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# *LIP1* Regulates Lipid Metabolism in *Saccharomyces cerevisiae*

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**ABSTRACT: *LIP1* is an acyl-CoA-dependent ceramide synthase vital in synthesizing sphingolipids. However, the function of *LIP1* in the other main lipids, such as phospholipids and neutral lipids, is still to be investigated. In this work, we evaluated the influence of the loss of *LIP1* on overall lipid metabolism, and we discovered that the deletion of *LIP1* had disrupted cell development, neutral lipid metabolism, and phospholipid homeostasis. As adequate phospholipid production is crucial for counteracting ER stress, we subjected the *LIP1* deletion cells to ER stress (DTT treatment). We discovered that the *LIP1* deletion cells were more vulnerable to ER stress than the wild. The levels of ER stress response genes levels were also elevated in *LIP1* deletion cells compared to the control. Thus, our study provides the new function of *LIP1* in ER stress response mechanisms and lipid homeostasis.**

**Keywords:** Ceramide, sphingolipids, phospholipids, neutral lipids, ER stress, DTT.

## INTRODUCTION

Sphingolipids have recently been shown to serve a crucial role in signal transduction and the maintenance of calcium homeostasis, in addition to their structural significance in lipid bilayer permeability and mobility (Cowart and Obeid 2007). As an essential precursor, ceramide is required for the production of sphingolipids. Ceramide synthases in *S. cerevisiae* are called *LAC1* and *LAG1* (Flor-Parra *et al.,* 2021; Gault *et al.,* 2010). These two enzymes contribute to the formation of ceramide in distinctive ways. *LAG1* is responsible for synthesizing long-chain ceramides, while *LAC1* is responsible for synthesizing very long-chain ceramides (Breslow, 2013).

Combining sphinganine, the structural backbone of complex sphingolipids, with fatty acids produces ceramides. Yeast produces sphinganine by a condensation reaction involving palmitoyl-CoA and serine. *LAC1* or *LAG1* produces ceramide molecules by acylating sphinganine with a fatty acid as the initial step (Schlarmann *et al.,* 2021). *LAG1/LAC1* interacting protein, also known as *LIP1* (Xu *et al*., 2021), is designed to facilitate the formation of a heteromeric complex between *LAG1 and LAC1* (Vallée and Riezman 2005). Without *LIP1*'s involvement, the ceramide synthase complex cannot operate (Vallée and Riezman 2005).

Any abnormalities in the ceramide synthesis can impact a cell's function and illness (Tani and Funato 2018). Biosynthesis and degradation of ceramides are closely regulated. Recent research suggests that an increase in the expression of complex ceramides may promote apoptosis, termed programmed cell death (Schlarmann *et al.,* 2021). This study revealed that the absence of *LIP1* impacted yeast lipid synthesis. To be more precise, the levels of phospholipids dropped while neutral levels surged. Chronic endoplasmic reticulum stress (ER stress) pathophysiology has been linked to altered phospholipid synthesis.

The viability of *lip1Δ* cells was inhibited under the influence of 2mM DTT in this study (external ER stressor). In addition, the absence of LIP1 increased the expression of genes implicated in the ER stress response pathway, namely HAC1 and IRE1 (Yemenici *et al*., 2022). In conclusion, the results of this study indicate that LIP1 regulates the synthesis of membrane and storage lipids and that LIP1 regulates the ER stress response. Moreover, the data suggest that LIP1 modulates the ER stress response.

## MATERIAL AND METHODS

**Chemicals and Reagents.** Difco supplied yeast extract, peptone, and bacteriological agar. Hi-Media provided the Yeast Nitrogen Base (YNB) Synthetic Whole Mixture. Merck provided 60 plates of thin-layer silica gel. Unless otherwise specified, Trizoland other chemicals were procured from Sigma. The ABO Biosystem cDNA synthesis kit was obtained. Avanti Polar Lipids furnished the lipid standards, and Merck supplied all solvents (Alabaster, AL).

**Strains and Growth conditions.** Yeast of the wild-type strain Saccharomyces cerevisiae wild [BY4741 (MATa; his3; leu20; met150; ura30)] Prof. Ram Rajasekaran

from the IFTRI in Mysuru donated*lip1Δ* cells. In the YPD medium (composed of 1% yeast extract, 2% peptone, and 2% glucose), wild and*lip1Δ* cells were cultivated aerobically in pre-cultures. Cells were inoculated in a synthetic complete (SC) medium of 0.2% complete synthetic mixture, 0.67 % yeast nitrogen base, and 2% glucose for experiments.

**Cell viability and growth.** Wild-type and mutant strains were grown in YPD media to study the cell viability at 30 ºC to mid-log phase. Then equal OD of cells was ($A_{600}$ nm) taken and serially with distilled water (up to 10) fold, spotted on SC plates containing 2% agar, and incubated for two days at 30°C.

**Extraction and separation of lipids using TLC.** Cells were cultured in SC medium up to the mid-log phase, and equal OD of cells was taken for lipid extraction. The pellet was added with chloroform and methanol (2:1 ratio) and added with an equal volume of acidified water (2% phosphoric acid) (Breil *et al.,* 2017). Phospholipids were separated using chloroform/methanol/acetic acid/water (85:15:10:3.5 v/v). The neutral lipids were separated using petroleum ether: diethyl ether: and acetic acid (70:30:1, v/v) as the solvent system. Individual phospholipid spots were scraped from TLC plates and were quantified by phosphorous estimation, and for neutral lipids, the individual spots were quantified using ImageJ software.

**Real-Time PCR.** Using the TRIzol procedure and the manufacturer's kit guidelines, total RNA was extracted from the yeast cells. In order to create the cDNA, 2 g of total RNA was used. High-capacity cDNA reverse transcription kit (Applied Biosystems) with 50 U/ml reverse transcriptases, 1X RT buffer, 1X random primer, and 4 mM dNTP mix was used, with the primers for the genes of interest. Table 1 lists the primers used in this investigation, created using Primer Express R software 3.0 (Applied Biosystems). The genes were amplified using PCR (Step One Plus TM Real-time PCR machine). The reaction is carried out under the following conditions: initial denaturation at 95 °C for 10 min, followed by 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C, and final extension at 60 °C for 20 s and 15 s at 95 °C. A loading control (Actin) was utilized. The double delta - Ct technique was used to calculate the fold change.

**Table 1: Primers used in this study.**

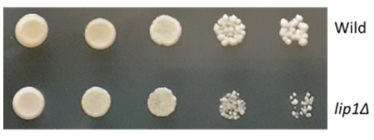
| Gene Name | Forward | Reverse |
|---|---|---|
| *HAC1* | CTGTACAATGGAGCCTGCGA | TGTTGTTGTCTACGGCAGGT |
| *IRE1* | TCGAACCTCTCCTGGTTTGC | ACGGTCACTGGAAGCGTATC |

**Confocal Microscopy.** As previously stated, yeast cells were cultivated and harvested after 24hrs. One ml of cells was put into a 1.5 ml tube and centrifuged for 5 minutes at 4000 rpm. The cells were rinsed with phosphate-buffered saline (1X) after being fixed with 2% formaldehyde. For membrane staining (excitation 482 nm/emission 504 nm), DiOC6 (1 mg/ml) was added. Nile red (20 g/ml) was added and incubated for 15 min at room temperature in the dark (excitation 480 nm/emission 510 nm) for lipid droplet analysis. Fluorescent images were taken using a Zeiss LSM 710 confocal microscope outfitted with a 100 /1.40 oil objective and an Axio Cam camera after three washes in 1X PBS.

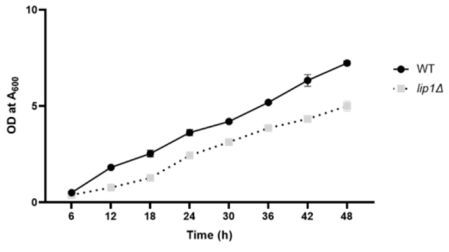
**Statistical analysis.** A two-way ANOVA test was used to examine the quantification of the data using the PRISM software version 6.0. The error bars show the mean ± standard deviation of three separate experiments. In the figures, significance was established as *p<0.5, **p<0.01, ***p<0.001.

## RESULTS AND DISCUSSION

**Deletion of *LIP1* hindered cell growth.** To determine the viability of *LIP1* deletion, cells were serially diluted and spotted on SC agar plates.




(A)

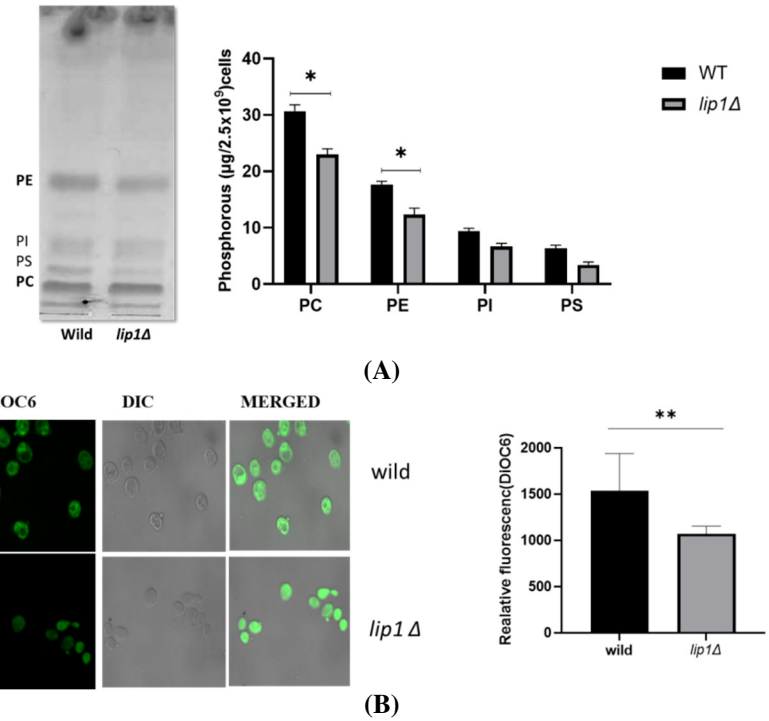



(B)

**Fig. 1.** Growth studies: **(A)** Yeast cells were grown in SC media up to the mid-log phase, and optical density was measured at 600 nm. 1.0 OD of cells were serially diluted (10-fold) and spotted on full synthetic plates at 30ºC for 48hrs (**B)** The growth pattern was studied by measuring the OD ($A_{600}$ nm) at frequent intervals until 48h, and the graph was plotted. The data represent the mean ± SD of triplicates from three independent experiments.

The *lip1*Δ strain showed decreased growth compared to the wild-type cells (Fig. 1A). The same pattern was observed in growth curve analysis as well, and *lip1Δ* cells growth was diminished in liquid media as well (Fig. 1B). Unlike *lip1Δ* cells, loss of its interacting partners (*LAG1* and *LAC1*) showed no such growth reduction (Mao *et al.,* 2000). These results indicate that *LIP1* is essential for proper cell growth.

**Alteration in Phospholipid alteration was observed in *lip1Δ*.**



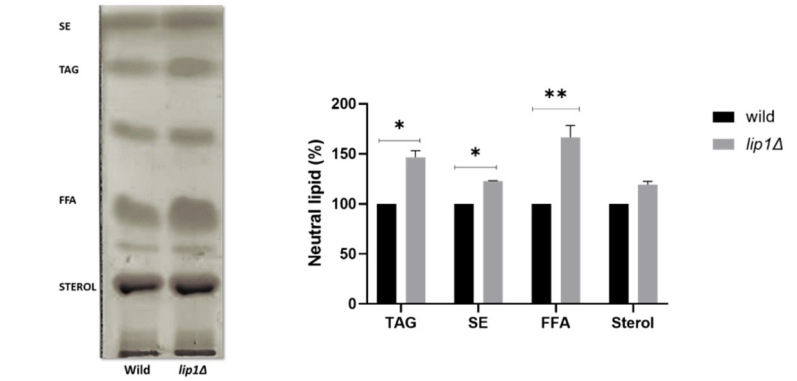

**(A)** The SC medium was used to grow the wild and *LIP1* deletion cells up to the mid-log phase, at which point an equal number of cells were harvested for lipid extraction. Thin-layer chromatography was used to separate the phospholipids; then, phosphorous estimation was used to quantify them. (**B**) DiOC6 (1 g/ml) staining was used to examine the morphology of the membrane. Pictures were captured using a scale bar of 10 m and the laser scanning confocal microscope LSM 710 (Zeiss) with emission and excitation at 482/504 nm. The findings were presented as mean SD following samples were repeated 3 times for statistical significance.

**Fig. 2**. Phospholipid quantification and membrane visualization**.**

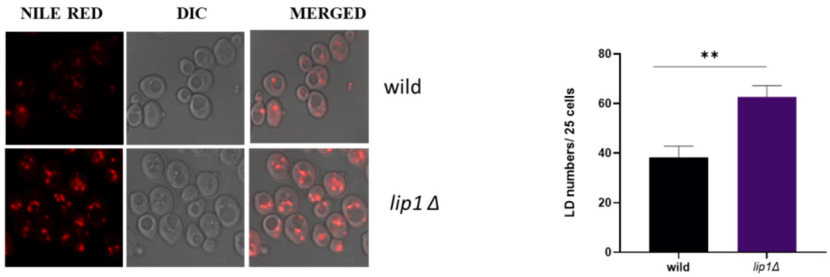
***LIP1* deletion modified the lipids in membranes.** We also looked into how *LIP1* affects lipid metabolism. When compared to wild cells, deletion of *LIP1* revealed a significant decrease in membrane lipids like phosphatidylcholine (PC), phosphatidylethanolamine (PE), Phosphatidyl Serine (PS), and Phosphatidyl Inositol (PI) (Coant *et al*., 2017) (Fig. 2A). DiOC6 staining was used to examine the change in membrane lipids. In our microscopic images, wild cells had normal membrane organization. In contrast, *lip1Δ* cells had membrane aberrancy and reduced fluorescence intensity (Fig. 2B). Based on these findings, we concluded that membrane lipids and membrane organization are modified when the ceramide synthase subunit *LIP1* is absent.

**Deletion of *LIP1* increased neutral lipids and lipid droplets.**

**Storage lipids are accumulated when the ceramide synthase subunit LIP1 is deleted.** Then, in lip1 cells, we also investigated neutral lipid profiles as lipid droplets (LDs), neutral lipids were stored by TAG and steryl esters (SE) and mobilized in response to energy demands. According to our findings, ire1 cells had significantly higher levels of triacylglycerol (TAG), Stearyl esters (SE), Free fatty acids (FFA), and sterol (Fig. 3A & B). As a result, we investigated how LDs formed in our strains. Compared to wild cells, LIP1 deletion strains showed an increase in the number and size of the LDs (Fig. 3A &B). These findings proved that LIP1 keeps neutral lipid homeostasis in control.



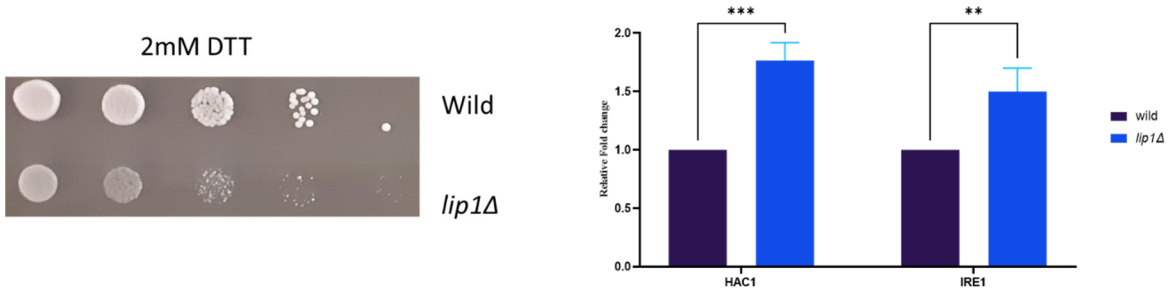

(A)




(B)

**Fig. 3. (A)** TAG levels and LD formation elevated with *LIP1* deletion. In *lip1Δ* cells, neutral lipids and lipid droplets accumulated. An equivalent number of yeast cells were taken to extract lipids after 24 hours of growth at 30 °C. The individual lipids were separated by thin layer chromatography. **(B)** Image J was utilized to estimate the lipids. The lipophilic dye Nile red (1 g/ml) was used to stain the cultured cells after entering the mid-log growth phase. The images were captured using a Zeiss LSM 710 laser scanning confocal microscope with an AxioCam camera and an oil objective with excitation and emission wavelengths of 480 nm and 510 nm, respectively—10 m bar scale. The whole data set reflects the average SD among three different experiments.

***lip1Δ* cells are susceptible to ER stress:**




(A) (B)

**Fig. 4.** Growth of lip1Δ was severely affected under ER stress. **(A)** Yeast cells were grown in SC media until the mid-log phase, and optical density was measured at 600 nm. 1.0 OD of cells were serially diluted (10 fold) and spotted on full synthetic plates with DTT (2mM) at 30ºC for 48hrs to monitor the growth **(B)** Q-RT PCR studied the mRNA expression of the ER stress response genes.

**DTT treatment hindered the growth of *lipl1Δ* cells.** DTT(Dithiothreitol) reduces disulfide bonds. Hence it disturbs the thiol interaction of proteins and induces the accumulation of misfolded proteins leading to ER stress (Hernández-Elvira *et al*., 2018; Read and Schröder 2021). Here we found that DTT treatment severely affected *liplΔ* cells compared to wild. Also, ER stress response genes such as *IRE1* and *HAC1* increased gene expression in *LIP1* deletion cells.

## CONCLUSIONS

Our results conclude that apart from the role of *LIP1* in ceramide synthesis, LP1 also plays a significant modulatory role in producing phospholipids and neutral lipids. Phospholipids mainly function as structural lipids, and their synthesis was downregulated, while the TAG and lipid droplets (LD) synthesis was upregulated. Loss of *LIP1* severely affected cell growth under ER stress and increased the expression of ER stress response genes, thus implying *LIP1*'s role in maintaining ER stress and protein quality control.

## FUTURE SCOPE

Upon ER stress, the unfolded protein response element gene *HAC1* splices and activates *IRE1* to mount an ER stress response. In the future, *HAC1* splicing in the loss of *LIP1* can also be investigated whether the complementation of *LIP1* recures the defective growth pattern, altered lipid levels, and ER stress induction observed in *lip1*Δcells.

**Author contributions:** Arul Mathivanan and Vasanthi Nachiappan designed the experiments. Arul Mathivanan performed the experiments. Arul Mathivanan and Vasanthi Nachiappan discussed the results and wrote the paper.

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**Conflict of Interest.** None.

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