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Identification of a phospholipase B encoded by the *LPL1* gene in *Saccharomyces cerevisiae*

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ABSTRACT

Phospholipids also play a major role in maintaining the lipid droplet (LD) morphology. In our current study, deletion of *LPL1* resulted in altered morphology of LDs and was confirmed by microscopic analysis. *LPL1*/*YOR059c* contains lipase specific motif GXSXG and acetate labeling in the *LPL1* overexpressed strains depicted a decrease in glycerophospholipids and an increase in free fatty acids. The purified Lpl1p showed phospholipase activity with broader substrate specificity, acting on all glycerophospholipids primarily at sn-2 position and later at sn-1 position. Localization studies precisely revealed that Lpl1 is exclusively localized in the LD at the stationary phase. Site directed mutagenesis experiments clearly demonstrated that the lipase motif is vital for the phospholipase activity. In summary, our results demonstrate that yeast Lpl1 exerts phospholipase activity, plays a vital role in LD morphology, and its absence results in altered LD size. Based on the localization and enzyme activity we renamed *YOR059c* as *LPL1* (LD phospholipase 1).

1. Introduction

All eukaryotic cells including yeast possess the cytoplasmic lipid droplets (LDs), which provide dense source of energy. These LDs consist of neutral lipids, triacylglycerol (TAG) and steryl esters (SE) and are surrounded by a single leaflet of phospholipids where a few proteins are embedded [1–5]. Some proteins residing on the lipid droplets are also present in endoplasmic reticulum and catalyze the synthesis or breakdown of core lipids [6,7]. In *Saccharomyces cerevisiae*, most of the LD proteins are enzymes involved in lipid biosynthesis. Erg6p (sterol methyltransferase), Slc1p (1-acylglycerol 3-phosphate O-acyltransferase) and Gat1p (glycerol 3-phosphate acyltransferase) were all identified in lipid droplet [8,9]. Understanding the biogenesis of lipid droplets during lipogenesis and lipolysis is crucial to understand lipid related disorders [10].

The biogenesis of lipid droplet in *Saccharomyces cerevisiae* is still in dispute and the enzymes involved in the synthesis of TAG and SE are predominantly localized in endoplasmic reticulum. Hence, it is believed that LD is generated from ER and budded off to cytoplasm. The final step of TAG biosynthesis is catalyzed by two different enzymes. The acyl-CoA independent acyltransferase Lro1p catalyzed the acylation of DAG to TAG using phospholipid as acyl donor and is exclusively localized in ER [11]. The other enzyme Dga1p, catalyzes the acyl CoA dependent acylation of DAG and is dually localized both in ER and LD [12,13]. Are1p and Are2p are localized exclusively in the ER, and involved mainly in SE synthesis but also contribute to the acyl CoA dependent TAG synthesis [14]. Interestingly, Are1p exhibited increased activity under anaerobic conditions and differs from Are2p in their substrate specificity [15,16].

The three TAG lipases Tgl3p, Tgl4p and Tgl5p and the two SE hydrolase Yeh1p and Tgl1p degrade TAG and SE to form DAG, sterol and FFA and are mainly localized on the LD surface [17,18]. Apart from this, mitochondrial localized Tgl2p [19] and plasma membrane localized Yeh2p [20] catalyze the degradation of TAG and SE respectively. Tgl2p and Tgl3p also hydrolyze DAG to MAG which is further degraded by the LD localized Yju3p to FFA and glycerol [21].

Similarly, phospholipases maintain the phospholipid homeostasis and remodeling [22,23]. Phospholipase B has been described in a wide range of eukaryotic species and plays a key role in phospholipid turnover. They release the fatty acids from both sn-1 and sn-2 positions without accumulation of lysophospholipids [24,25]. The genes that encode the phospholipase B in yeast are *PLB1*, *PLB2*, and *PLB3* and catalyze a similar type of reaction but differ in their substrate preference and localization. Plb1 is localized in the ER, vesicles and plasma membrane and is also secreted into the extracellular space and primarily utilizes PC and PE as substrates *in vivo* [26]. Plb3 is found in vesicles, vacuoles, as well as cytosol and primarily uses PI as a substrate *in vivo* [27]. Plb2 is a homolog of Plb1, confers resistance to 1-palmitoyl lysophosphatidylcholine toxicity when overexpressed; however it does not exhibit the transacylase activity like Plb1 [28]. Triple deletion of *PLB1, PLB2* and *PLB3* displayed better growth than wild type cells which concluded that they are not essential for cell viability [29,30]. Nte1, a PC specific phospholipase B was also identified which controls

Abbreviations: LD, lipid droplet; PL, phospholipid; FFA, free fatty acid; PC, phosphatidylcholine; DDM, dodecyl maltoside

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the PC abundance in the ER and also regulates the transcription of phospholipid biosynthetic genes [31,32]. However, to date no phospholipase has been reported in LDs.

In the present study, we characterized the yeast open reading frame *YOR059c* which contains a typical consensus sequence of GXSXG and harbors a predicted αβ hydrolase domain. *In vitro* experiments evidenced that it was able to hydrolyze all phospholipids preferentially at sn-2 position followed by sn-1. Overexpression of the protein led to substantial reduction of total phospholipids accompanied with accumulation of FFA. Here we report that *LPL1* encodes a broad substrate phospholipase B and is localized in lipid droplet and we termed the gene *YOR059c* as *LPL1*—lipid droplet phospholipase 1.

2. Materials and methods

2.1. Chemicals and media

Yeast extract, peptone, and bacteriological agar were purchased from Difco. Radiochemicals: [^{14}C]acetate was supplied by American Radiolabeled Chemicals. TopFluor-LPE (1-(dipyrrometheneboron difluoride)undecanoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), NBD-PC (1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl}-*sn*-glycero-3-phosphocholine), NBD-PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl), NBD-PE (1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl}-*sn*-glycero-3-phosphoethanolamine) NBD-PA (1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphate), NBD-PG (1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] and NBD-PS (1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl}-*sn*-glycero-3-phosphoserine) were supplied by Avanti Polar Lipids. Thin-layer Silica Gel 60 plates were purchased from Merck. BODIPY 493/503 purchased from Invitrogen. Dodecyl maltoside (DDM) and phenylmethylsulfonylfluoride were purchased from Sigma. All other chemicals were purchased from Sigma unless specifically mentioned.

2.2. Plasmids, strains and culture conditions

Plasmids used in this study are listed in Table 1. The wild type *Saccharomyces cerevisiae* strain (BY4741-MATa; HIS3Δ1; LEU2Δ0; MET15Δ0; URA3Δ0) and *lpl1Δ* from wild background were used in this study and obtained from Euroscarf. Cultures were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) medium. Protein expression was induced by growing the cells aerobically at 30 °C in synthetic medium without uracil (SM-U) in the presence of 2% galactose for 24 h. *Escherichia coli* cells were maintained in Luria Bertani medium composed of 0.5% yeast extract, 1% tryptone, and 1% NaCl with 100 mg/l ampicillin.

2.3. Construction of plasmid that overexpresses LPL1 and its GFP tag

Yeast genomic DNA was prepared by standard protocol and used as a template [33]. The gene corresponding to *LPL1* was amplified from the genomic DNA of wild type *Saccharomyces cerevisiae* using the following primers. 5′-ATATGGATCCATGACTTCGGATAAACACCT TT-3′ and 5′-ATAT CTCGAGATTACTCTTGTGCATCAAGTATG-3′ as forward and reverse primers. The amplification was carried out under the following conditions: initial denaturation of the template at 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s (denaturation), 55 °C for 1 min (annealing), and 72 °C for 1 min (extension). The final extension was done at 72 °C for 10 min. The amplified product was cloned into pYES2/NT-C vector (Invitrogen) at BamHI and XhoI restriction sites. The resultant plasmids containing the gene were confirmed by double digestion and sequencing. For the construction of *LPL1*-GFP plasmid, the *LPL1* gene was double digested with BamHI and XhoI from plasmid pYES2/NTC and the fragment was gel-purified and inserted into the BamHI and XhoI sites of pUG34 plasmid. The yeast expression plasmids pYES2/NT-*LPL1* and pUG34-*LPL1* were transformed into *Saccharomyces cerevisiae* strain by standard lithium acetate method [34].

Table 1
Plasmids used in this study.

Plasmids	Description	Source
pYES2-NT/C	GAL promoter, URA3 marker	This study
pYES2-NT/C-*LPL1*	*LPL1* with N-terminal His tag	This study
pYTY103	*ERG6* promoter, *ERG6*-mRFP	Ryogo Hirata
pRS316	ADH1 promoter, *Sec12*-mRFP	Ryogo Hirata
pUG34	Met25 promoter, HIS marker	Cathal Wilson
pUG34-*LPL1*	Met25 promoter, EGFP-*LPL1*	This study

2.4. Purification of His-tagged Lpl1p from membrane fractions

Yeast cells were grown in the presence of galactose and suspended in lysis buffer containing 50 mM Tris, pH 6.5, 1 mM $MgCl_2$, 1 mM EDTA, 1% glycerol and 0.1 mM phenylmethylsulfonyl fluoride. Cells were lysed using glass beads and unbroken cells were removed by centrifugation at 5000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 90 min to obtain the membrane fractions. The membrane fraction was solubilized with the lysis buffer containing 20 mM DDM and incubated at 4 °C for 60 min and centrifuged at 150,000 *g* for 60 min. The solubilized membrane fraction was then loaded on to Ni^{2+}-NTA column chromatography and the protein purified with elution buffer (50 mM Tris, 150 mM NaCl, 250 mM imidazole and 3 mM DDM). Eluted, purified protein fractions were analyzed by 12% SDS–PAGE followed by Coomassie Brilliant Blue staining. Protein expression was confirmed by immunoblotting using anti-His antibodies at a dilution of 1:5000 (v/v). Protein concentrations were determined by the Bradford assay with bovine serum albumin as a standard.

2.5. Incorporation of [^{14}C]acetate into yeast lipids

The yeast transformants (pYES2/NT-*LPL1* and pYES2/NTC) were grown to late log phase in 5 ml of SM-U containing 2% glucose. Lipid labeling was done by inoculating 0.4 OD (A_{600}) of the cells into a fresh medium containing 2% galactose and 0.5 μCi/ml of [^{14}C]acetate and grown for 24 h at 30 °C until the cells reached an OD of 20 (A_{600}). Lipids were extracted using the Bligh and Dyer method [35]. Briefly the cells were vortexed with glass beads, 600 μl of chloroform/methanol (1:2, v/v) and washed with 200 μl of 2% orthophosphoric acid. Lipids from chloroform extract were separated using Silica Gel TLC plates. For neutral lipids, petroleum ether/diethyl ether/glacial acetic acid (70:30:1, v/v) was used as a solvent system and for phospholipids, chloroform/methanol/ammonia (65:25:5, v/v) was used in the first dimension and chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, v/v) in the second dimension. After the separation, TLC plates were exposed and developed by phosphor imager, individual lipid spots were scraped off from the plate and their radioactivity determined using liquid scintillation counter.

2.6. In vitro phospholipase assay

Phospholipase activities of purified protein fractions were assayed by measuring the release of fluorescently labeled products from NBD labeled phospholipids. The assay mixture contained the assay buffer (50 mM Tris, pH 6.5, 1 mM DTT), 2 μg purified protein, and 5 μM of NBD-PC/NBD-PE/NBD-PS/NBD-PA/NBD-PG/TopFlour-LPE (5 μl of sonicated vesicles in 5 mM CHAPS) in a final volume of 100 μl. The reaction was carried out at 30 °C for 30 min and terminated by lipid extraction with chloform–methanol–2% orthophosphoric acid. Fluorescent lipids were resolved by single dimension TLC on silica plates using

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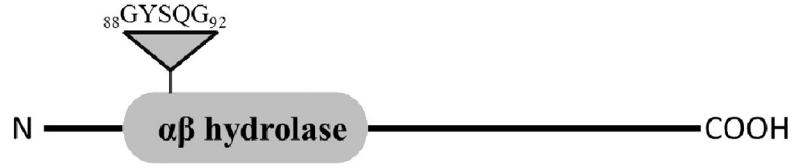

B

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YOR059C         ----------------MTSDKHLFVLIHGLWGNYTHMESMRTILSTTLKKEDVNDDMIYFL    45
O.sativa        ---AEEEAQAPGGGVGPEPDHLVVMVHGIVGSAADWKFGAE-----QFEKLLSDKVIVHR    66
A.thaliana      DESGNVDLTVDFPGERTEPTHLVVMVNGLIGSAQNWRFAAK-----QMLKKYPQDLLVHC    115
                                .  **.*::.*: *.  . .            :   :.:: .

YOR059C         PKQNAMFKTFDGIEIIGYRTLIEVCEFIRDYKDGKITKLSVMGYSQGGLVARFMIGKMLT    105
O.sativa        SNRNMYKLTLDGVDVMGERLAQEIVEETN--KRPQIRKISFVAHSVGGLVARYAIGRLYR    124
A.thaliana      SKRNHSTQTFDGVDVMGERLAEEVRSVIK--RHPSLQKISFVGHSLGGLIARYAIGRLYE    173
                 ::*    *:**::::* *   *: .  .  :  .: *:*.:.:* ***:**: **::

YOR059C         EF---------------------KELFEDIEPQLFITMATPHLGVEFYNPTGIAYKSALY    144
O.sativa        PPKQTSQSS-------QNLNNTNKGTIHGLEAVNFITVASPHLGSRGNKQVPFLFGFTAI    177
A.thaliana      QESREELPHNSDDIGDKCSIEEPKARIAGLEPVYFITSATPHLGSRGHKQVPLFSGSYTL    233
                                  *  :  :*   *** *:**** .  : . :

YOR059C         SAL-RTLGSTILGKSGREMFIANSSNNIL---V----KLSQGEYLEALSLFKWRIAFANV    196
O.sativa        ETFASYIIHLIFGKTGKHLFLTDNDDGKPPLLLRMVDDWGGVQFMSALKVFKRRVAYSNV    237
A.thaliana      ERLATRM-SGCLGKTGKHLFLADSDGGKPPLLLRMVKDSRDLKFISALQCFKRRIAYANT    292
                . :   :    :**:*:.:*::::..       :    .    :::.**. ** *:*::*.

YOR059C         KNDRTVAFYTAFITDCDPFIDFDNKLKYTFEEKIPGSGYKGILPKIVDLNALNVNSHAPT    256
O.sativa        GHDHIVGWRTSSIRRNSELPKW----------TDSG-SKIYPHIVYEELSKAET----    280
A.thaliana      SFDHLVGWSTSSIRRHNELPKL----------QRGPVNEKYPHIVNVEAPDTASNYEE    340
                  *: *.: *: *   . : .                .  .   *:**  :  .. :

YOR059C         KPTKTYKKWGRTILIILVATFLILPIALVMNGLGTAYSYIVTCKYRKMLSNGILHNEVRG    316
O.sativa        MNQCTD--VADVDSCML-EERL-------LRGLKLVSWEKVDVSFHNSKVRSAAHSVIQV    330
A.thaliana      DHSRTK--ADRFKNLDM-EEEM-------IRELTKLSWERVDVSFRGTLQRFLAHNTIQA    390
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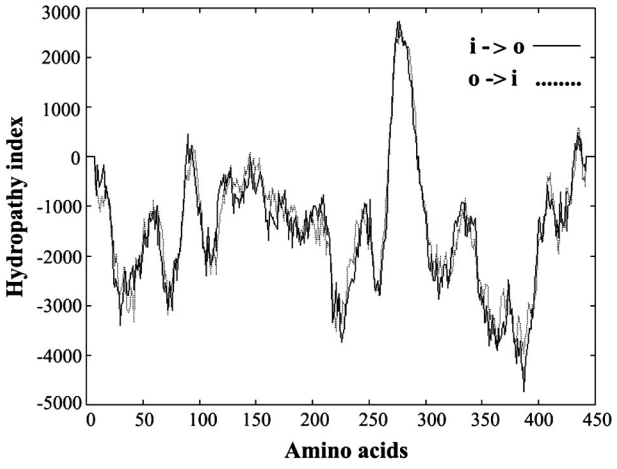

Fig. 1. Domain structure, multiple sequence alignment and hydropathy plot of Lpl1p. A. Domain structure of Lpl1p. The domains are retrieved from the conserved domain database at NCBI and pfam. It was found to have αβ hydrolase fold (pfam00561), lipase fold (pfam 00151). B. BLAST analysis of protein with non-redundant protein database available at NCBI was performed. Multiple sequence alignment of *YOR059c* (NP_014702.1) is homologous to *Annnnnnn thaliana* (NP_001031014.1) and *Oryza sativa* indica group (EEC73719.1). *C*, Hydropathy plot was performed for Lpl1p using TMPRED. i→o, inside to outside; o→i, outside to inside.

chloroform/methanol/28% ammonia (65:25:5, v/v) as solvent system. NBD-containing molecules were visualized using a PhosphorImager Typhoon (Amersham Biosciences) and quantified by Quantity One software. Enzyme activity was determined using different substrate concentrations. We used GraphPad Prism version 5 to calculate the Michaelis–Menten kinetics.

2.7. Localization of Lpl1p by confocal microscopy

Using a confocal laser scanning fluorescence microscope, *LPL1*-EGFP cells were co-expressed either with *Erg6*-*m*RFP or *Sec12*-mRFP and were illuminated at 488 and 570 nm with an argon laser to excite EGFP and mRFP respectively. For microscopy with Nile red staining, cells

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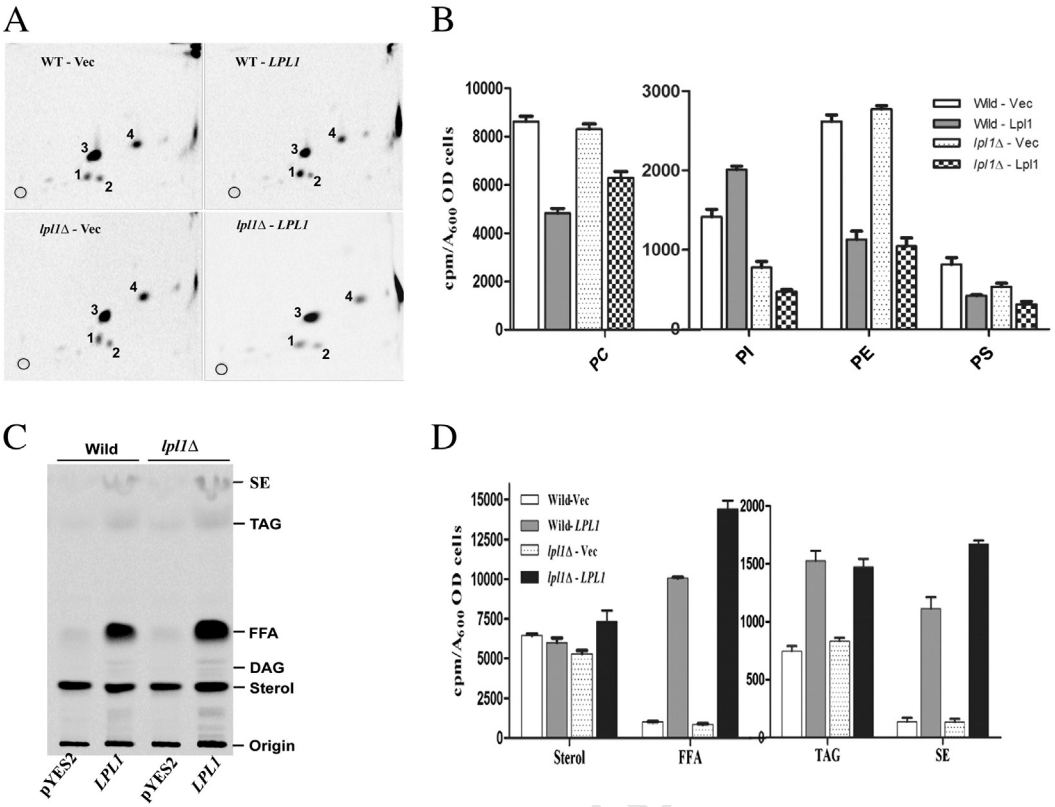

Fig. 2. Incorporation of [^{14}C]acetate into yeast lipids. A. Yeast cells overexpressing Lpl1 and the vector control were grown for 24 h in galactose medium in the presence of 0.5 μCi of [^{14}C] acetate. The lipids were extracted from cells (A_{600} − 20) and resolved on two-dimensional silica TLC using solvent systems, chloroform/methanol/ammonia (65:25:5) in the first dimension and chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5) in the second dimension. 1, phosphatidylinositol (PI); 2, phosphatidylserine (PS); 3, phosphatidylcholine (PC); 4, phosphatidylethanolamine (PE); O represents the origin. B. The amount of [^{14}C]acetate incorporated into individual phospholipids is represented as the cpm/A_{600} of cells/24 h of labeling. C. Incorporation of [^{14}C]acetate into neutral lipids. Lipids were separated on TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v). D. The amount of [^{14}C]acetate incorporated into neutral lipids is represented as the cpm/A_{600} of cells/24 h of labeling. For the quantitation of lipids, each value represents the mean ± SD of three independent experiments.

expressing the YOR059c-EGFP were fixed and stained for LDs using 4% formaldehyde plus Nile red dye for 30 min. After washing with PBS twice, cells were examined using fluorescence confocal microscope with 63× oil objective from Zeiss LSM710 microscope.

2.8. For LD staining

The wild type BY4741 strain and the *lpl1*Δ deletion strain were grown on YPD media until the stationary phase; then both the strains were transformed with the empty vector pYES2/NTC or overexpressing *LPL1*, and grown on synthetic defined media without uracil until the stationary phase. A total of 1.5 ml of the yeast cells was harvested and resuspended in 2% paraformaldehyde for fixation. After washing with distilled water, BODIPY 493/503 (0.5 μg/ml) was added to the cell suspension and incubated for 20 min at room temperature. After two washes with PBS buffer, cells were resuspended in 50 μl of PBS and observed with a fluorescence microscope (Zeiss LSM710, with 100× oil objective) directly after staining.

2.9. Site-directed mutagenesis

Mutations (**S_{90}An G_{88}A**X**S_{90}A**X**G_{92}A**) in *LPL1* were introduced using the following primers: forward primer for S90A, 5′-CGTAATGGGA TAT**GCG**CAAGGCGGATTAG-3′ and reverse primer 5′-CTAATCCGCCTT GCGCATATCCCATTACG-3′; forward primer for **G_{88}A**X**S_{90}A**X**G_{92}A**, 5′-CACCAAACTAAGCGTAATG**GCA**TAT**GCG**CAA**GCC**GGATT AGTGGCCCGA TT-3′ and reverse primer 5′-AATCGGGCCACTAATCCGGCTTG CGCATA TGCCATTACGCTTAGTTTGGTG-3′. The underlined base pairs indicated the sites of mutation. The reaction mixture contained pYES2/NT-*LPL1* template (100 ng), primers (125 ng each), 0.2 mM dNTPs, reaction buffer and enzymes. Amplification was done using the following conditions: denaturation of the template at 95 °C for 2 min followed by 18 cycles at 95 °C for 20 s (denaturation), 60 °C for 10 s (annealing) and 68 °C for 30 s (extension). The final extension was carried out at 68 °C for 5 min. Once the amplification was completed, the reaction product was incubated with DpnI at 37 °C for 1 h to digest the non-mutated plasmid and transformed into *Escherichia coli* DH5α strain. Mutation of the gene was confirmed by sequencing and it was expressed and purified from yeast for enzymatic assays.

3. Results

3.1. Lpl1p belongs to the αβ hydrolase family

Lpl1p is a 450 amino acid protein with a predicted molecular weight of 51.1 kDa and a pI of 9.81. Based on sequence similarity and conserved domain search against NCBI non-redundant protein database, the *LPL1* gene product with unknown function was found to possess a predicted αβ hydrolase domain. The structural motif of the gene, 88G*X*S*X*G92 (Fig. 1A) forms a highly conserved stretch of amino acids as it is in the majority of the known lipases, phospholipases, lysophospholipases, esterases, and serine proteases [36]. To determine the relationship of Lpl1p with its close homologues among prokaryotes and higher eukaryotes, BLAST analysis was performed. Using Lpl1p as a sequence query, many sequences were identified from different organisms, and most of them belonged to fungi, bacteria and plants but no homologue

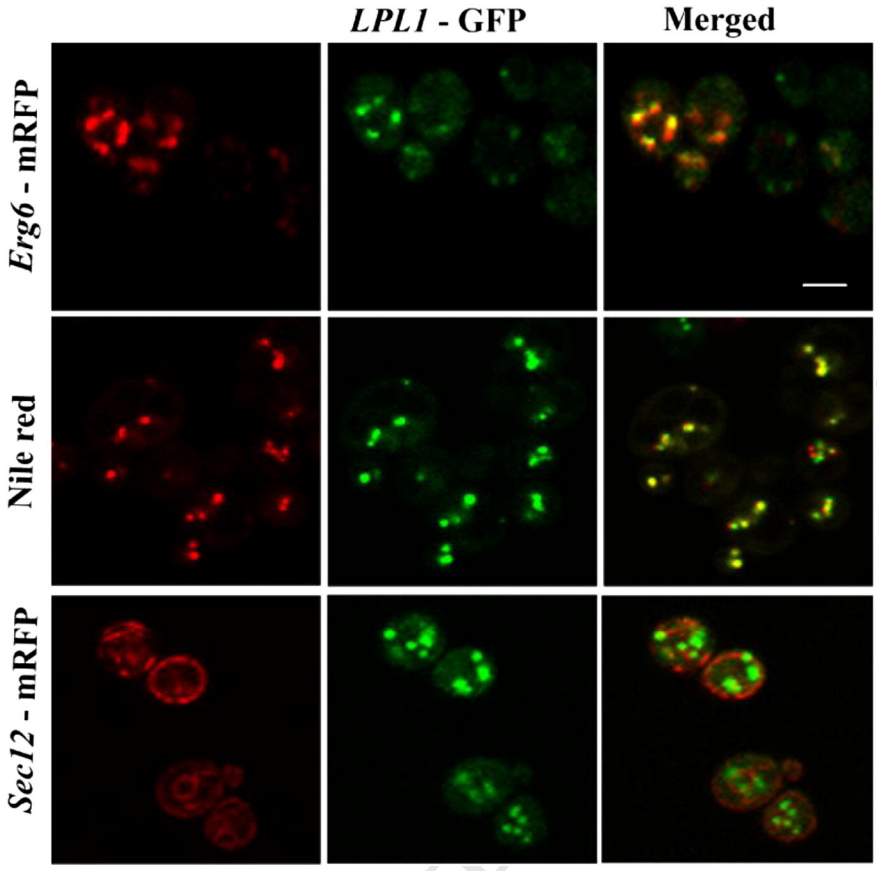

Fig. 3. Subcellular localization of Lpl1p. Cells expressing the *LPL1*-EGFP fusion gene were co-expressed either with LD marker protein *ERG6*-mRFP or ER marker protein *Sec12*-mRFP and examined under a fluorescence microscope as described in Materials and methods. For Nile red staining, cells expressing the *LPL1*-EGFP fusion protein were stained with Nile red and examined. Bar, 5 μm.

sequence was found in animals. Multiple sequence alignment results revealed Lpl1p shares 31% homology with *A. thaliana* hydrolase and 28% with *Oryza sativa* hypothetical protein. The conserved amino acid stretch of GXSXG is indicated in the box (Fig. 1B). The TMPRED analysis revealed a predicted single transmembrane helix structure which implied that the protein might be associated with the membrane (Fig. 1C).

3.2. LPL1 overexpression led to accumulation of free fatty acids and reduced phospholipids

Wild type and *lpl1Δ* strain were transformed with the yeast expression vector pYES2/NTC-*LPL1* or the empty vector and grown up to stationary phase on synthetic defined media, without uracil (SM-U) using galactose as a carbon source. The lipid profile of wild and mutant cells was monitored using [^{14}C]acetate steady state labeling. The most striking difference was the reduction of total phospholipids in the *LPL1* overexpressed strains (Fig. 2A and B). On the other hand, neutral lipid profile exhibited an increase in the triacylglycerol and steryl ester (SE) levels and a significant accumulation of free fatty acids in the overexpressed strains (Fig. 2C and D). From the results, we can conclude that the Lpl1p is involved in the turnover of phospholipids and releases free fatty acids in overexpressed cells. Part of the released FFA gets incorporated into neutral lipids resulting with slight increase in SE and TAG.

3.3. Lpl1p is localized in lipid droplet

Before understanding the functional role of *LPL1*, we determined its subcellular localization. Overexpression of the Lpl1 protein (His tagged) in yeast, was found to be exclusively localized in the membrane fraction (data not shown). Hence we expressed *LPL1* in a pUG34 plasmid as N-terminally tagged green fluorescent (EGFP) fusion protein. The marker proteins *ERG6*-mRFP for the LDs [37] and *Sec12*-mRFP for ER were co-expressed together with the Lpl1 fusion proteins and the cells grown to exponential phase in synthetic media devoid of histidine and uracil. Confocal results precisely revealed that *LPL1*-EGFP is localized exclusively in the spherical structured organelle and it merged well with *ERG6*-mRFP, which indicated that the protein is localized in LDs. To confirm this further, fluorescence microscopy experiments were undertaken by staining the exponentially grown *LPL1*-EGFP cells with Nile red, a liphophilic dye specific for LDs and the results confirmed that Lpl1p is co-localized in LD. On the other hand, *Sec12*-mRFP was not co-localized with *LPL1*-EGFP (Fig. 3).

3.4. LPL1 encodes for phospholipase/lysophospholipase activity

To validate the biochemical function of *LPL1*, gene was overexpressed in *Saccharomyces cerevisiae*, and the membrane fraction was solubilized using DDM. Protein was purified to homogeneity and the solubilized fraction was used for enzymatic assays. The expression of Lpl1p was confirmed by immunoblot with anti-His tag antibodies (Fig. 4A and B). We performed the lipase assay using the purified protein and the phospholipid substrates that were fluorescently labeled (NBD) at sn-2 position. Interestingly, we found that Lpl1p was able to hydrolyze all the phospholipids preferentially at sn-2 position releasing FFA except the phosphatidylglycerol where sn-1 position was preferred and the fluorescent LPG was released (Fig. 4C). In case of PE, no FFA was observed because both PE and FFA have the same Rf value in this solvent

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system. In order to overcome this, we used head group-labeled NBD-PE rather than sn-2 labeled and found the release of NBD-LPE (Fig. 4D). Time and protein dependent enzymatic assays were performed to confirm the phospholipase activity (Fig. 4E to M). The protein after hydrolyzing PE, released the LPE which was further degraded in a protein and time dependent manner, which indicated that the protein also possessed the lysophospholipase activity and it was confirmed by TopFlour-LPE hydrolyzing assay (Fig. 4N). The presence or absence of calcium ions did not affect the phospholipase activity of Lpl1p (data not shown). Purified enzyme was used to calculate the specific activity towards various phospholipid substrates. The highest activity was found for PE followed by PA with the apparent Km value of 0.86 ± 0.13 and 0.97 ± 0.23 respectively (Table 2). From all the above data we concluded that the protein possessed both phospholipase and lysophospholipase activity.

3.5. Phospholipase activity of Lpl1p is required for LD homeostasis

The protein is localized in the lipid droplet (LD) and possessed phospholipase activity; hence we wanted to elucidate its function using knock out gene. Cells were stained with BODIPY 493/503 and examined by fluorescence microscopy. Our results revealed that the *lpl1*Δ strain exhibited an altered morphology of LDs in particular, smaller in size than wild type when grown in synthetic medium containing galactose as a carbon source. To check whether the loss of phospholipase activity of Lpl1p is responsible for the observed phenotype, we tested complementation of the mutant with functional Lpl1p. When the gene was overexpressed in mutant strain, the protein rescued the LD morphology to normal size. In addition, the size of LDs was further increased in overexpressed cell than vector control which denoted that Lpl1p decreased the availability of phospholipid for monolayer formation thereby the LDs coalesced with one another to form giant LDs (Fig. 5A and B). These data demonstrated that functional complementation of the *lpl1Δ* mutant is required for lipid homeostasis.

3.6. Site directed mutagenesis in the catalytic site abolished the phospholipase activity of Lpl1p

The serine residues of the GXSXG motif are important for lipase/esterase activity [38]. To validate whether the phospholipase activity of the protein was mainly due to the serine residue, we performed the site directed mutation of S90A and $G_{88}AXS_{90}AXG_{92}A$, expressed and purified by affinity column chromatography. Expression of the mutated protein was confirmed by Immunoblot with anti-His monoclonal

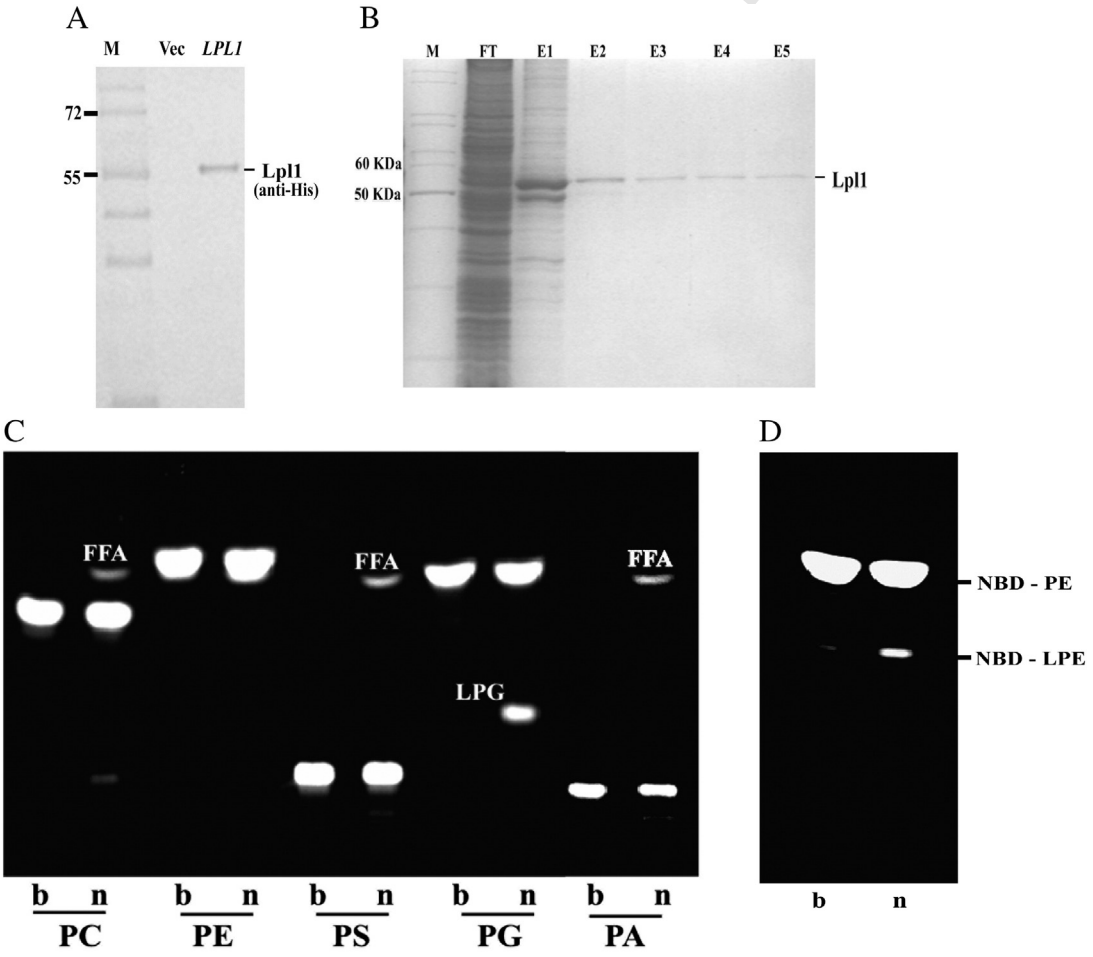

Fig. 4. Purification and characterization of Lpl1p. A. Immunoblot analysis with anti-His antibodies was carried out. Lane 1, marker; lane 2, vector transformed; lane 3, *LPL1* transformed. B. Purification of Lpl1 protein from yeast, using nickel-NTA affinity column chromatography. The proteins were resolved on 12% SDS-PAGE and stained with Coomassie Brilliant Blue. M, marker; FT, flow through; E1 to E5, eluted fractions of purified protein. C. Phospholipase assay was carried out with purified protein using various phospholipid substrates under standard assay conditions. Reaction product was resolved on a TLC using chloroform:methanol:ammonia (65:25:5, v/v/v) solvent systems. b, boiled enzyme; n, native enzyme. D. PE hydrolysis assay with purified Lpl1p. Hydrolysis of phospholipid substrates was analyzed in a time and protein dependent manner, E & F for PE, G & H for PS, I & J for PA, K & L for PG and M for time dependent hydrolysis of PC. N. LPE hydrolysis assay by purified Lpl1 using TopFlour labeled LPE. For the quantitation of enzyme products, each value represents the mean ± SD of three independent experiments.

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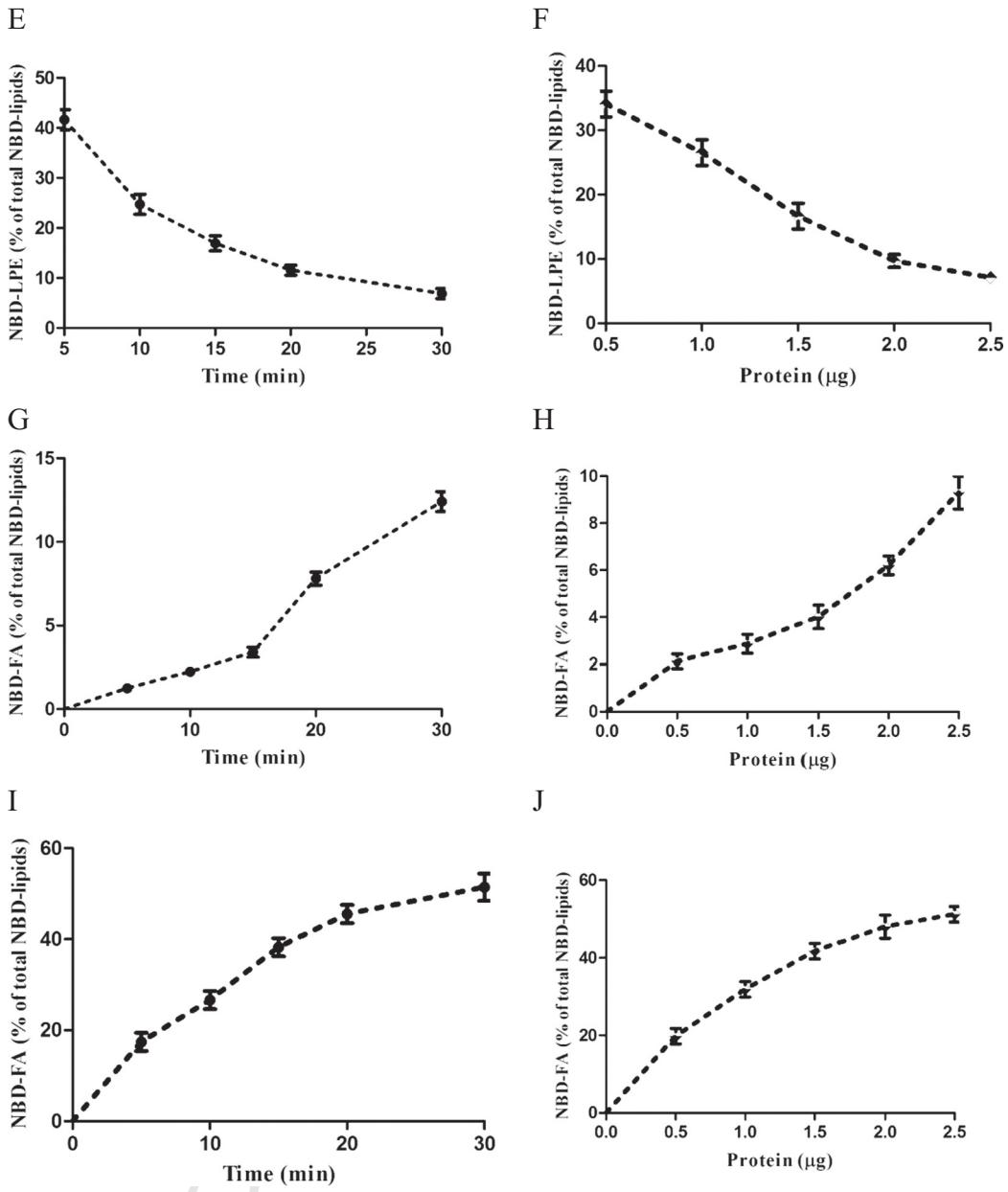

Fig. 4. (*continued*).

antibody (Fig. 6A). Lpl1p highly preferred PE, so we used NBD-PE as a substrate for enzymatic assay. The functional protein exhibited the phospholipase activity with PE and released the LPE, whereas the mutation of serine and $G_{88}AXS_{90}AXG_{92}A$ mutation completely abolished the phospholipase activity of Lpl1p (Fig. 6B). On the other hand, active site mutation only partially abolished the phospholipase activity towards PS and PA (data not shown). These results suggested that the active site serine is necessary for the esterase property of Lpl1p and this activity is only due to Lpl1p and not because of any other contaminants.

4. Discussion

Lipid droplet is a highly dynamic sub-cellular organelle playing a key role in lipid trafficking, storage, turnover and many other biological processes [39,40]. A phospholipid monolayer surrounds a neutral lipid core in LDs and is decorated by LD localized proteins. In yeast, more than 50% of the LD surface phospholipid is PC followed by PI and PE [5]. This phospholipid monolayer plays a major role in regulating the LD size and its alteration leads to abnormal morphology of the organelle [41]. To date, no phospholipases have been identified in LDs and in our current study, we reported that Lpl1p belongs to the family of serine lipase and exhibits phospholipase B activity and is localized in LDs.

Absence of *LPL1* gene reduced PI level alone and overexpression of *LPL1* in the *lpl1Δ* reduced the total phospholipid level. Overexpression of *LPL1* in the wild type cells also decreased PC, PE and PS, whereas PI showed an increase, and we are unable to explain why PI alone showed a varied pattern. Furthermore, *in vitro* analysis of the purified protein confirmed phospholipase/lysophospholipase activity. Lpl1p acts on both PL and LPL and so the strain overexpressing Lpl1p does not accumulate lysophospholipids. Site directed mutation studies revealed that, serine in the GXSXG motif is important for enzyme activity. So far, the major phospholipases identified in yeast are namely Plb1, Plb2, Plb3 and Nte1 and they all hydrolyzed the substrates at both sn-1 and sn-2 positions [26–28,30]. Interestingly, Lpl1 deacylated the

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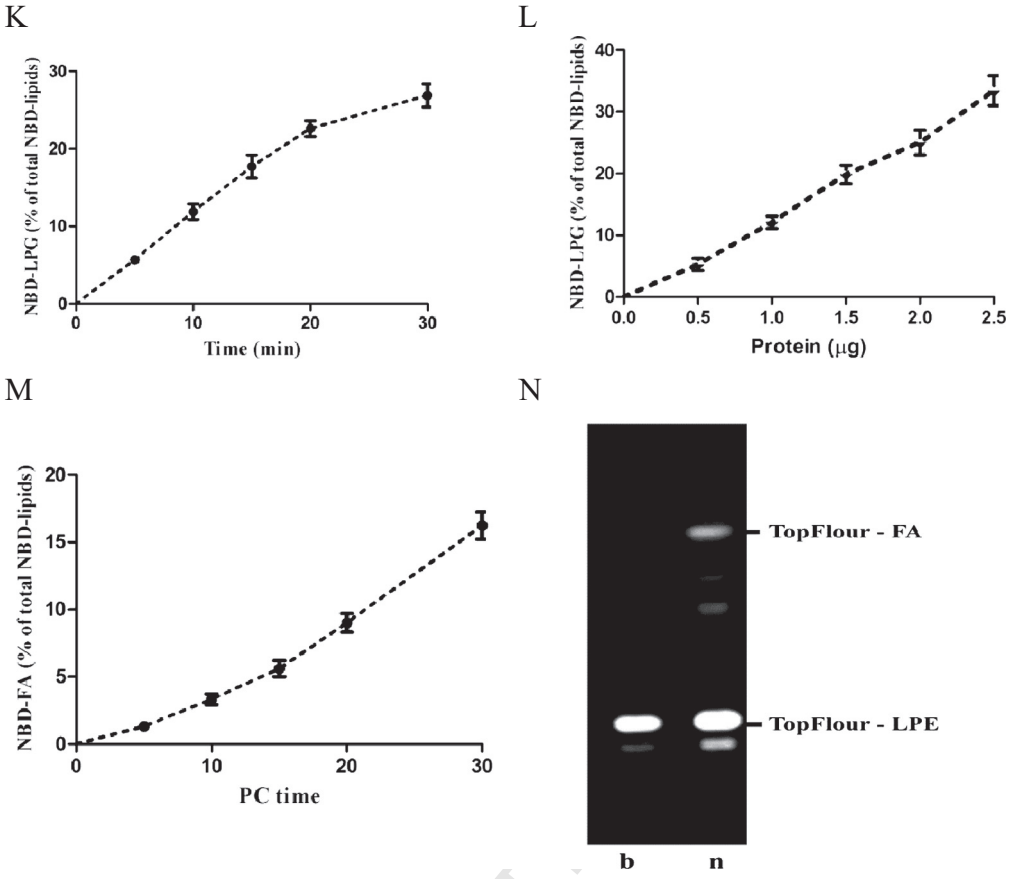

Fig. 4. (*continued*).

phospholipid substrates primarily at sn-2 position followed by sn-1 position. Substrate preference for Lpl1p is PE > PA > PG > PC > PS. Enzyme showed very less preference towards PI and CL which was confirmed by cold assay (data not shown).

Neutral lipid profile displayed the accumulation of free fatty acids in the overexpressed cells than the vector control which supported the lipase activity of the enzyme. From the results we concluded that Lpl1p deacylated the phospholipids of LDs. It is well known that the DAG acylating enzyme Dga1p is localized both in ER and lipid droplet [13], but significant incorporation of the released FFA from phospholipids into neutral lipids was not observed in our study and we found only a slight increase in TAG, DAG and SE level. Earlier it has been claimed that the accumulation of free fatty acids is toxic to the cell [42]. Normally when the cells are fed with exogenous FFA, it is either activated to acyl CoA for β-oxidation or involved in the synthesis of storage lipids and during these conditions size and number of LD are increased to prevent FFA accumulation [43]. In our study on the contrary FFA gets accumulated but we did not observe any toxic effect on the cell growth (data not shown).

Identification of protein localization is crucial for characterizing the protein function. According to the transmembrane prediction tool, Lpl1 has a predicted single transmembrane domain. Immunoblot analysis of yeast expressing *LPL1* revealed that the protein is associated with membranes and not in cytosol. Earlier data, based on the LD proteomic studies revealed that Lpl1p is dually localized both in LD and ER [6]. But our microscopic studies revealed that Lpl1p and Erg6p were mainly associated with the spherical structures, likely LDs on stationary phase. Cells co-expressing *LPL1* with the ER protein Sec12 displayed, that both the proteins were localized in distinct places. A recent study reported that, Loa1p showed a dynamic localization and when the cells were grown up to the stationary phase, Loa1p was mainly associated with LDs, but in the early log phase, Loa1p was with ER [44]. Hence, different stages of the cell growth might show different localization, and we did the study only at the stationary phase and we only observed that Lpl1p is localized in LD.

A genome-wide RNA interference studies in *Drosophila* illustrated that genes encoding enzymes for phospholipid biosynthesis play a major role in determining LD size and number. The phospholipid composition in LD is important for maintaining its morphology [45]. Knockdowns of the Kennedy pathway enzymes catalyzing PC synthesis resulted in abnormal LD phenotypes which denoted a key role for PC in LD expansion. Knockdown of Cct1 (phosphocholine cytidylyltransferase), the rate-limiting enzyme in PC synthesis of the Kennedy pathway, showed giant LD morphology [41], and this might be due to the limited amount

Table 2
In vitro phospholipase activity of Lpl1p.

Substrate	V_{max}	K_m	V_{max}/K_m
	μmol/min/mg	mM	ml/min/mg
PE	4.55 ± 0.49	.86 ± 0.13	5.23
PA	4.05 ± 0.35	0.97 ± 0.23	4.17
PG	3.25 ± 0.28	1.12 ± 0.12	2.90
PS	1.05 ± 0.15	0.92 ± 0.24	1.14
PC	1.65 ± 0.21	0.83 ± 0.15	1.98

Various phospholipid substrates were used and the specific activity calculated for the purified protein. We arrived at K_m and V_{max} values using GraphPad Prism version 5 and Michaelis–Menten approximation. Values were mean ± SD of three independent experiments.

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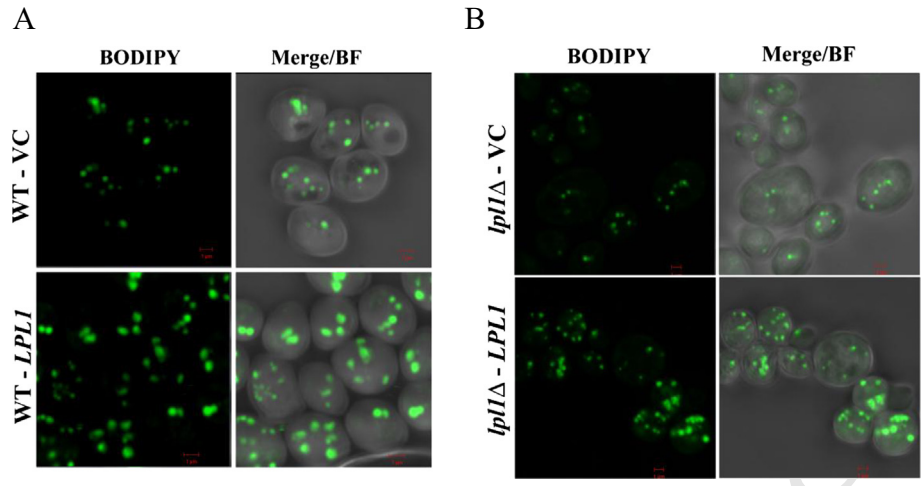

Fig. 5. LD morphology studies in vector control and overexpressed cells. Strains transformed with the empty vector or with the yeast expression vector pYES2/NTC-*LPL1* were grown on synthetic defined media using galactose as carbon source. BODIPY 493/503 stained cells were observed under a fluorescence microscope as described in Materials and methods. A. Wild vector control and overexpressed cells. B. *lpl1Δ* vector control and overexpressed cells. Bar, 1 μM.

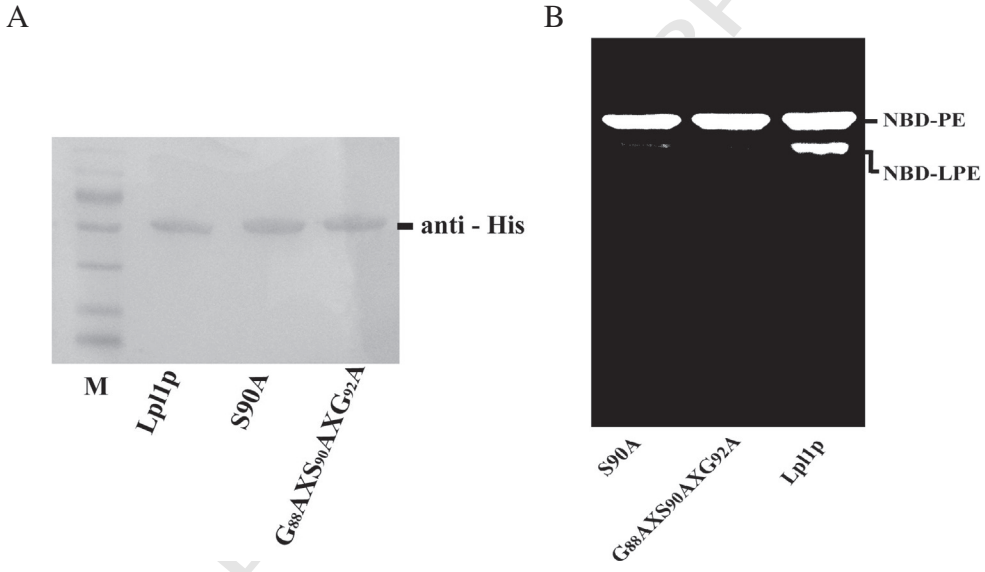

Fig. 6. Effect of site directed mutation on Lpl1p for phospholipase activity. A. Immunoblot analysis of wild type and mutant protein of Lpl1p was performed using anti-His monoclonal antibody. B. Purified functional and mutant proteins were used for phospholipase assay under standard assay conditions.

of PL for LD formation. Similarly, the *LPL1* overexpressed strain displayed giant morphology in LD, whereas *lpl1*Δ cells showed tiny LDs, emphasizing that *LPL1* has a vital role in determining LD morphology. In summary, our studies revealed a vital role of *LPL1* in maintaining the structure of LDs, and the protein encodes for phospholipase B activity. To our knowledge, this is the first report for the existence of broad substrate specific phospholipase on LD surface in *Saccharomyces cerevisiae*.

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