SEI1 deletion resulting in strongly reduced GFP-Ldo45 signal (Fig. 6 B). This phenotype is consistent with Sei1 being an Ldo interaction partner because loss of a physically interacting component often results in subsequent destabilization of the remaining partner protein. We confirmed the MS data by Western blotting and found that both Ldb16 and Sei1 were efficiently coisolated with GFP-tagged Ldo16 (Fig. 6 D, lane 4) as well as with Ldo45 (not depicted). Collectively, these results indicate that both Ldo16 and Ldo45 are linked to seipin (Teixeira et al., 2018).

**Antagonistic roles of seipin and LDO machineries**

Seipin is crucial for regular LD biogenesis and has been found mutated in patients suffering from Berardinelli-Seip congenital lipodystrophy (Magré et al., 2001). This disease has an intriguing combination of symptoms, presenting with virtually complete absence of subcutaneous adipose tissue, but with ectopic fat accumulation and a metabolic syndrome associated with high prevalence of diabetes (Agarwal and Garg, 2004). Despite the obvious importance of seipin in cellular biology and in human health, its exact molecular role is currently unclear. Two leading concepts suggest either a structural role of seipin in LD biogenesis or a role in regulation of phospholipid metabolism (Szymanski et al., 2007; Fei et al., 2008, 2011a; Cartwright and Goodman, 2012). Across species, seipin mutants generally display morphological alterations of LDs (Szymanski et al., 2007; Fei et al., 2008; Boutet et al., 2009). Yeast cells with deletions of SEI1 or LDB16 typically have aggregated LDs, similar to the morphology phenotype we observe upon overexpression of LDO16 or LDO45 (Fig. 4, A and B), suggesting that Ldo proteins could function in an antagonistic manner to seipin. To explore the idea of opposing phenotypes between the seipin complex and the LDO machinery, we turned to inositol-depletion conditions that lead to formation of supersized LDs in seipin mutants (Wang et al., 2014a). We tested whether that was also the case for Ldo overexpression and found that,
indeed, overexpression of either Ldo protein resulted in formation of supersized LDs in \( \Delta sei1 \) cells and in cells overexpressing Ldo proteins result in enlarged MDH signal as well as in the hollow appearance of the signal of the LD surface protein Faad-GFP. Bar, 5 µm. (B) Schematic representation of a visual screen in which GAL1p-LDO16 or GAL1p-LDO45 cells were crossed with a library expressing all known LD proteins with an N-terminal GFP tag, grown on glucose (GAL1p repression) or galactose (GAL1p induction/overexpression) and imaged. 15 proteins were identified that failed to target efficiently to LDs exclusively upon Ldo45 overexpression. Full list of hits is provided in Table S1. (C) Example of targeting phenotype of a hit from the screen described in B. Strains GFP-Erg7 GAL1p-LDO16 and GFP-Erg7 GAL1p-LDO45 from the screen and a GFP-Erg7 GAL1p-SEI1 control strain were imaged in the presence of glucose (repression) or galactose (overexpression). LDs were labeled with MDH. Upon overexpression of Ldo45 as well as repression of SEI1, Erg7 fails to localize to LDs. Bar, 5 µm.

Figure 7. Antagonistic roles of LDO and seipin machineries. (A) Indicated cells were cultured in synthetic medium lacking inositol and analyzed by fluorescence microscopy. Supersized LDs in \( \Delta sei1 \) cells and in cells overexpressing Ldo proteins result in enlarged MDH signal as well as in the hollow appearance of the signal of the LD surface protein Faad-GFP. Bar, 5 µm. (B) Schematic representation of a visual screen in which GAL1p-LDO16 or GAL1p-LDO45 cells were crossed with a library expressing all known LD proteins with an N-terminal GFP tag, grown on glucose (GAL1p repression) or galactose (GAL1p induction/overexpression) and imaged. 15 proteins were identified that failed to target efficiently to LDs exclusively upon Ldo45 overexpression. Full list of hits is provided in Table S1. (C) Example of targeting phenotype of a hit from the screen described in B. Strains GFP-Erg7 GAL1p-LDO16 and GFP-Erg7 GAL1p-LDO45 from the screen and a GFP-Erg7 GAL1p-SEI1 control strain were imaged in the presence of glucose (repression) or galactose (overexpression). LDs were labeled with MDH. Upon overexpression of Ldo45 as well as repression of SEI1, Erg7 fails to localize to LDs. Bar, 5 µm.
Discussion

We report on the identification of a specialized LD subpopulation with a unique protein composition that is positioned next to the NVJ. Both characteristics are determined by the subpopulation residents Ldo16 and Ldo45, two proteins derived from overlapping genes with the latter formed by a unique, intergenic splicing event. Although Ldo45 is crucial for correct targeting of the subpopulation resident Pdr16, Ldo16 is involved in recruiting LDs to the NVJ.

Formation of subpopulations within the cellular LD pool might offer an expansion of the functional capacity of this organelle and/or enhance its flexibility in responding to environmental cues. How such heterogeneity of the cellular LD pool is established and maintained, however, is unknown. The morphology phenotype of Ldo16/45 overexpression strains as determined by fluorescence and immunoelectron microscopy shows that Ldo proteins induce clustering of LDs on vacuolar and ER membranes. Indeed, tethering to distinct partner organelles might be a simple and efficient way to induce LD heterogeneity by permanently locking them in specialized environments with unique features. At the same time, binding to a membrane surface generates a unique subdomain on the tethered LD that might attract specific proteins and/or lipids, the functions of which could further propagate into imprinting permanent molecular LD identity. Pdr16 is a lipid-transfer protein that has previously been suggested to function in the context of organelle contact sites and potentially can act as a tethering molecule (Šimová et al., 2013; Moldavski et al., 2015; Selitrennik and Lev, 2016). In addition, Pdr16 has been shown to inhibit fat mobilization from LDs via a phosphatidylinositol-4-phosphate–dependent mechanism (Ren et al., 2014), which is consistent with Pdr16 potentially acting as a rheostat on lipid exchange between the vacuole and LDs. Thus, we hypothesize that confinement to distinct cellular landmarks by tethering might be a general mechanism for imprinting LD identity and function. It is currently unclear whether Ldo16/45 directly act as molecular LD tethers or whether Ldo proteins promote formation of LD contact sites indirectly via downstream effectors. Candidates for downstream factors acting on LD recruitment to membrane surfaces are other proteins enriched in this unique LD subpopulation (Table S1) and components identified in our genome-wide screen for mutants affecting the LD clustering phenotype upon Ldo45 overexpression (Fig. 6 B).

One effector identified in this screen is seipin, an LD biogenesis component located at contact sites between LDs and the ER. Seipin has previously been reported to prevent equilibration of LD and ER surface components, thus affecting general LD identity as compared with the ER (Gripping et al., 2015). The interplay we find between Ldo45 and (to a lesser extent) Ldo16 with seipin may offer a clue to their function. Under physiologic conditions, LDs are enriched on the NVJ specific LD subpopulation and hence may be modulating seipin activity only in specific LDs, resulting in altered entry of certain proteins and potentially enabling creation of an LD subpopulation with altered surface composition.

Seipin is conserved from yeast to human. According to basic primary sequence comparison, Ldo proteins are restricted to Saccharomycetes. Of note, one Saccharomycyes strain has an LDO45 gene corresponding to the spliced version of S. cerevisiae LDO45 (GenBank: KOH48639.1), highlighting the functional importance of that component. Remote homology searches with HHsearch identify TMEM159/promethin as a protein with strong similarity to Ldo45 (Fig. S2 C). Promethin is found in numerous species, including human and fly. In fungi, occurrence of promethin is mutually exclusive with Ldo45, supporting an orthologous role for the two proteins. The closest proteins in terms of primary sequence to promethins are the oleosins, which are key structural LD proteins in plants (D’Andrea, 2016). Because promethin is up-regulated in liver cells during lipid storage conditions (Yu et al., 2004), and seipin has been identified as high-score interactor of promethin in a high-throughput affinity–purification MS approach (https://thiobiogrid.org/166968/publication/the-bioplex-network-of-human-protein-interactions-additional-unpublished-ap-ms-results.html), we consider it a promising candidate for an Ldo homologue.

More broadly, by aiming to identify the molecular identity of a unique LD subpopulation and the mechanisms governing it, we have discovered a new LD protein formed by an intergenic splicing reaction and have elucidated a function for two previously uncharacterized proteins. Our results suggest a conserved mechanism for imprinting identity into organelles that underlies our bodies’ ability to maintain energy homeostasis.

Materials and methods

Strains and plasmids
Saccharomyces cerevisiae strains used in this study are described in Table S2. Plasmids are described in Table S3. Plasmids Sec63-RFP-URA and P4636-RFP-PTSI-URA were provided by J. Gerst (Weizmann Institute of Science, Rehovot, Israel). Plasmid pBS35-mCherry-hygromycin was provided by N. Barkai (Weizmann Institute of Science, Rehovot, Israel). Plasmid MTS-RFP-URA was provided by J. Nunnari (University of California, Davis, Davis, CA). Yeast strains were constructed from the laboratory strain BY4741 (Brachmann et al., 1998). Cells were genetically manipulated using a transformation method that includes the usage of Li-acetate, polyethylene glycol, and single-stranded DNA (Longtine et al., 1998; Janke et al., 2004; Gietz and Woods, 2006).

Primers for manipulations and validation were designed using Primers4–Yeast (Yofe and Schuldiner, 2014).

Yeast culturing and microscopy
Yeast cells were cultured overnight in synthetic minimal medium (0.67% [wt/vol] yeast nitrogen base with ammonium sulfate, 2% [wt/vol] glucose, amino acid supplements) at 30°C. Subsequently, cells were diluted and grown until reaching midlogarithmic phase. For experiments performed in the stationary phase, samples were kept undiluted. Cells were moved to glass-bottom, 384-well microscope plates (Matrical Bioscience) coated with Concanaevalin A (Sigma-Aldrich). After 20 min, wells were washed twice with medium or with PBS to remove nonadherent cells. Different dyes were used for labeling organelles: For vacuolar lumen staining, CellTracker Blue CMAC Dye (10 µM; Invitrogen). Yeast cells were imaged at room temperature using a VisiScope Confocal Cell Explorer system composed of a Zeiss Yokogawa spinning disk scanning unit (CSU-W1) coupled with an inverted IX83 microscope (Olympus). Single–focal-plane images were acquired with a 60x oil lens (NA 1.4) and were captured using a PCO-Edge sCMOS camera, controlled by VisiView software (GFP [488 nm], RFP [561 nm], or BFP [405 nm]).
Automated library preparation
Query strains for screens (Pdr16-Cherry, Erg6-Cherry, GFP-Ldo45, GAL1p-LDO45, and GAL1p-LDO16) were constructed on a synthetic genetic array ready strain and were integrated into yeast libraries using the synthetic genetic array method (Tong and Boone, 2006; Cohen and Schuldiner, 2011). A RotOr bench-top colony array instrument (Singer Instruments) was used to handle libraries (Tong and Boone, 2006; Cohen and Schuldiner, 2011). Strains from opposing mating types harboring the desired genomic manipulations (mutation/deletion/ tag, etc.) were mated, and diploid cells were selected. Sporulation was induced (by moving the yeast to nitrogen starvation media for 7 d), and the haploid cells were selected using canavanine and thiamine (Sigma-Aldrich). By moving the haploid cells to plates containing selections for the combination of manipulations desired, a final library containing the genomic traits was created. Representative strains of the resulting screening libraries were validated by manual microscopy and check PCR.

High-throughput microscopy
Libraries were screened at room temperature using an automated, inverted fluorescence microscopic ScanR system (Olympus), during midlogarithmic growth (Breker et al., 2013). Images were acquired using a 60x air lens (NA 0.9) with excitation at 490/20 nm (GFP) or 572/35 nm (RFP). After acquisition, images were manually reviewed using the ImageJ analysis program.

Immunoelectron microscopy
Cells were fixed, embedded in gelatin, and cryosectioned as in Griffith et al. (2008). Sections were then immuno-labeled using rabbit anti–GFP (chromatin immunoprecipitation [ChIP] grade ab290; Abcam), followed by protein A–gold detection. Sections were imaged in a FEI CM100bio electron microscope at 80 KV, equipped with a digital camera (Morada; Olympus).

Protein proximity assay (split-DHFR assay)
Pdr16 was C-terminally tagged with one half of a methotrexate-resistant variant of the DHFR enzyme, and the resulting strain was crossed with a library of strains in which each strain expressed one protein C-terminally tagged with the other half of the enzyme (Tarassov et al., 2008). Diploid cells were then moved to plates containing methotrexate, which inhibits the endogenous DHFR, whereas the mutated enzyme variant remains functional (Tarassov et al., 2008). Large colonies form if the tagged proteins are far apart. Colony size was analyzed using the Balony software (Young and Loewen, 2013).

Preparation of whole cell extracts and Western blot
Cells expressing GFP tagged proteins and control cells were harvested by centrifugation, and proteins were extracted by NaOH or TCA extraction (Kushnirov, 2000; Ast et al., 2013). Samples were analyzed by SDS-PAGE and Western blotting using an anti–GFP antibody (ChIP grade ab290; Abcam). Membranes were either probed with a secondary antibody coupled to horseradish peroxidase (0545; Sigma-Aldrich) for visualization by enhanced chemiluminescence, or with a secondary antibody coupled to horseradish peroxidase (0545; Sigma-Aldrich), followed by protein A–gold detection. Sections were imaged in a FEI CM100bio electron microscope at 80 KV, equipped with a digital camera (Morada; Olympus).

GFP affinity chromatography and MS
Cells expressing GFP-tagged proteins and untagged control cells were grown to midlogarithmic phase in synthetic minimal medium, harvested, washed in distilled water, and resuspended in lysis buffer (50 mM Tris HCl, pH 7, 150 mM NaCl, and protease inhibitors [complete EDTA-free cocktail; Roche]). Subsequently, samples were snap-frozen in liquid nitrogen and ground using a ball mill (30 s at 30 Hz; Retsch). Samples were resuspended in lysis buffer supplemented with 1% digitonin (Sigma-Aldrich) and incubated at 4°C for 1 h. After centrifugation at 19,000 rpm in an SW41 swing-out rotor for 30 min at 4°C, GFP-trap (Chromotek) was added, and samples were incubated for 1 h at 4°C. Three washes were performed with lysis buffer, followed by two additional washes in PBS.

For analysis by Western blot, bound proteins were eluted by the addition of 0.2 M glycine, pH 2.5. Samples were neutralized by the addition of 10% (vol/vol) 1 M Tris, pH 9.4, and analyzed by SDS-PAGE and Western blotting using an anti–GFP antibody (ChIP grade ab290; Abcam), and antibodies directed against Sei1, Ldb16 (both provided by P. Carvalho, University of Oxford, Oxford, England, UK), and histone H3 (ChIP grade ab1791; Abcam).

For analysis by MS, samples were subjected to in-solution, on-bead, tryptic digestion. 8 M urea in 0.1 M Tris, pH 7.9, was added onto PBS-washed beads and incubated for 15 min at room temperature. Proteins were reduced by incubation with dithiothreitol (5 mM; Sigma-Aldrich) for 60 min at room temperature, and alkylated with 10 mM iodoacetamide (Sigma-Aldrich) in the dark for 30 min at room temperature. Urea was diluted to 2 M with 50 mM ammonium bicarbonate. 250 ng trypsin (Promega) was added and incubated overnight at 37°C, followed by addition of 100 ng trypsin for 4 h at 37°C. Digestions were stopped by addition of trifluoroacetic acid (1% final concentration). After digestion, peptides were desalted using Oasis HLB µEvaporation format (Waters), vacuum-dried, and stored at −80°C until further analysis. Ultra-liquid chromatography/MS-grade solvents were used for all chromatographic steps. Each sample was loaded using splitless nano-ultraperformance liquid chromatography (10 kpsi nanoAcquity; Waters). The mobile phase was a) H2O + 0.1% formic acid, and b) acetonitrile + 0.1% formic acid. Desalting of the samples was performed online using a reversed-phase Symmetry C18 trapping column (180 µm internal diameter, 20 mm length, 5 µm particle size; Waters). The peptides were then separated using a T3 high-strength silica nanocolumn (75 µm internal diameter, 250 mm length, 1.8 µm particle size; Waters) at 0.35 µl/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4–20% b in 55 min, 20–90% b in 5 min, maintained at 95% for 5 min, and then back to initial conditions. The nano-ultraperformance liquid chromatography was coupled online through a nano-electrospray ionization emitter (10 µm tip; New Objective) to a quadrupole orbitrap mass spectrometer (Q Exactive HF; Thermo Scientific) using a FlexIon nanospray apparatus (Proxeon). Data were acquired in data-dependent acquisition mode, using a top-20 method. MS1 resolution was set to 120,000 (at 400 m/z), mass range of 300–1650 m/z, automatic gain control of 3e6, and maximum injection time was set to 20 msec. MS2 resolution was set to 30,000, quadrupole isolation 1.7 m/z, automatic gain control of 1e6, dynamic exclusion of 60 s, and maximum injection time of 60 ms. Raw data were imported into the Expressiost software version 10.5 (Genedata) and processed as described (Shalit et al., 2015). The software was used for retention-time alignment and peak detection of precursor peptides. A master peak list was generated from all MSMS events and sent for database searching using Mascot v2.5.1 (Matrix Sciences). Data were searched against the S. cerevisiae sequences UniprotKB appended with common laboratory-contaminant proteins. Fixed modification was set to carbamidomethylation of cysteines, and variable modifications were set to oxidation of methionines and deamidation of N or Q. Search results were then filtered using the PeptideProphet algorithm (Keller et al., 2002) to achieve a maximum false-discovery rate of 1% at the protein level. Peptide identifications were imported back to Expressionist software (P. Carvalho, University of Oxford, Oxford, England, UK), and histone H3 (ChIP grade ab1791; Abcam).
to annotate identified peaks. Quantification of proteins from the peptide data was performed using an in-house script (Shalit et al., 2015). Data were normalized based on the total ion current. Protein abundance was obtained by summing the three most-intense, unique peptides per protein. A two-sided Student’s t test, after logarithmic transformation, was used to identify significant differences across the biological replica. Fold changes were calculated based on the ratio of arithmetic means of the case versus control samples.

**Isolation of an LD-enriched fraction and phospholipid analysis by thin-layer chromatography**

Cells were grown on minimal medium at 30°C overnight, back-diluted, and left to grow until they reached mid-logarithmic phase. Harvested cells were resuspended in DTT buffer (100 mM Tris-HCl, pH 9.4, and 10 mM DTT) and incubated for 20 min (30°C). Cells were collected by centrifugation and washed with Zymolyase buffer without enzyme (20 mM potassium-phosphate buffer, pH 7.4, and 1.2 M sorbitol). Subsequently, cells were resuspended in Zymolyase buffer and incubated in the presence of Zymolyase for 30 min. The enzyme was removed with a washing step using Zymolyase buffer without enzymes, and cells were resuspended in breaking buffer (10 mM Tris-HCl, pH 6.9, 0.2 mM EDTA, and 12% Ficoll 400) supplemented with 2 mM phenylmethylsulfonyl fluoride (Sigma-Alrich). Cells were broken using a doune homogenizer, and the resulting homogenate was subjected to a clarifying spin. Supernatants were adjusted to a volume of 6.7 ml using breaking buffer and transferred to ultracentrifugation tubes (331372; Beckman Coulter). Samples were overlaid with the same volume of breaking buffer and subjected to ultra-centrifugation in an SW41 swing-out rotor (28,000 rpm). The resulting white, floating layer enriched with LDs was collected using a bent glass pipette. Thin-layer chromatography analysis was performed on crude LDs using a developing solvent of chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5, vol:vol:vol:vol:vol). Lipids were visualized using copper sulfate (6.25 mM in 9.4 ml phosphoric acid) at 110°C for 30–60 min. Bands were identified using phospholipid standards (Avanti Polar Lipids; Sigma-Alrich).

**Online supplemental material**

Fig. S1 characterizes the LD subpopulation located adjacent to the NVJ. Fig. S2 shows mechanistic aspects of LDO machinery function. Table S1 provides a list of hits from all screens performed in this study. In Table S2, all strains used in this study are described. Table S3 shows all plasmids used in this study.

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