High-confidence proteome analysis of yeast lipid droplets identifies additional droplet proteins and reveals connections to dolichol synthesis and sterol acetylation

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Running title: High-Confidence Yeast Lipid Droplet Proteome

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Abbreviations: DTT, dithiothreitol; ER, endoplasmic reticulum; cis-IPTase, cis-isoprenyl transferase; FPP, farnesyl pyrophosphate; GFP, green fluorescent protein; H/L, heavy/light ratio; IPP, isopentyl pyrophosphate; LD, lipid droplet; MDH, monodansyl pentene; SE, sterol ester; SILAC, stable isotope labeling by amino acids in cell culture; TG, triglyceride.
ABSTRACT

Accurate protein inventories are essential for understanding an organelle’s functions. The lipid droplet (LD) is a ubiquitous intracellular organelle with major functions in lipid storage and metabolism. LDs differ from other organelles because they are bounded by a surface monolayer that presents unique features for protein targeting to LDs. Many proteins with varied functions have been found in purified LD fractions by proteomics. While these studies have become increasingly sensitive, it is often unclear which of the identified proteins are specific to LDs. Here we used protein correlation profiling to identify 35 proteins that specifically enrich with LD fractions of Saccharomyces cerevisiae. Of these candidates, 30 fluorophore-tagged proteins localize to LDs by microscopy, including six proteins—several with human orthologs linked to diseases—that we newly identify as LD proteins (Cab5, Rer2, Say1, Tsc10, YKL047W, and YPR147C). Say1, a sterol deacetylase, and Rer2, a cis-isoprenyltransferase, are enzymes involved in sterol and polyrenol metabolism, respectively, and we show their activities are present in LD fractions. Our results provide a highly specific list of yeast LD proteins and reveal that the vast majority of these proteins in yeast are involved in lipid metabolism.

Keywords: lipid droplets • proteome • lipid metabolism • lipids • protein targeting • sterol • dolichol • polyrenol synthesis • sterol acetylation
The lipid droplet (LD) is a cytoplasmic organelle that is ubiquitous among eukaryotic cells and also found in some prokaryotic cells (1-3). Long thought to be mostly inert, the LD is now recognized as a *bona fide* organelle with dynamic size, number, distribution, and protein composition (4). LD proteins include a family of structural proteins (5), many enzymes involved in lipid metabolism, and an assortment of proteins with other functions. Protein localization to the LD can be regulated by many factors. For example, development regulates histone localization (6), and phospholipid content regulates CCT1 localization (7). A thorough understanding of protein composition is an essential step for understanding the functions of the LD.

The LD has a unique architecture of neutral lipid core bounded by a phospholipid monolayer. The surfactant monolayer imposes specific structural requirements on proteins localized to the LD. For example, it prohibits transmembrane proteins with luminal domains and favors structures that localize to the interface by dipping segments into the hydrophobic phase, such as proteins with hydrophobic sequences (8-9) or amphipathic helices (5). LD biogenesis and growth uniquely depend on neutral lipid synthesis because the organelle core contains primarily triacylglycerols (TGs) and sterol esters (SEs). The composition varies by cell type and nutritional status. In yeast, amounts of TGs and SEs are roughly equal (10).

LDs are often found in close apposition to other organelles, including peroxisomes (11), mitochondria (12-14), endosomes (15), phagosomes (16), and especially the endoplasmic reticulum (ER) (17-18), which is likely their site of origin (reviewed in 19, 20). In fact, some proteins that appear to target the LD may actually target ER membranes closely apposed to the LD, and the actual target can be difficult to distinguish at the resolution of confocal light microscopy (∼300 nm). The close association of LDs with other organelles makes it challenging to purify them biochemically. Additionally, their hydrophobic nature offers a potential sink for non-LD proteins whose topologies are disrupted during the mechanical fractionation process. These artifacts, combined with the high sensitivity of mass spectrometry (MS), often yield LD proteomes with low specificity.
We sought to determine a high-confidence proteome of the yeast *Saccharomyces cerevisiae* LD, an established model for LD studies (21). Although comprehensive lists of yeast LD proteomes have been reported (11, 22), there is little overlap between the lists. Additionally, it is unclear which of the candidate proteins identified by proteomics are specific to LDs, since many have not been examined by microscopy. We sought to overcome the specificity limitations of LD proteomes by using protein correlation profiling (PCP), a quantitative method of determining purification profiles of proteins compared with organelle markers, based on high-resolution MS. PCP was successfully used to create specific inventories of many organelles (23-24), including LDs in *Drosophila melanogaster* cells (25). We reasoned that *bona fide* LD proteins should fulfill two criteria. They should be enriched in the LD purification fraction by PCP, and they should localize to LDs by microscopy. We used PCP to generate a high-confidence list of 35 proteins that specifically co-purify with the yeast LD. By cross-referencing with fluorescence microscopy in this study or previous reports, we verified that 30 of these proteins are *bona fide* LD proteins. We showed that two proteins (Faa1 and Hfd1) previously identified in yeast LD proteomes in fact do localize to LDs. Additionally, we identified six new LD proteins (Cab5, Rer2, Say1, Tsc10, YKL047W, and YPR147C), and we assessed enzymatic activities for two of these proteins, Say1 and Rer2, at LDs.
MATERIALS AND METHODS

Strains, media and materials

*S. cerevisiae* strain BY4741 (*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was used as wild type. Yeast strains were routinely transformed using lithium acetate. Cells were cultured in synthetic complete medium with dextrose (SCD) containing 2% dextrose, 0.67% yeast nitrogen base (BD Biosciences), amino acids (Sunrise Science), and ammonium sulfate. Cells were grown for 2 days at 30°C to stationary growth phase for all experiments.

Protein localization

C-terminally tagged GFP strains were created using published cloning cassettes (26). PCR primers were designed using Primer3 (http://bioinfo.ebc.ee/cgi-bin/primer3/primer3web_results.cgi) and purchased from Elim Biopharm. Yeast were stained with 1:1000 (v/v) monodansyl pentane (MDH) (Abgent) for LD identification (27) and allowed to settle on concanavalin A–coated coverslips for 10 min. They were then mounted on slides, and images were acquired by using a Nikon ECLIPSE Ti 2000 microscope, with Yokogawa CSU-X1 spinning disk and Hamamatsu ImagEM electron multiplier CCD camera, and image acquisition and mechanical control by Micro-Manager. Solid-state lasers at excitation/emission of 405/460 nm, 491/520 nm, and 561/595 nm were used. Images were deconvolved (Huygens SVI) and cropped (Image J). The fraction of LDs with GFP was determined manually by counting numbers of LDs with and without GFP signal colocalization. The fraction of GFP colocalizing with LDs was determined by a custom CellProfiler (http://www.cellprofiler.org) pipeline (available by request) and Python software script that calculated the fraction of total GFP intensity that colocalized with MDH punctae on a per cell basis.
Protein localization was confirmed by cellular fractionation and immunoblotting with mouse monoclonal anti-protein disulfide isomerase (PDI) (Abcam), anti-GFP (Roche), and rabbit polyclonal anti-hemagglutinin (HA) (Upstate) antibodies. For crude fractionation (e.g., in experiments of Figures 5 and 6), spheroplasted cells were dounce-homogenized in 200 mM sorbitol, 10 mM HEPES-KOH pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and 1 mM EDTA. Cell lysates centrifuged at 300g for 30 min and then 100,000g for 30 min. LDs were collected from the top fraction with a tube slicer (Beckman Coulter), and the remaining supernatant (cytosol) and pellet (membranes) were collected.

**Thin layer chromatography (TLC)**

Lipids were extracted by disrupting cells by bead-beating in water:CHCl₃:MeOH (0.3:1:1), collecting the single phase, and drying it under N₂ gas. Dried lipids were resuspended in chloroform, separated on silica gel TLC plates (Whatman or Analtech) by using hexane:ethyl ether:acetic acid (80:20:1), and detected by charring with cuprous sulfate. Bands were identified by comparison to standards.

**LD purification**

Stable isotope labeling by amino acids in cell culture (SILAC) was performed (28-29). Cells were pelleted, washed with water, and then incubated in 0.1 M Tris-Cl pH 9.0, 10 mM dithiothreitol (DTT) at 30°C for 10 min. Cells were washed by and resuspended in 20 mM KH₂PO₄, pH 7.4, 1.2 M sorbitol to 0.5 g/mL and digested with 4 mg/g zymolyase 100T (MP Biomedicals) at 30°C for 2 h. Cells were pelleted and washed with and resuspended in 5 mL 20 mM HEPES, pH 7.4, 0.6 M sorbitol, 1 mM EDTA, and EDTA-free protease inhibitor pellet (Roche), and homogenized in a dounce homogenizer for 40 strokes. Homogenized cells were spun at 300g for 30 min, 20,000g for 30 min, and then 100,000g for 30 min with
the pellet collected at each centrifugation step. The final supernatant was then subjected to sucrose gradient (3 mL of each 1, 0.75, 0.5, 0.25, 0.125, and 0.063 M sucrose in TBS buffer) centrifugation overnight at maximum speed in a SW41 rotor. LDs were collected from the top (3 mL) with a tube slicer (Beckman Coulter) and other fractions (~6 mL) were collected by pipette. The six gradient fractions and three pellets from the initial high-speed spins were analyzed by MS.

**Liquid chromatography MS/MS analysis**

Each peptide fraction was separated by reversed-phase chromatography on a Thermo Easy nLC 1000 system connected to a Q Exactive mass spectrometer (Thermo) through a nano-electrospray ion source. Peptides were separated on 15-cm columns (New Objective) with an inner diameter of 75 µm packed with 1.9 µm C18 resin (Dr. Maisch GmbH). 120-min chromatographic runs were used, and peptides were eluted with a linear gradient of acetonitrile from 5 to 30% in 0.1% formic acid for 95 min at a constant flow rate of 250 nl/min. The column temperature was kept at 35°C. Eluted peptides were applied directly into the mass spectrometer by electrospray ionization. Mass spectra were acquired on the Q Exactive in a data-dependent mode to automatically switch between full-scan MS and up to 10 data-dependent MS/MS scans. The maximum injection time for full scans was 20 ms with a target value of 3,000,000 at a resolution of 70,000 at m/z = 200. The 10 most-intense multiple-charged ions (z ≥ 2) from the survey scan were selected with an isolation width of 3Th and fragmented with higher energy collision dissociation, with normalized collision energies of 25. Target values for MS/MS were set to 1,000,000 with a maximum injection time of 120 ms at a resolution of 17,500 at m/z = 200. To avoid repetitive sequencing, the dynamic exclusion of sequenced peptides was set to 20 s.

The resulting MS and MS/MS spectra were analyzed by using MaxQuant (version1.3.0.2), utilizing its integrated ANDROMEDA search algorithms (30, 31). Peak lists were searched against local
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databases for *S. cerevisiae* (obtained from the *Saccharomyces* Genome Database, Stanford University; 6641 entries, July 26, 2012) with common contaminants added. The search included carboxamidomethylation of cysteine as fixed modification, and methionine oxidation and N-terminal acetylation as variable modifications. Maximum allowed mass deviation for MS peaks was set to 6 and 20 ppm for MS/MS peaks. Maximum missed cleavages were 2. The false discovery rate was determined by searching a reverse database. Maximum false-discovery rates were 0.01 both on peptide and protein levels. Minimum required peptide length was six residues. Proteins with at least two peptides (one of them unique) were considered identified. The “match between runs” option was enabled with a time window of 2 min to match identification between replicates. Soft-clustering was performed with the Mfuzz software package in the programming language R. The cluster number C was set at 9, and cluster stability m = 1.6.

Criteria for defining LD proteins

To define a high-confidence LD protein set, we applied four criteria to filter candidates. First, we set an arbitrary cutoff point for rank-ordered membership in the LD cluster as the top 136 proteins (see Supplementary Table 2 for a list of all proteins with a LD cluster value >0.1). We chose this arbitrary cut off because it included the majority of previously identified LD proteins in two experimental replicates. Second, proteins had to be above the arbitrary cutoff in both experimental replicates. Third, proteins had to have a calculable heavy:light ratio (H/L) in at least eight fractions so that we were confident that their cluster membership value was not strongly influenced by missing data points. Finally, the remaining proteins were filtered based on a purification profile that closely matched that of 12 proteins, well-established to localize to the LD because they were detected in all published LD proteomes and confirmed to localize to the LD by microscopy in other studies (Fig. 3A). In the case of our analysis, these stringent criteria used the thresholds of H/L <0.06 in fractions 1–3, <0.16 in fractions 4–6, <0.2 in fraction 7, and
<0.371 in fraction 8 (Fig. 1E). Validating our rationale, using these same filtration criteria on the remaining 857 proteins in the LD fraction identified only an additional four proteins as potential LD proteins (not shown).

**cis-Isoprenyl transferase (cis-IPTase) assay**

Cells were crudely fractionated as above. To reduce cytoplasm in the LD fraction, LDs in 1.5-ml Eppendorf tubes were rinsed twice by addition of 1 volume homogenization buffer, spun at 13,000 rpm in a tabletop centrifuge, and a needle and syringe were inserted below the floating LDs to remove 1 volume cytoplasm. Pellets were rinsed twice and then resuspended in 250 µL yeast cis-IPTase reaction buffer (60 mM HEPES, pH 8.5, 5 mM MgCl₂, 2 mM DTT, 2 mM NaF, 2 mM sodium orthovanadate). Yeast reaction mixtures contained 100 µg protein, 50 µM farnesyl pyrophosphate (FPP) and 45 µM 50-60 mCi/mmol [14]C-isopentyl pyrophosphate (IPP) (American Radiolabeled Chemicals). Reactions were incubated at 30°C for 1 h and quenched by adding 2 ml of CHCl₃:MeOH (2:1). Products were separated from unreacted water-soluble IPP by partition through addition of 0.8 ml of 0.9% NaCl in water. The organic phase was washed three times with CHCl₃:MeOH:H₂O (3:48:47) and dried under nitrogen. The dried sample was resuspended in hexane and loaded onto silica gel TLC plates, run in hexane:ethyl acetate (80:20), and dried. Plates were exposed to a phosphor screen for 3–4 days. The band corresponding to an iodine-labeled dolichol standard was scraped from the TLC plate, resuspended in scintillation counting liquid, and counted in a scintillation counter.

**Cholesteryl acetate deacetylase assay**
Cells were fractionated as described above in lysis buffer of 200 mM sorbitol, 10 mM HEPES-KOH pH7.5, 100 mM NaCl, 5 mM MgCl₂, and 1 mM EDTA. Samples (300 µg of microsome or 300 µg of cytoplasm and LD protein) were assayed for cholesteryl acetate deacetylase activity as described (32). Cell fractions were incubated with 0.143 µCi [14C]-cholesteryl-acetate (American Radiolabeled Chemicals) in 26 nmol total cholesteryl acetate for 1 h at 30°C. The assay was stopped by adding 1.5 vol of CHCl₃. MetOH (3 volumes) was added, and the reaction was vortexed until clear. Ortho-phosphoric acid (2.8% in water, 1.5 volumes) was added and the samples were centrifuged for 2 min. at 13,000 rpm. The upper aqueous layer was discarded. Samples were re-extracted with 1.5 volumes CHCl₃ and 3 volumes acidified water. The lower organic layer was collected and dried under nitrogen. The dried sample was resuspended in CHCl₃:MetOH (1:1), loaded onto silica gel TLC plates, run in petroleum ether:diethyl ether:acetic acid (70:30:2), and dried. Plates were exposed to a phosphor screen for 2–3 days and compared to iodine-labeled standards.
RESULTS

PCP yields a distinct LD purification profile

LDs were isolated from wild-type yeast grown at 30°C to stationary phase, where LDs are most abundant. To obtain the fractions for PCP, we purified LDs from cells labeled with heavy, non-radioactive isotope-containing lysine (stable isotope labeling by amino acids in cell culture, SILAC (28)) with sequential differential centrifugation and a sucrose density gradient. We collected samples from pellets of each initial centrifugation (fractions 1–3) and six layers of the sucrose density gradient (fractions 4–9). Using peptides identified in the various fractions, we found that the sequential centrifugations pelleted primarily the following: unbroken cells and agglomerated membrane in fraction 1; vacuolar, nuclear, ER, plasma, endosomal, and Golgi membranes in fraction 2; and transport vesicles, endosomal membranes, and Golgi complex in fraction 3. The sucrose density gradient separated the remaining cytoplasm into six additional fractions. We combined each of these samples with equal amounts of protein from the LD fraction (fraction 9) of unlabeled cells and analyzed the combined samples by liquid chromatography coupled online to electrospray ionization and high-resolution tandem MS (Fig. 1A). We detected 16,589 peptides from 2,165 proteins in all fractions. 1,377 proteins were identified in the LD fraction.

We calculated heavy:light ratios (H/L) for each protein in every fraction. Of the 1,377 proteins in the LD fraction, 993 yielded heavy:light ratios (Supplemental Table S1). To compare individual proteins, we set the maximum H/L to 1 and normalized the ratios in other fractions to this H/L. To directly compare different organelle purification profiles, we plotted a representative profile for a protein from
each of the major cellular organelles, choosing proteins that were only annotated to a single intracellular compartment in the *Saccharomyces* Genome Database (Fig. 1B). We found that *bona fide* LD proteins (i.e., detected in multiple proteomic studies and confirmed by light microscopy) peaked in fraction 9 with very little representation in other fractions. ER proteins, which are especially difficult to separate from LDs during purification, similarly peaked in fraction 9, but also had smaller peaks in fractions 2 and 3. Several other organelles had abundant proteins in the LD fraction, highlighting the need for a technique, such as PCP, to determine specificity.

We used soft clustering to generate clusters of typical purification profiles with each protein assigned a membership value for each cluster (33). The membership value provides a measurement of the similarity between each protein’s purification profile and the cluster, thereby providing a measure of the likelihood that a protein belongs to a certain cluster. Soft clustering identified a group of LD proteins (Fig. 1C, bottom center) as well as clusters containing mostly proteins from other organelles (e.g., mitochondria (center) and ER (center right)) (See Supplemental Table S2 for all proteins in the LD cluster with H/L >0.1).

To assess reproducibility of the LD PCP measurements, we repeated the purification and analysis and compared the membership value for the LD cluster of each identified protein. The results were highly reproducible, with a Pearson’s correlation of 0.77 between the two PCP experiments (Fig. 1D). We performed the same comparison with a third biological replicate and found that it correlated well with both previous PCPs ($r = 0.79$ and $r = 0.76$, respectively), again showing the reproducibility of our technique and dataset.

Because PCP results yield membership values for a particular cluster, the analysis requires choosing an arbitrary cutoff. We applied such a cutoff to the LD cluster (rationale described in Experimental Procedures) and filtered the list to include proteins that were within this arbitrary cutoff in the original PCP and a biological replicate. This yielded a cluster of 88 proteins that reproducibly purified in the LD fraction (filtration schematic in Fig. 1E). We next applied stringent filtering criteria to this data
set to minimize false-positive results and separated the dataset into the highest-confidence LD proteins (Fig. 1F, red) and suspected non-LD proteins (Fig. 1F, blue). This analysis yielded a list of 35 highest-confidence LD proteins (Table 1).

**Verification of LD protein localization**

We next determined which proteins in the high-confidence PCP list are *bona fide* LD proteins, as confirmed by localization to LDs by live-cell fluorescence microscopy (Fig. 2B-D, quantified in Fig. 2E-F). We considered a protein a *bona fide* LD protein if it localized to the LD, regardless of whether it localized elsewhere in the cell. Of the 35 identified proteins, 19 were previously identified as LD proteins by fluorescence microscopy (annotated in Table 1 and Fig. 2A). Three proteins (Faa1, Hdf1 and Gtt1) were identified in multiple other yeast LD proteomes, but their localizations to the LD was not explored by fluorescence microscopy. We tagged these proteins at the C-terminus with GFP to assess LD localization in stationary growth phase by colocalization with MDH, a blue LD vital dye. Hfd1-GFP exhibited predominantly LD localization while Faa1-GFP appeared to be visible in additional cellular compartments, likely the ER (representative images shown in Fig. 2B, quantified in Fig. 2E-F). Gtt1-GFP was predominantly found in other cellular compartments (Fig. 2C), and quantification revealed that very few LDs had GFP signal (Fig. 2E). However, the fraction of GFP signal that colocalized with LDs was comparable to other *bona fide* LD proteins (Fig. 2F).

Seventeen proteins identified in our PCP proteome were not previously annotated to the LD or, in some cases, were only found in a single report of the LD proteome. Seven of these proteins were previously localized to the LD by microscopy (Dga1, Ldh1, Pgc1, Tgl4, Tgl5, Ubx2, YOR059C). Of the 10 remaining proteins, three (Ecm29, Nte1, Taz1) did not show LD localization when we examined C-terminal-GFP fusion proteins by microscopy, suggesting that they purify with the LD fraction but do not localize to this organelle. We were unable to image Pho81 because we could not express the tagged
protein. However, six proteins (Cab5, Rer2, Say1, Tsc10, YKL047W, and YPR147C) were newly identified as localizing to LDs (Fig. 2D, 2E-F).

Comparison of PCP proteome to reported yeast LD proteomes

Of the 959 proteins with a calculable H/L in the LD fraction, we found 35 (<4%) that specifically purify with the LD, according to our filtering criteria (see Experimental Procedures) (Table 1). We compared our LD protein list with other reported proteomes that attempted to threshold their lists and found that the current proteome overlaps most (95%) with the one reported by Athendstaedt et al. (34), which utilized earlier and less sensitive proteomics technology. In contrast, there was considerably less overlap with LD proteomes generated more recently with more sensitive MS techniques (Grillitsch et al. (22), 55%; Binns et al. (11), 30%) (Fig. 3A).

Twelve proteins (Ayr1, Eht1, Erg1, Erg6, Erg7, Faa1, Faa4, Fat1, Pet10, Scl1, Tgl1 and Tgl3) were detected in the current work and all reported proteomes. Of note, 11 of these are known to be lipid metabolic enzymes.

We also analyzed our results in relationship to protein abundance within cells, using data from Ghaemmaghanmi et al. (35) (Fig. 3B). High abundance proteins are overrepresented in previously reported yeast LD proteomes, suggesting more contamination of the LD fraction. Our results show substantially fewer high-abundance proteins, consistent with less contamination. As expected with the outstanding sensitivity of current MS instruments, we detected low-abundance proteins with the current proteome.

Functions of bona fide LD proteins
We annotated the functions of the 30 bona fide LD proteins, based on available data in the literature (Fig. 4A). Interestingly, all proteins with known functions (83%) are involved in lipid metabolism. These include many proteins involved in ergosterol metabolism (Fig. 4B) and fatty-acid esterification and TG metabolism (Fig. 4C). The only de novo TG synthetic enzyme that we failed to detect in our LD proteome was Pah1, although it was reported to localize to the LD (36). Four of the six newly identified LD proteins connect LDs with new possible lipid metabolic functions. Cab5 is involved in coenzyme A synthesis (37), an important cofactor for many lipid synthesis reactions. Tsc10 catalyzes the second step in the synthesis of phytosphingosine, a long-chain base for sphingolipid production (38). Rer2 and Say1 function in dolichol and sterol metabolism, respectively, and are discussed below. Five proteins (Pet10, Yim1, YOR059C, and the newly identified YKL047W and YPR147C) do not have clear functions in yeast (see Table 1) but, based on our findings, are also likely to be involved in lipid metabolism. Indeed, the mammalian ortholog of YPR147C was recently reported to have cholesterol esterase activity (39).

Of the six newly identified LD proteins, we selected Rer2 and Say1 for further investigations because they have known enzymatic functions in sterol metabolism, and their connections to LDs have not been well explored.

**Rer2 is active in the LD fraction**

Rer2 is a cis-isoprenyltransferase (cis-IPTase) involved in dolichol synthesis (40). It is a 286-amino acid protein with a predicted globular structure. Cis-IPTases condense successive IPPs with FPP to create polyprenols, such as dolichol, the lipid anchor for sugars used in N-linked glycosylation. Because many enzymes in N-glycan biosynthesis were recently identified at the Drosophila LD (25), we sought to better understand the LD localization of Rer2.
The two known cis-IPTases in yeast are Rer2 and Srt1. Another protein, Nus1, has significant homology to Rer2 and Srt1 but does not have cis-IPTase activity (41). Both Srt1 (42) and Nus1 (22, 37, 43) have been localized previously to the LD. We found that Rer2 also localizes to the LD, as we detected Rer2-GFP in both membrane and LD fractions (Fig. 5A) and saw colocalization of Rer2-GFP with ER marker Sec61-mCherry and MDH (Fig. 5B). Deleting Rer2 affects neutral lipids as rer2Δ cells showed a specific increase (> twofold) in SE levels (Fig. 5C) that was rescued by expression of the human Rer2 homolog, dehydrodolichyl diphasphate synthase.

To assess whether Rer2 is active at LDs, we examined cis-IPTase activity in cell extracts by incubating cells with FPP and 14C-IPP and measuring incorporation into polyprenols by TLC. We found that wild-type cells had cis-IPTase activity in both membrane and LD fractions (Fig. 5D). srt1Δ cells had similar cis-IPTase activities, while rer2Δ cells had very little cis-IPTase activity in either membrane or LD fractions, indicating that Rer2 is the major source of polyprenol synthesis at the ER and the LD in stationary phase yeast.

Say1 is active in the LD fraction

In yeast, sterol metabolism is intricately linked between LDs and the ER. We therefore further investigated Say1, a sterol metabolic enzyme that we newly identified as a LD protein. While Say1 has been previously localized to the ER, we detected Say1-GFP in both the ER and LD fractions (Fig. 6A). Additionally, Say1-GFP colocalized with ER marker Sec61-mCherry and MDH showing that it has both ER and LD localization (Fig. 6B). say1Δ yeast do not have grossly altered levels of SE or TG (data not shown).

Other studies propose that yeast have a cycle of sterol acetylation and de-acetylation. Atf2 promiscuously acetylates sterols so they can be secreted for detoxification, and Say1 specifically de-acetylates acetylated sterols, such as ergosterol and its synthetic intermediates, so they can be retained in
the cell (44). To measure Say1 de-acetylase activity, we fractionated cells, incubated with radiolabeled cholesteryl acetate, and monitored the appearance of free cholesterol. We detected sterol deacetylase activity in both ER and LD fractions (Fig. 6C and D) when we overexpressed Say1-HA under control of a \textit{GAL1}-promoter. Thus, it appears that Say1-mediated sterol de-acetylation is present at the LD. We did not detect sterol deacetylase activity in either wild-type (cells not overexpressing Say1-HA) or \textit{say1Δ} cells. The lack of detectable Say1 activity in wild-type cells is consistent with a previous report (44).
DISCUSSION

A yeast LD proteome generated by protein correlation profiling

In the current study, we applied PCP to the analysis of yeast LDs and identified 35 proteins that reproducibly and stringently co-purify in the LD fraction. Of the 35 proteins identified, 30 of these proteins localize to the LD by microscopy, in our studies and others (Fig. 2 and Table 1), identifying them as *bona fide* LD proteins. Importantly, we identify six proteins (Cab5, Rer2, Say1, Tsc10, YKL047W, YPR147C) that have not previously been localized to the LD. Further, we show that Say1 and Rer2 are lipid metabolic enzymes that are active in LD fractions. Cab5 is involved in coenzyme A biosynthesis with Cab2, Cab3, and Cab4 (37). Although none of Cab2-4 was identified as an LD protein in our PCP proteome, we detected Cab4-GFP at the LD by fluorescence microscopy (not shown). Using fluorescence microscopy, we also verified stationary-phase LD localization of two proteins (Faa1 and Hfd1) previously identified in multiple LD proteomes and in our proteome but never visually shown to be at the LD.

Our results differ somewhat from previous reports of yeast LD proteomes (see Fig. 3). The first reported LD proteome identified only 19 proteins (34), in part due to technological limitations of MS at the time. However, it was likely highly specific and most closely overlaps with the current results. Two subsequent proteomes benefited from enhanced MS sensitivity, but had limited confirmation and likely included a number of contaminants, particularly of highly abundant proteins. The lack of overlap between the proteomes may also reflect differences in culture conditions (Binns et al. (11)), minimal medium with oleate (Grillitsch et al. (22)), rich medium with and without oleate (Athendstaedt et al. (34)), rich medium without oleate or differences in purification methods. The 12 proteins identified in all proteomes (Ayr1,
Eht1, Erg1, Erg6, Erg7, Faa1, Faa4, Fat1, Pet10, Slc1, Tgl1 and Tgl3) are likely to be constitutive LD proteins, since they were identified regardless of specific growth conditions.

Although our PCP identified 35 proteins that specifically co-purify with the LD, we were unable to verify LD localization by fluorescence microscopy for five proteins (Fig. 2 and Table 1). For some of these proteins, GFP-tagging might interfere with their targeting to the LD. For Gtt1, microscopy revealed that clusters of Gtt1-GFP localize near some LDs, offering an explanation for why Gtt1 biochemically purifies with LDs. Taz1, a cardiolipin remodeling enzyme found primarily in mitochondrial membranes (45), might also co-purify with LDs due to organelle associations.

Our proteome failed to identify several proteins that are most likely bona fide LD proteins (e.g., Atf1 (46), Loa1 (47), Pah1 (36), Pdr16 (48) or Srt1 (42)). This may reflect the dynamic nature of protein localization to the LD [we only examined stationary phase cells grown in minimal medium, and nutrient carbon source affects protein targeting to the LD (22)], limitations of organelle fractionation, or our stringent filtration criteria. For example, our criteria potentially filtered out proteins that were in the LD and other non-ER cellular fractions, as it did for Pdr16, which localizes to the LD and cell periphery (48). Atf1 and Srt1 were not detected in the LD fraction, whereas Loa1 was only detected in a single replicate. Pah1 was found in the LD fraction but not included because it lacked sufficient H/L ratios in multiple fractions, as our criteria demanded. Because there is no “gold standard” list of LD proteins, we cannot analyze our data for measurements, such as specificity or false-discovery rate. Nonetheless, due to our stringent criteria, we believe our results reflect a list that favors high specificity.

Our PCP analysis of the yeast LD proteome revealed some notable differences from a PCP analysis of LDs in Drosophila S2 cells (25). Although both PCP studies found that the majority of LD proteins function in lipid metabolism, we found considerably more LD proteins (>100) in Drosophila cells. The additional proteins are involved in ER organization, protein degradation, and N-glycan biosynthesis, suggesting additional functions and complexity for LDs in fly versus yeast cells. Other differences might relate to the different relationships between the ER and LDs in these cell types. In
yeast, LDs are often more directly connected or exist as a subdomain of the ER (17), whereas in S2 cells, there are distinct LD populations: those that are connected or not connected to the ER (9). The closer association of LDs and ER in yeast may explain why this study required additional filtration criteria to yield a LD-specific list of proteins.

A specific list of yeast LD proteins presents the opportunity to determine how these proteins target to the LD. For example, of the twelve proteins found in all reported proteomes, six have predicted transmembrane domains (Erg1, Erg7, Fat1, Slc1, Tgl1, and Tgl3), with the topology of Tgl1 experimentally verified (49). It is unclear how a protein with a transmembrane domain can localize to a membrane monolayer at LD surfaces. In yeast, perhaps many of these transmembrane proteins are in an ER microdomain that is closely associated with the LD and indistinguishable by biochemical fractionation or light microscopy. LDs appear to be a subdomain of the ER in yeast, and LD-ER bridges have been found in yeast and a number of other organisms (9, 17, 50-51).

Most of the newly identified yeast LD proteins (Cab5, Rer2, Say1, Tsc10, and YPR147C) have human orthologs and thus the localization to LDs might also be important for functions of these proteins in humans. The functional homolog of Rer2, dehydrodolichyl diphasphate synthase (DHDDS or hCIT), has been linked to retinitis pigmentosa (52). Human coenzyme A synthase (COASY) is a bifunctional enzyme (phosphopantetheine adenylyltransferase and dephospho coenzyme A kinase activities) that catalyzes the last step of coenzyme A synthesis, like Cab5 (53). While sterol acetylation is a yeast specific process, Say1 is orthologous to ary lacetamide deacetylase (AADAC) (44), an enzyme putatively involved in TG hydrolysis (54). Tsc10 is functionally homologous to 3-ketodihydrosphingosine reductase (KDSR or FVT1), known to be active at the cytosolic face of the ER (55) and implicated in tumor processes (56). YPR147C is a highly conserved protein with a GXSXG lipase motif that affects lipid storage in Drosophila (57) and cholesterol ester storage in macrophages (39).

Identification of Rer2 and Say1 as LD-localized enzymes in yeast

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Our findings show that the cis-IPTase Rer2 localizes in part to LDs. cis-IPTase activity is an essential step in dolichol biosynthesis and, in yeast, is catalyzed by Rer2 and Srt1, with Nus1 as a potential cofactor (41). Both Srt1 and Nus1 have been reported at the LD (11, 22, 42-43). We found that the cis-IPTase Rer2 localizes to the ER and LDs both biochemically and by fluorescence microscopy and has similar specific activities in each compartment. The major role of ER-localized Rer2 is to generate dolichol for glycosylation, and yeast lacking LDs (lacking all four enzymes of neutral lipid synthesis (58)) still make dolichol (not shown). Therefore, LD-localized Rer2 may be more important for synthesizing dolichol destined for storage pools or other cellular functions.

It is unclear how Rer2 localizes to LDs. Rer2 does not have predicted transmembrane sequences although it behaves as an integral membrane protein (40). Thus, there are no theoretical topological problems for its localization to the LD. In our experimental conditions, Rer2 appears to be the major cellular cis-IPTase. A previous report showed that Srt1 was most highly expressed in stationary phase and Srt1-dependent activity was detected in an rer2Δ background when Srt1 was overexpressed and only in stationary phase (42). However, we found little cis-IPTase activity in the rer2Δ in stationary phase, leaving questions about the cellular role of Srt1.

Yeast cells with Rer2 deletion exhibited an increase in SEs. While stressed or slow-growing yeast cells often accumulate LDs, the accompanying neutral lipid accumulation is usually both SE and TG. We suspect that rer2Δ cells likely have a SE-specific accumulation because FPP, a Rer2 substrate, may be channeled into sterol synthesis and SEs. Rechanneling of FPP is consistent with the finding of several unidentified radiolabeled lipid species synthesized by rer2Δ cells in cisIPTase activity assays (data not shown).

We also found that an enzyme involved in a sterol detoxification system, Say1, targets to the LD. A current model suggests that Say1 works in concert with Atf2. Atf2 promiscuously acetylates exogenous and endogenous sterol molecules, which are then secreted unless they are recognized and de-acetylated by
Say1 (44). The topology of ER-localized Say1 showed that the enzyme has a single transmembrane domain (44). Such a topology should not exist at the LD monolayer because it would put a hydrophilic protein domain in the hydrophobic core of the LD. This suggests that LD-localized Say1 is a component of ER that is tightly associated with LDs, a possibility that is the consistent with the apparent connections of ER and LDs in yeast (17).

Rer2 and Say1 join a list of lipid metabolic enzymes that localize to LDs. While both Say1 and Rer2 are present and active at the LD and ER, similar to Ayr1 (59), Gpt2 (60), and Slc1 (61), not all proteins are active in all subcellular populations. For example, Dga1 (62), Erg6 (63), Erg7 (64), Erg27 (65), and Yju3 (66) are predominantly active at the LD, which may reflect that these proteins have a higher LD:ER localization ratios. In contrast, Erg1 is strongly present in both the ER and LD but only active at the LD (67). The significance of some enzymes differing in activity in the ER versus LDs is unclear.
Acknowledgments - We thank Crystal Herron, Gary Howard and John Carroll for editorial assistance, Beth Cimini, Ellen Edenberg, Manuele Piccolis, and Andrew Nguyen for technical assistance, and Natalie Krahmer, Charles Waechter, and Jeffrey Rush for advice. This work was supported by NIH grants RO1-GM09984 (to R.F.), GM097194 (T.C.W.), and The G. Harold and Leila Y. Mathers Charitable Foundation (to T.C.W). E.C was supported in part by a grant from NSF.
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High-Confidence Yeast Lipid Droplet Proteome


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High-Confidence Yeast Lipid Droplet Proteome


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FIGURE LEGENDS

**Fig. 1.** Identification of LD proteins in *S. cerevisiae* using PCP. A: Schematic of PCP workflow. B: Purification profiles of representative proteins for different organelles. C: Soft clustering of all fractions of a LD purification. Proteins identified in the LD fraction that were identified in at least five of the nine fractions were analyzed. A normalized H/L was used. Clusters that showed enrichment of proteins of a certain organelle or function are indicated. Proteins with a minimal membership value of 0.1 for LD cluster are shown in supplemental Table S2. D: Reproducibility of LD proteome data between experiments. Pearson correlation = 0.77. E: Schematic of data filtration to create high-confidence LD protein list. F: Proteins identified with high confidence in the LD PCP cluster of two biological replicates. Proteins in red passed additional stringent filtration criteria whereas those in blue did not.

**Fig. 2.** Identification and verification of LD proteins. A: Logic flow diagram for identification of LD proteins. Numbers in parentheses refer to the number of proteins in each category. *Proteins that were not imaged. B: Verification of LD localization of 2 proteins previously found in multiple proteomes but not previously localized to the LD by microscopy. C: Microscopy of a protein that reproducibly purifies with LDs but does not appear to localize to the LD. D: Microscopy of four newly identified LD proteins. E: Quantification of the fraction of LDs that colocalize with GFP on a population basis. Sec61 and Erg6 are negative and positive controls, respectively. >150 LDs/genotype were quantified. F: Quantification of the fraction of GFP signal that colocalizes with LDs on a per cell basis. Sec61 and Erg6 are negative and positive controls, respectively. >50 cells/genotype were quantified. Error bars are standard deviation. GFP tagged proteins were colocalized with MDH, a LD marker vital dye. Cells were grown in SCD media to stationary phase. The scale bar is 3.5um on merged images and 0.7um on inset images.
Fig. 3. Comparison of current LD proteome with previously reported yeast LD proteomes. A: Venn diagram showing overlap of LD-annotated proteins between the current work and three previously reported yeast LD proteomes that attempted specificity. B: Current work using PCP for identification of LD proteins has fewer high-abundance proteins than previously reported yeast LD proteomes. Protein abundances were from Ghaemmaghanmi et al. 2003.

Fig. 4. Functional annotation of identified LD localized proteins. A: All validated LD proteins with known functions are involved in LD metabolism. Newly verified proteins are in italics. Newly identified proteins are underlined. B: Proteins that co-purify with LDs include many enzymes in sterol metabolism. Two are newly identified (Rer2 and Say1). C: Proteins that co-purify with LDs include most enzymes in TG metabolism. *activity shown in vitro, presumed to be minor function in vivo. Proteins marked in red were identified and microscopically verified in the current work. Proteins marked in blue were not identified in our proteome but have been microscopically verified in other works.

Fig. 5. Rer2 is present and active at the LD. A: Rer2-GFP is present at the LD and in membranes by western blot. Rer2-GFP labeled cells were centrifuged at 100,000 g. LDs were collected by slicing centrifuge tubes. The upper fraction (containing LDs) was rinsed and the rinsed upper fraction, lower fraction (containing cytoplasm), and pellet (containing membranes), were probed with anti-GFP (for Rer2), anti-phosphoglycerate kinase (PGK) (for cytoplasm), and anti-dolichol phosphate mannose synthase (DPM1) (for ER) antibodies. The lipids were extracted and separated by TLC. TG was identified by co-migration with a standard. B: Rer2-GFP is present at the LD and ER by spinning disk confocal microscopy. Rer2-GFP shows a reticular and punctate pattern that colocalizes with ER marker Sec61-mCherry and LD stain MDH. C: rer2Δ have increased levels of sterol esters as determined by TLC. Expression of DHDDS (human homolog of Rer2) rescues the sterol ester accumulation. Unidentified
lipids are marked with an *. D: Rer2 is active at the LD. Cell extracts, as in A, were given FPP and 14C-IPP, which were incorporated into polyprenols by cis-IPTases in both the membrane and LD fractions. cis-IPTase activity is nearly wild-type in an srt1Δ strain and nearly missing in an rer2Δ strain, suggesting that Rer2 is the major cis-IPTase in both microsomes and LDs. BY4741 is the wild type strain for the srt1Δ strain and SS328 is the wild type strain for the rer2Δ strain. E: Quantification of D, normalized to wild type whole cell activity. Data are the mean +/- standard deviation of n=4–8 samples. There were no statistical differences between individual fractions in srt1Δ and BY4741. Whole cell and membrane cis-IPTase activity is significantly reduced (p<.05) in rer2Δ when compared to SS328. WC, whole cell. M or mem., membrane. C, cytoplasm.

Fig. 6. Say1 is present and active at the LD. A: Say1-GFP is present at the LD and in membranes by western blot. pGal-Say1-GFP cells were fractionated as in Fig. 5 and probed with anti-HA (for Say1), anti-PGK (for cytoplasm), and anti-DPM1 (for ER) antibodies. Lipids were extracted and separated by TLC. TG was identified by co-migration with a standard. B: Say1-GFP is present at the LD and ER by spinning disk confocal microscopy. Say1-GFP shows a reticular and punctate pattern that colocalizes with ER marker Sec61-mCherry and LD stain MDH. C: Say1 is active at the LD. Cell extracts were given 14C-Cholesteryl acetate (CA) which was de-acetylated into free cholesterol in both membrane and LD fractions by cells grown in galactose and overexpressing Say1-HA under a GAL1 promoter. CA deacetylase activity was below the limit of detection in wild type or say1Δ cells. WC, whole cell. M or mem., membrane. C or cyt., cytoplasm.

Supplemental Fig. 1. – Proteome dataset

Supplemental Fig. 2. – Proteins with membership value of >.1 in LD fraction
## TABLE 1. Identification of 35 proteins that specifically purify with the lipid droplet

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th>Function</th>
<th>Proteomes Present</th>
<th>Microscopically Localized to LD</th>
<th>Biochemically Localized to LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>YIL124W</td>
<td>AYR1</td>
<td>Acyl-DHAP reductase, catalyzes lyso-PA formation</td>
<td>A,B,G</td>
<td>(68)</td>
<td>(59)</td>
</tr>
<tr>
<td>YDR196C</td>
<td>CAB5</td>
<td>Dephospho-CoA kinase involved in coenzyme A synthesis</td>
<td></td>
<td>Current Work</td>
<td></td>
</tr>
<tr>
<td>YOR245C</td>
<td>DGA1</td>
<td>Diacylglycerol acyltransferase, catalyzes DAG to TAG</td>
<td>G</td>
<td>(68)</td>
<td>(62)</td>
</tr>
<tr>
<td>YHL030W</td>
<td>ECM29</td>
<td>Proteasome assembly</td>
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<td></td>
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<tr>
<td>YBR177C</td>
<td>EHT1</td>
<td>Acyl-CoA:ethanol acyltransferase</td>
<td>A,B,G</td>
<td>(68)</td>
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<td>YGR175C</td>
<td>ERG1</td>
<td>Squalene epoxidase, enzyme in ergosterol synthesis</td>
<td>A,B,G</td>
<td>(67)</td>
<td>(67)</td>
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<td>YLR100W</td>
<td>ERG27</td>
<td>3-keto sterol reductase, enzyme in ergosterol synthesis</td>
<td>B,G</td>
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<td>(65)</td>
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<td>YML008C</td>
<td>ERG6</td>
<td>24-C-sterol methyltransferase, enzyme in ergosterol synthesis</td>
<td>A,B,G</td>
<td>(67)</td>
<td>(63)</td>
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<td>YHR072W</td>
<td>ERG7</td>
<td>Lanosterol synthase, enzyme</td>
<td>A,B,G</td>
<td>(64)</td>
<td>(64)</td>
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<tr>
<td>GenBank Accession</td>
<td>Enzyme</td>
<td>Function</td>
<td>Interactant</td>
<td>Notes</td>
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<tr>
<td>YOR317W FAA1</td>
<td>Fatty acyl-CoA synthetase, activates imported fatty acids</td>
<td>A,B,G</td>
<td>Current Work</td>
<td></td>
<td></td>
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<td>YMR246W FAA4</td>
<td>Fatty acyl-CoA synthetase, activates imported fatty acids</td>
<td>A,B,G</td>
<td>(68)</td>
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<tr>
<td>YBR041W FAT1</td>
<td>Fatty acyl-CoA synthetase, activates imported fatty acids</td>
<td>A,B,G</td>
<td>(68)</td>
<td></td>
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<tr>
<td>YIR038C GTT1</td>
<td>Glutathione transferase</td>
<td>B,G</td>
<td>(22)</td>
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<tr>
<td>YMR110C HFD1</td>
<td>Fatty aldehyde dehydrogenase</td>
<td>B,G</td>
<td>Current work</td>
<td></td>
<td></td>
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<td>YBR204C LDH1</td>
<td>Serine hydrolase, weak TG lipase activity</td>
<td>G</td>
<td>(69)</td>
<td></td>
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</tr>
<tr>
<td>YML059C NTE1</td>
<td>Phospholipase B</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>YDL193W NUS1</td>
<td>Putative prenyltransferase involved in dolichol synthesis</td>
<td>A,G</td>
<td>(43)</td>
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<tr>
<td>YKR046C PET10</td>
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<td>A,B,G</td>
<td>(68)</td>
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<tr>
<td>YPL206C PGC1</td>
<td>Phosphatidyl glycerol phospholipase C, catalyzes PG to DAG</td>
<td>G</td>
<td>(70)</td>
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<td></td>
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<tr>
<td>YGR233C PHO81</td>
<td>Cyclin-dependent kinase inhibitor</td>
<td></td>
<td></td>
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<tr>
<td>YBR002C RER2</td>
<td>Prenyltransferase involved</td>
<td>Current work</td>
<td>Current work</td>
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<tr>
<td>Genes</td>
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<tr>
<td>YGR263C</td>
<td>SAY1</td>
<td>Sterol deacetylase</td>
<td>A,B,G</td>
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<td>YDL052C</td>
<td>SLC1</td>
<td>Acyltransferase, catalyzes lyso-PA to PA</td>
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<td>YPR140W</td>
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<td>Lyso-PC acyltransferase, catalyzes lyso-PC to PC</td>
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<td>YKL140W</td>
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<td>YMR313C</td>
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<tr>
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<td>TGL4</td>
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<td>G</td>
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<tr>
<td>YOR081C</td>
<td>TGL5</td>
<td>Lipase, catalyzes TAG to DAG and LPA to PA</td>
<td>G</td>
<td></td>
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<tr>
<td>YBR265W</td>
<td>TSC10</td>
<td>3-ketosphinganine reductase, involved in sphingosine synthesis</td>
<td>G</td>
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<td>YML013W</td>
<td>UBX2</td>
<td>Involved in ER-associated protein degradation, regulates LD homeostasis.</td>
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<tr>
<td>YKL094W</td>
<td>Monoacylglyceride lipase, catalyzes MAG to glycerol</td>
<td>A,G</td>
<td>(68) (66)</td>
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<td>Unknown</td>
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</table>

Proteins were identified as described in the results and are annotated for ORF, gene name, presence in other proteomes, and previous localization to the LD by microscopy or biochemistry. Proteomes are abbreviated as A = (37), B = (11), and G = (22). Proteins were considered previously identified if they were previously microscopically or biochemically localized to the LD or if they were previously identified in more than one proteome. Newly identified proteins are bolded in the table. Proteins that have not been verified by microscopy to localize to the LD are in italics.
High Confidence Yeast Lipid Droplet Proteome

Figure 1

A

K8

Heavy

Fractions Mixed

K0

Unlabeled

LDs

MS Analysis

B

Mito. (Lpd1)

Cyt. (Pgk1)

Golgi (Rud3)

Nuc. (Dst1)

ER (Sec63)

LD (Erg6)

Normalized Ratio H/L

Fraction

C

Normalized Ratio H/L

Fraction

Proteasome

Cytoplasm

Ribosome

Mitochondria

Endoplasmic Reticulum

LD

D

Membership Value in LD Cluster (PCP2)

Membership Value in LD Cluster (PCP1)

Correlation = 0.77

E

H/L in LD Fraction

n = 1002

Arbitrary Cutoff

n = 136

In Cutoff of 2nd Replicate

n = 88

H/L in 8 Fractions

n = 68

Filtered LD Purification Profile

n = 35

F

Normalized Ratio H/L

Fraction
Figure 3

High Confidence Yeast Lipid Droplet Proteome

A

Current Work  
\( n = 30 \)  
Athendstaedt et al., 1999  
\( n = 19 \)

Grillitsch et al., 2011  
\( n = 76 \)

Binns et al., 2006  
\( n = 100 \)

B

Histogram showing frequency distribution of molecules/cell in different studies.

- Current Work
- Grillitsch et al., 2011
- Binns et al., 2000
- Athendstaedt et al., 1999
Figure 5

High Confidence Yeast Lipid Droplet Proteome