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**Saccharomyces cerevisiae** lipid droplet associated enzyme Ypr147cp shows both TAG lipase and ester hydrolase activities

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**Saccharomyces cerevisiae** Ypr147cp was found localized to lipid droplets but the physiological role of Ypr147cp remains unknown. Sequence analysis of Ypr147cp revealed an αβ hydrolase domain along with the conserved GXSXG lipase motif. Recombinant Ypr147cp showed both triacylglycerol lipase and ester hydrolase activities. Knock out of YPR147C led to accumulation of TAG in yrpr147cΔ when compared to wild type (WT). In addition, transmission electron microscopic analysis of yrpr147cΔ cells revealed a greater number of lipid bodies, justifying the increase in TAG content, and the phenotype was rescued upon overexpression of YPR147C in yrpr147cΔ. Moreover, the lipid profiling confirmed the accumulation of fatty acids derived from neutral and phospholipids in yrpr147cΔ cells. Based on these results, Ypr147cp is identified as a lipid droplet associated triacylglycerol lipase along with an ester hydrolyzing capacity.

Key Words: Alpha Beta Hydrolase Domain (ABHD); Lipid Droplet (LD); Triacylglycerol (TAG)

**Introduction**

Sterols and triglycerols (TAG) are the two main classes of storage lipids in yeast. An excess amount of these two major classes of lipids are modified and stored within yeast lipid droplets (LD) as TAG and steryl esters (SE) (Grillitsch et al., 2011). While sterols contribute to the permeability and fluidity of constituent biological membranes, TAG is involved in the diverse functions of the cell (Kohlwein, 2010; Sharma, 2006; Turkish and Sturley, 2007). Besides acting as an energy repository in the form of fatty acids (FA), the cleavage products of TAG, diacylglycerides (DAG) and monoacylglycerides (MAG) serve as precursors of membrane phospholipids. Furthermore, excess free fatty acid induced lipotoxic conditions are neutralized by enhanced TAG synthesis. Since the precursors and degradation products of TAG are acting as secondary messengers in diverse signaling pathways, both the synthesis and breakdown of TAG are under stringent control mechanisms (Coleman and Lee, 2004; Kurat et al., 2006). The stored TAG and SE are utilized from the LDs as per the cellular requirements. TAG and SE hydrolyzing enzymes are highly conserved across the phyla and *Saccharomyces cerevisiae*, being a reliable model system, provided a platform for the screening of such hydrolyzing enzymes. Many hydrolytic enzymes possessing multiple domains with diverse substrate specificities are identified from *S. cerevisiae*. Some of these enzymes are known orthologs of mammalian lipases while the others are found to be redundant in *S. cerevisiae* (Grillitsch and Daum, 2011). Tgl3p, the TAG lipase of *S. cerevisiae* was reported to have a conserved GXSXG lipase motif and a well conserved HXXXXD motif attributing acyltransferase activity. Tgl3p was found to be localized in lipid droplets and exhibited DAG hydrolytic activity along with definite lysophosphatidylethanolamine (LPE) acyltransferase activity in vitro (Athenstaedt and Daum, 2003; Rajakumari and Daum, 2010; Schmidt et al., 2013).

Following this, Tgl4p and Tgl5p were reported with no transmembrane domain but having the conserved lipase motif (Athenstaedt and Daum, 2005). Tgl4p is the multifunctional enzyme involved in lipid metabolism. Along with Tgl3p, Tgl5p also displayed an HXXXXD motif but preferentially acted as lysophosphatidicacid (LPA) acyltransferase. Both GSXSG and HXXXXD motifs were found to be acting independently. Mutational studies of the lipase motif did not affect the acyltransferase...
activity of Tgl5p (Rajakumari and Daum, 2010). The other three yeast enzymes, Yeh1, Yeh2 and Tgl1, constitute a separate class of membrane anchored lipases and contribute to sterol ester hydrolysis in vivo (Jandrositz et al., 2005; Köfle et al., 2005).

All these identified enzymes were found to be localized, either in LDs (Athenstaedt and Daum, 2003; Jandrositz et al., 2005; Thom et al., 2011), mitochondria (Ham et al., 2010), peroxisomes (Debely et al., 2011; Thom et al., 2008), or vacuoles (Epple et al., 2001; Teter et al., 2001). In addition, the screening of knock out strains with different enzyme combinations still showed low, but persistent, TAG mobilization (Ploier et al., 2013). The only characteristic property shared by all the known lipases is the presence of a conserved motif GXSXG in the active site with serine playing a vital role in catalysis. This catalytic triad is also found in an α/β hydrolase domain (ABHD) containing enzymes (Brady et al., 1990; Kumar et al., 2016; Schrag and Cygler, 1997; Wiszotzkey et al., 2003). LD hydrolase Ldh1p also had a GXSXG motif and was found to exert TAG lipase and esterase activities. Ldh1a showed the accumulation of nonpolar and phospholipids in lipid droplets (Debely et al., 2011) Ypr147cp was found to be localized to LD but the physiological function was putative (Currie et al., 2014). This prompted studying the molecular characterization of YPR147C and its role in lipid metabolism. Here, we report the functional identification of the lipid droplet enzyme Ypr147cp of Saccharomyces cerevisiae. Ypr147cp exerts both triacylglycerol lipase and short chain ester hydrolyse activities.

**Materials and Methods**

**Growth conditions of yeast strains.** The Saccharomyces cerevisiae strains used as the host system were wildtype BY4741 (WT), the desired gene YPR147C was overexpressed in WT (OE), ypr147Δ, and YPR147C was expressed in Δ (Δ+), and only the pYES2/CT vector was cloned in WT (V). WT and ypr147Δ cells were grown either in YPD medium containing 1% yeast extract, 2% peptone, and 2% dextrose weight/volume (w/v), or synthetic minimal medium (SC+Ura) containing 0.67% yeast nitrogen base (YNB), supplemented with the complete supplement mixture 0.192% appropriate amino acids without uracil, 2% dextrose, and 0.015% uracil (w/v). Recombinant yeast strains, i.e., OE, Δ+, and V, were cultured in synthetic minimal medium (SC-Ura) containing 0.67% YNB, supplemented with the complete supplement mixture 0.192% appropriate amino acids without uracil and dextrose. Induction was carried out in SC-Ura media with 2% raffinose and 3×YP medium with 6% galactose to a final concentration of 1×YP+2% galactose. All cells were cultured in liquid media at 30°C and 180 rpm. As per Gelperin et al., the induction parameters and media conditions were replicated (Gelperin et al., 2005; Kumar et al., 2016).

**Phylogenetic analysis.** Phylogenetic analysis was done with the sequences retrieved from NCBI. Sequence comparison of Ypr147cp were done with orthologs of Human C2orf43/hLDAH (GI: 74761484), Mouse MGI:1916082/mLDAH (GI: 81913659), Drosophila melanogaster CG9186 (GI:74872031), Arabidopsis thaliana AT3G11620 (GI:332641553) and homologs of yeast (TGL1p/YKL140W (GI:464877), TGL2p/YDR058C (GI:1729921), TGL3p/YMR313C (GI:1730604), TGL4p/YKR089C (GI:549643), TGL5p/YOR081C (GI:4676509), EEB1/YPL095C (GI:2501571), EHT1/YBR177C (GI:586309), LDH1/YBR204C (GI:34194085), CVT17p/AUT5/YCR068W (GI:37999929), AYR1p/YIL124W (GI:731868), LPX1/YOR084W (GI:74765695), YEH1/YLL012W (GI:74766447), YEH2/YLR020C (GI:74766452), Yju3p (GI:6322756), LPL1/YOR059C (GI:4767471), ROG1/YGL144C (GI:1723926), MGL2/YMR210W (GI:2501570), and YPR147C (GI:74764609). The phylogenetic tree was developed with the maximum likelihood method using MEGA6 (Tamura et al., 2013). The tree is drawn to 0.2 scale and the analysis involved 22 protein sequences.

**Recombinant Ypr147cp expression and purification.** To determine the hydrolytic activity, the YPR147C gene was overexpressed in the WT background and purified to perform enzyme analysis. YPR147C was cloned in the pYES2/CT vector and transformed into DH5α cells. Only vector pYES2/CT and vector with insert were transformed into Δ and WT cells individually by using the Frozen-EZ Yeast Transformation II kit (Zymo Research, USA) following the manufacturer’s protocol. The expression of the recombinant YPR147C in WT and Δ was performed following Gelperin et al. (2005) protocol. In brief, a single colony of the positive clone was inoculated into 5 ml of SC-Ura +2% dextrose and was grown overnight at 30°C. 1 ml of overnight culture was inoculated into 25 ml of SC-Ura +2% raffinose and continued to grow overnight at 30°C with proper shaking. The overnight 25 ml culture was diluted to 200 ml of SC-Ura +2% raffinose media at a rate of 0.3 OD at 600 nm and incubated until OD reached 1.2. Then cells were induced by the addition of 100 ml of 3×YP+6% galactose to a final concentration of 1×YP+2% galactose, and then grown for 12 h. Full-length protein was expressed with a C-terminal 6xHis-tag using the pYES2/CT vector in S. cerevisiae and was successfully purified from whole cell extracts by an Ni-NTA agarose column. In brief, the yeast cells were lysed for 10 min on a bead beater with an equal volume of lysis buffer (50 mM Tris-HCl (pH 8.0), 1% glycerol, 1 mM PMSF and 5 mM MgCl₂). The lysate was centrifuged for 20 min at 12,000 × g at 4°C and the supernatant was collected. The supernatant was incubated with pre-equilibrated (50 mM Tris-HCl (pH 8.0), 0.3 M NaCl and 10 mM imidazole) Ni-NTA resin. After incubation, the resin was packed in the column and was washed with 5 column volumes of wash buffer (50 mM Tris-HCl (pH 8.0), 0.3 M NaCl and 25 mM imidazole). Subsequently, the bound protein was eluted with 50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 250 mM imidazole. The molecular weight of the purified protein was analysed by SDS PAGE.

**Esterase activity.** Measurement of esterase activity was performed by adopting the standard protocol for p-Nitrophenyl acetate (pNPA), p-Nitrophenyl butyrate (pNPB) (Ploier et al., 2013), and for p-Nitrophenyl palmitate (pNPP) substrates (Kanwar et al., 2005). Measure-
ment of the esterase activity was carried out with purified enzyme, crude extracts of WT, spr147cΔ and OE strains using p-nitrophenyl substrates of acetate, butyrate, octanate, deconate, dodecanate, palmitate, stearate, and oleate. The screened substrates were further analyzed for enzyme kinetics, pH and temperature optima. Controls were set with the pYES2/CT vector overexpressed and purified under the same conditions. All the assays were performed in triplicate and the mean values were recorded. Michaelis-Menten kinetics were analyzed using Graph Pad Prism version 5.

**TAG lipase assay.** TAG lipase activity was assayed using the Lipase Activity Assay Kit (K722-100; Biovision, Mountain View, CA, USA) following the manufacturer’s protocol. In brief, lipase hydrolyzes the triglyceride substrate to form glycerol which is measured at 570 nm, by monitoring an enzymatically linked change in the OxiRed probe absorbance. A TAG lipase activity assay of Ypr147cp enzyme was performed with 7.55 μg of purified enzyme (Subramanian et al., 2013).

**Lipid extraction.** Lipids from the WT and Δ strains were extracted according to Schneiter and Daum (2006). The residue was dissolved in 4 ml of chloroform and transferred into a pre-weighed vial. The solvent was evaporated under a nitrogen gas purge and the weight of the vial along with the residue was noted.

**Separation of lipid classes.** Different lipid classes were separated on LC-Silica Sep Pak cartridges (3 ml, 500 mg, Supelco) according to Lynch and Steponkus (Lynch and Steponkus, 1987). An appropriate amount of total lipid extract was dissolved in 1 ml of chloroform and transferred to the Sep-Pak cartridge. The cartridge was sequentially eluted with 10 ml of chloroform for neutral lipids. After draining the first solvent, 15 ml of acetone:methanol (9:1, v/v) was added to elute the glycolipids and ceramides. Finally, 15 ml of methanol was added into the cartridge to elute the phospholipids. All three fractions were dried under a nitrogen purge and used immediately. Extra fractions were reconstituted with a small volume of chloroform (for neutral lipid) and chloroform: methanol (2:1, v/v) (for other fractions) under nitrogen and stored at −20°C until further analysis.

**Separation of neutral lipid subclasses.** Different neutral lipid subclasses were further separated on LC-Silica Sep Pak cartridges (3 ml, 500 mg, Supelco). An appropriate amount of neutral lipid fraction from the last step was redissolved in 1 ml of hexane. This was transferred to the Sep-Pak cartridge and another 3 ml of hexane was sequentially added to elute hydrocarbons. After draining the first solvent hexane, 6 ml of hexane:diethyl ether (99:1, v/v) was added to elute steryl esters. Another 5 ml of hexane:diethyl ether (95:5, v/v) was sequentially added to elute triglycerides, and 5 ml of hexane:diethyl ether (92:8, v/v) to elute free fatty acids. All fractions, except the hydrocarbons, were evaporated under a nitrogen purge and used immediately, or stored by reconstituting with a small volume of chloroform under nitrogen at −20°C until further FAMEs analysis.

**Preparation of FAMEs.** Fatty acid methyl esters (FAMEs) were prepared by 2% H₂SO₄, 2 ml of H₂SO₄ with 100 ml methanol. FAMEs were extracted by the addition of 2 × 2 ml aliquots of hexane and vortexing. The two layers were allowed to separate and the upper hexane layer was collected and subjected to gas chromatography analysis for the identification and quantification of fatty acids.

**Gas chromatographic analysis of FAMEs.** Analysis of FAMEs was performed using an Agilent 6890N gas chromatography instrument coupled with an Agilent MS-5975 iner XL mass selective detector (Agilent Technologies) in the Electron Impact (EI) mode. Separation of fatty acids was achieved by injecting 2 μl of the FAMEs on to (88% - Cyanopropyl) aryl-polysiloxane column, HP88 (Agilent J & W Scientific, 30 × 0.25 mm × 0.25 μm). Splitless injection was performed with a constant carrier gas (helium) at a flow rate of 1 ml/min. The inlet temperature and transfer line temperatures were set at 200°C and 180°C, respectively. The temperature programming was as follows: initial isotherm of 80°C held for 1 min, raised to 90°C at the rate of 1°C/min, 90–250°C at a rate of 6.1°C/min with a hold of 15 min at the final temperature. The MS ion source temperature was 230°C and the Quadruple temperature was 150°C. Peak identification of fatty acids in the analyzed samples was carried out by a comparison of the chromatogram with the mass spectral library (NIST), and against the retention times and mass spectra of Supelco 37 component FAME mix (Sigma-Aldrich, St Louis, MO, USA). Statistical analysis of the data was carried out following a paired t-test. The level of significance was evaluated from a p-value of 0.05.

**Electron microscopy.** For the transmission electron microscopy, overnight cultures of WT, Δ, and OE, cells were harvested at 0.5 OD at A600. The pellet was washed with 0.1 M phosphate buffer (pH 7.4) and again centrifuged. The cells were then fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4°C and washed with a buffer. After the wash, samples were postfixed in 1% OsO₄ (osmium tetroxide) for 1 h at 4°C. The samples were dehydrated in an ascending grade of acetone, infiltrated and embedded in Araldite CY 212 (TAAB, UK). Thick sections (1 μm) were cut with an ultramicrotome, mounted onto glass slides, stained with aqueous toluidine blue, and observed under a light microscope for gross observation of the area and quality of the cell fixation. For the electron microscope examination, thin sections of grey-silver colour interference (70–80 nm) were cut and mounted onto 300 mesh copper grids. Sections were stained with alcoholic uranyl acetate and alkaline, lead citrate, washed gently with distilled water and observed using a Tecnai transmission electron microscope (Fei Company, The Netherlands) at an operating voltage 200 KV (Wright, 2000). Images were digitally acquired by GATAN camera (Megaview III, Fei Company) using iTEM software (Sift Imaging System, Münster, Germany) attached to the microscope.

**Growth sensitivity and growth curve assays.** The effect of the YPR147C gene knockout and overexpression on the growth of yeast cells was examined by using a growth sensitivity assay as described previously (Golla et al.,
Ypr147cp was reported by Currie et al. (2014) to be localized to lipid droplets, but its functionality was not clearly indicated. To understand the biochemical functionality of \textit{YPR147C}, it was cloned and expressed in \textit{S. cerevisiae}. The present study reveals the TAG lipase and ester hydrolase activities of recombinantly expressed Ypr147cp based on the experimental data.

**In silico analysis**

Ypr147cp (gi|74676409|sp|Q06522.1) sequence analysis showed that the protein has 304 amino acids containing an \(\alpha/\beta\) hydrolase domain (ABHD) ranging from 23–293 amino acids (IPR029058), and was also depicted as a lipid droplet associated hydrolase domain spanning 9–293 amino acids (PTHR13390). The GXSXG motif, which is the general substrate binding site of TAG lipases and ester hydrolases, is found conserved between 107–111 amino acids (Fig. 1). The conserved catalytic triad residues of D and H are also indicated (\(\varnothing\)).

**Phylogenetic analysis**

The phylogenetic tree diverged into three major clades with (i) yeast LD proteins Tgl3p, Tgl4p, Tgl5p and Ayr1p, (ii) Ypr147cp grouped with known LDAH proteins of Human, Mouse (25% identity), \textit{Drosophila} (24% identity), along with an uncharacterized \textit{Arabidopsis} ortholog (24% identity). The only yeast homolog paired with Ypr147cp is the Ldh1p (18% identity) with similar functionality, and shown by shaded boxes. In addition, catalytic triad residues of D and H are also indicated (\(\varnothing\)).

**Results**

Ypr147cp was reported by Currie et al. (2014) to be localized to lipid droplets, but its functionality was not clearly indicated. To understand the biochemical functionality of \textit{YPR147C}, it was cloned and expressed in \textit{S. cerevisiae}. The present study reveals the TAG lipase and ester hydrolase activities of recombinantly expressed Ypr147cp based on the experimental data.
Ypr147cp shows in vitro TAG lipase and hydrolytic activity. (iii) the major group which contained all other known homologs of yeast lipases (Fig. 2).

Ypr147cp shows in vitro TAG lipase and hydrolytic activity

To determine the hydrolytic activity, YPR147C was overexpressed and purified to perform enzymatic assays. For this purpose, full-length protein was expressed with a C-terminal His-tag using pYES2/CT vector in S. cerevisiae and was successfully purified from whole cell extracts using an Ni-NTA agarose column (Supplementary Fig. S1). This affinity purified fraction showed TAG lipase activity of 14.97 ± 1.81 nmol/min/mg of protein against control. The purified recombinant Ypr147cp enzyme was used for esterase activity with pNPA, pNPB as well as pNPO, pNPD, pNPDD, pNPP, and pNPS as substrates. Ypr147cp showed activity with pNPA and pNPB substrates only. Esterase activity was screened at different pHs and was found to be an optimum at pH 7.5 for both pNPA and pNPB substrates, respectively (Fig. 3). The esterase activity was also monitored at different temperatures and both the substrates showed optimum activity at 30°C (Fig. 3). Ypr147cp cleaved pNPA with a K_m of 12.07 ± 0.92 mM, V_max of 0.87 ± 0.05 s⁻¹, and a k_cat/K_m of 154 mM⁻¹s⁻¹, and pNPB with a K_m of 14.79 ± 1.15 mM, V_max of 14.79 ± 1.15 s⁻¹, with a k_cat/K_m of 65 mM⁻¹s⁻¹ (Table 1). Control assays with the only pYES2/CT vector overexpressed and purified showed no activity with p-nitrophenyl substrates. Based on these results, Ypr147cp confirms both TAG lipase and very short chain ester hydrolase activities.

GC-MS lipid profile of WT and ypr147cΔ

Comparison of the lipid profiles of ypr147cΔ and the WT strain revealed significant variations among fatty acids derived from certain lipid classes. The total lipid content showed an increase of arachidic acid C20:0 (p = 0.009), 9Z-docosenoic acid C22:1(13) (p = 0.005), and, in contrast, cetoleic acid C22:1(11) content significantly decreased (p = 0.008) along with oleic acid/elaidic acid C18:1(9) (p = 0.017). Apart from this, other total lipids did not show any significant variation (Fig. 4A). Phospholipids showed a significant increase in palmitic acid C16:0 (p = 0.018) in ypr147cΔ strain, whereas oleic acid/elaidic acid C18:1(9) decreased (p = 0.022) (Fig. 4B) respectively, when compared with WT.

Neutral lipid class of ypr147cΔ strain significantly accumulated arachidic acid C20:0 and 9Z-docosenoic acid C22:1(13) with p = 0.011 and p = 0.032 respectively, while cetoleic acid C22:1(11) (p = 0.009) was found decreased significantly when compared to WT (Fig. 4C).

Similarly steryl esters of ypr147cΔ showed variation significantly among cetoleic acid C22:1(11) (p = 0.015) and C14:0 tetradecanoic acid (myristic acid) (p = 0.016) when compared to WT. In addition, steryl ester C16:1(9) palmitoleic acid content increased significantly (p = 0.013) in the ypr147cΔ strain. Other steryl esters were found to be varying, but the changes were not significant (Fig. 4D).

Electron microscopy revealed the accumulation of lipid droplets in the ypr147cΔ strain

Electron microscopy confirmed normal appearing ± 1 lipid droplets in wild type cells, whereas 6 ± 1 LD’s accumulated in ypr147cΔ cells showed a significant variation (p = 0.029). However, there was no significant variation in the diameter (0.31 ± 0.041 μm) of the LD’s of WT and ypr147cΔ grown under similar conditions (Fig. 5). This data clearly indicates the possible role of YPR147C in the lipid droplet metabolism of S. cerevisiae.

Table 1. Kinetic parameters of Ypr147cp. Showing the Michaelis-Menten kinetics (K_m, V_max and k_cat/K_m) of Ypr147cp ester hydrolase activity with pNPA and pNPB as substrates.

| Substrate | K_m (mM) | V_max (s⁻¹) | k_cat/K_m (mM⁻¹s⁻¹) |
|-----------|----------|-------------|---------------------|
| pNPA      | 12.07 ± 0.92 | 0.87 ± 0.05 | 154                 |
| pNPB      | 14.79 ± 1.15 | 0.44 ± 0.08 | 65                  |
Ypr147cp-TAG lipase and Ester hydrolase

There was no effect of YPR147C loss in cultures spotted onto the SC-Agar plates and imaged after 48 h incubation at 30°C. The growth of WT and ypr147cΔ cells was also monitored for 20 h and both WT and ypr147cΔ cells were found growing normally (Fig. 6A). Similarly, the overexpression of YPR147C did not affect the growth of WT, ypr147cΔ when compared with WT cells containing an empty vector (Fig. 6B), both on plates and in liquid cultures.

Discussion

Lipases play an important role in maintaining lipid homeostasis in cells. YPR147C was previously reported as LD localized protein of Saccharomyces cerevisiae but was not functionally characterized. The Ypr147c protein sequence showed a lipase catalytic domain and it belongs to the α/β hydrolase family. Ypr147cp was found aligning with Drosophila melanogaster CG9186 (Thiel et al., 2013), Mus musculus MGI: 1916082 and Homo sapiens C2orf43 (Goo et al., 2014), and showed a similar lipase activity profile.

The present study highlights the TAG lipase activity of Ypr147cp along with very short chain esterase activity. The Ypr147cp $K_m$ and $V_{max}$ values are comparable to the activity of Ldh1p with a pNPB substrate (Thoms et al., 2011). This is also in agreement with the results of the cellular TAG analysis of the WT, OE, ypr147cΔ and Δ+ strains. Deletion of YPR147C led to an increase in the TAG content and the TAG levels in ypr147cΔ were rescued by recombinant YPR147C expression in the ypr147cΔ strain, justifying the role of Ypr147cp in TAG turnover. However, TAG turnover is not only dependent on the main lipases (Tgl3p, Tgl4p and Tgl5p) but also taken care by a set of lipases and hydrolases with lower activities. Interestingly, yeast lacking the major lipases Tgl3p, Tgl4p and Tgl5p, along with Ayr1p and Ldh1p, still showed TAG mobilisation indicating the presence of other functional enzymes. The lipolytic activity shown by Ypr147cp might

**Fig. 4.** GC MS estimation of total fatty acids derived from different classes of lipids in WT and ypr147cΔ strains.

Total fatty acids derived from different lipid classes of Saccharomyces cerevisiae WT and ypr147cΔ strains were estimated and are represented as (A) total lipid, (B) phospholipids, (C) neutral lipid, and (D) steryl esters. Significant variation of lipid classes are represented with “*” and the data represented as mean ± SD of replicate values.
also play a role in the TAG homeostasis.

There was a marked increase in the neutral lipid class of 20:0 and 22:1(13) in ypr147cΔ which is in line with the accumulation of nonpolar lipids in Ldh1Δ. Though in minor amounts, VLCFAs (C_{22:0}, C_{22:1}, C_{20:0}, C_{20:1}) were also known to be components in the synthesis of sphingolipids (Cerantola et al., 2007) which are the major components of plasma membranes in *S. cerevisiae*. Accumulation of VLCFA in ypr147cΔ suggests the involvement of Ypr147cp in the supply of fatty acids to membranes. In addition, human Lipid Droplet—Associated Serine Hydrolase (hLDAH) was found regulating CE homeostasis in human embryonic kidney-293 cells, macrophages and was also involved in cholesterol mobilization (Goo et al., 2014). Ypr147cp, also being a lipid droplet associated hydrolase exhibited variations among steryl esters.

Interestingly, the localization of Ypr147cp to lipid droplets was known but the orientation of Ypr147cp on a LD membrane was not studied. A transmembrane region occupying the central position of the protein sequence was predicted (Supplementary Fig. S2 and GXS^{109}XG is found towards the N-terminal, and the other two conserved residues of catalytic triad D (255) and H (289) were located on the other side of the transmembrane domain. Hence, Ypr147cp might be oriented with both N and C terminals inside the LD while attached to the LD membrane (Supplementary Figs. S3A and B) similar to Yeh1 orientation (Köffel et al., 2005). Moreover the transmembrane domain was predicted only in *Drosophila* (Thiel et al., 2013) and yeast proteins but not in human and mouse orthologs. Since LDs derive from the endoplasmic reticulum by inclusion of neutral lipids, it would also be interesting to understand the role of the transmembrane domain in integrating with the monolayer membranes of LDs.

Our data clearly suggest that Ypr147cp is a TAG lipase along with an ester hydrolyzing capacity, associated with lipid droplets, and the accumulation of lipid droplets in ypr147cΔ strongly suggests a role of Ypr147cp in LD lipid metabolism.

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Fig. 5. Transmission electron microscopic analysis of yeast lipid droplets.
WT strain lipid droplets are fewer in number, whereas ypr147cΔ shows an increase in the number of lipid droplets, and YPR147C OE rescued the phenotype of WT showing a fewer number of lipid droplets.

Fig. 6. Effect of overexpression or deletion of YPR147C on cell growth.
(A) Showing wild-type (WT) BY4741 and ypr147cΔ exponentially growing cells monitored for 24 h by recording the Optical Density at 600 nm using a plate reader at 30°C. (B) WT and ypr147cΔ cultures spotted onto SC+ Ura Agar plates and imaged after 48 h incubation at 30°C. (C) Represents the growth curve of WT cells containing only pYES2/CT vector (WT+Vector), YPR147C overexpressed in WT (OE) and YPR147C overexpressed in ypr147cΔ cells (∆+) using the same vector, monitored in respective liquid media for 36 h. (D) Stamping data showing no growth difference between WT+Vector, OE and ∆+ when spotted onto the SC-Ura Agar plates and imaged under the same growth conditions.
Conflict of Interest

The authors declare that there is no conflict of interest.

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Supplementary Materials

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References

Athenstaedt, K. and Daum, G. (2003) YMR313c/TGL3 encodes a novel triacylglycerol lipase located in lipid particles of Saccharomyces cerevisiae. J. Biol. Chem., 278, 23317–23323.
Athenstaedt, K. and Daum, G. (2005) Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast Saccharomyces cerevisiae are localized to lipid particles. J. Biol. Chem., 280, 37301–37309.
Brady, L., Brzozowski, A. M., Chen, Z. S., Doedson, E., and Doedson, G. (1990) A serine protease triad forms the catalytic centre of a triacylglycerol lipase. Nature, 343, 767.
Cerantola, V., Vionnet, C., Eiebisher, O. F., Jenny, T., Knudsen, J. et al. (2007) Yeast sphingolipids do not need to contain very long chain fatty acids. Biochem. J., 401, 205–216.
Coleman, R. A. and Lee, D. P. (2004) Enzymes of triacylglycerol synthesis and their regulation. Prog. Lipid Res., 43, 134–176.
Currie, E., Guo, X., Christiano, R., Chitraju, C., Kory, N. et al. (2014) High confidence proteomic analysis of yeast LDs identifies additional droplet proteins and reveals connections to dolichol synthesis and sterol acetylation. J. Lipid Res., 55, 1445–1477.
Debelyy, M. O., Thoms, S., Connerth, M., Daum, G., and Erdmann, R. (2011) Involvement of the Saccharomyces cerevisiae hydrolase Ldh1p in lipid homeostasis. Eukaryotic Cell, 10, 776–781.
Epple, U. D., Suriapranata, I., Eskelinen, E.-L., and Thumm, M. (2001) Aut5p/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. J. Biol. Chem., 276, 134–142.
Gelperin, D. M., White, M. A., Wilkinson, M. L., Kon, Y., Kung, L. A., Kon, Y., L. et al. (2005) Biochemical and genetic analysis of the yeast proteome demonstrates that genes encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis. Molecular and Cellular Biology, 25, 1655–1668.
Kohlewein, S. D. (2010) Triacylglycerol homeostasis: insights from yeast. J. Biol. Chem., 285, 15663–15667.
Kumar, N., Thunuguntla, V., Veeramachaneni, G., Guntupalli, S., and Bondidi, J. S. (2016) Molecular characterization of human ABHD2 as TAG lipase and ester hydrolase. Biochim. Biophys. Acta, 83, 761–767.
Lynch, D. V. and Steponkus, P. L. (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (Secale cereale L. cv Puma). Plant Physiology, 83, 761–767.
Ploier, B., Scharwey, M., Koch, B., Schmidt, C., Schasse, J. et al. (2013) Screening for hydrolytic enzymes reveals Ayr1p as a novel triacylglycerol lipase in Saccharomyces cerevisiae. J. Biol. Chem., 288, 36061–36072.
Rajakumari, S. and Daum, G. (2010) Janus-faced enzymes yeast Tgl3p and Tgl5p catalyze lipase and acyltransferase reactions. Molecular Biology of the Cell, 21, 501–510.
Schmidt, C., Athenstaedt, K., Koch, B., Ploier, B., and Daum, G. (2013) Regulation of the yeast triacylglycerol lipase tgl3p by formation of nonpolar lipids. J. Biol. Chem., 288, 19939–19948.
Schneiter, R. and Daum, G. (2006) Extraction of yeast lipids. Yeast Protoc., 41–45.
Schrag, J. D. and Cysler, M. (1997) Lipases and αβ-hydrolyase fold. Methods in Enzymology, 284, 85–107.
Sharma, S. C. (2006) Implications of sterol structure for membrane lipid composition, fluidity and phospholipid asymmetry in Saccharomyces cerevisiae. FEMS Yeast Research, 6, 1047–1051.
Subramanian, M., Metya, S. K., Sadaf, S., Kumar, S., Schwudke, D. et al. (2013) Altered lipid homeostasis in Dro sophila InsP3 receptor mutants leads to obesity and hyperphagia. Disease Models & Mechanisms, 6, 734–744.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30, 2725–2729.
Teter, S. A., Eggertson, K. P., Scott, S. V., Kim, J., Fischer, A. M. et al. (2001) Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. J. Biol. Chem., 276, 2083–2087.
Thiel, K., Heier, C., Haberl, V., Thul, P. J., Oberer, M. et al. (2013) The evolutionarily conserved protein CO9816 is associated with lipid droplets, required for their positioning and for fat storage. J. Cell Sci., 126, 2198–2212.
Thomas, S., Debelyy, M. O., Nau, K., Meyer, H. E., and Erdmann, R. (2008) Lpx1p is a peroxisomal lipase required for normal peroxisome morphology. FEBs J., 275, 504–514.
Thoms, S., Debelyy, M. O., Connerth, M., Daum, G., and Erdmann, R. (2011) The putative Saccharomyces cerevisiae hydrolase Ldh1p is localized to lipid droplets. Eukaryotic Cell, 10, 770–775.
Turkish, A. and Sturley, S. L. (2007) Regulation of triglyceride metabolism. I. Eukaryotic neutral lipid synthesis: “Many ways to skin ACAT or a DGAT”. American Journal of Physiology-Gastrointestinal and Liver Physiology, 292, G953–G957.
Wisotzkey, R. G., Johnson, A. N., Takaesu, N. T., and Newfeld, S. J. (2003) αβ hydrolyase2, a predicated gene adjacent to mad in Drosophila melanogaster, belongs to a new global multigene family and is associated with obesity. Journal of Molecular Evolution, 56, 351–361.
Wright, R. (2000) Transmission electron microscopy of yeast. Microscopy Research and Technique, 51, 496–510.