



Research Article
Genomics and Bioinformatics

The multiple roles of lipid metabolism in yeast physiology during beer fermentation

Diego Bonatto¹ 

¹Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia da UFRGS, Laboratório de Biologia Molecular e Computacional, Departamento de Biologia Molecular e Biotecnologia, Porto Alegre, RS, Brazil.

Abstract

The ability of brewing yeasts (*Saccharomyces cerevisiae* and *Saccharomyces pastorianus*) to cope with the toxic effects of ethanol during beer fermentation depends on the modulation of lipid and lipid droplets (LDs) biosynthesis, which affects membrane fluidity. However, it has been demonstrated that lipids and LDs can modulate different biological mechanisms associated to ethanol tolerance, including proteostasis and autophagy, leading to the hypothesis that lipid and LDs biosynthesis are integrative processes necessary for ethanol tolerance in yeast. Supporting this hypothesis, a transcriptome and systems biology analyses indicated the upregulation of autophagy, lipid biosynthesis, and proteostasis (ALP)-associated genes in *lager* yeast during beer fermentation, whose respective proteins interact in a shortest-pathway ALP network. These results indicated a cross-communication between various pathways linked to inter-organellar autophagy, lipid metabolism, and proteostasis (ALP) during *lager* beer fermentation, thus highlighting the importance of lipids for beer fermentation.

Keywords: *Lager* yeasts, lipid metabolism, systems biology, transcriptomics, ethanol tolerance.

Received: October 12, 2021; Accepted: July 17, 2022.

Introduction

Nowadays, high gravity (HG) and very high gravity (VHG) fermentation technologies are often used in the brewing industry for beer production. This reduces the consumption of water during the brewing process and increases the ethanol yield, thus maximizing the brewing efficiency and reducing the production costs and energy demand (Puligundla *et al.*, 2011; 2019). However, the accumulation of large amounts of ethanol (>5% v/v) due to the fermentation of HG/VHG wort drastically alters the yeast's physiology (Hallsworth, 1998) and promotes an ethanol stress response that induces molecular mechanisms associated with the heat shock response (Odumeru *et al.*, 1992; Piper, 1995).

The ability of different yeast strains to cope with the toxic effects of ethanol depends on the modulation of cell membrane fluidity by alteration of the ratio of incorporated saturated and unsaturated fatty acids and the ergosterol content (Ding *et al.*, 2009). It is well established that membrane-associated lipids have a strong influence in beer brewing, affecting the fermentative capacity and ethanol tolerance of *Saccharomyces cerevisiae* (*ale* yeast) and *Saccharomyces pastorianus* (*lager* yeast) (Ahvenainen, 1982; Mishra and Kaur, 1991). In wine yeast strains, the high concentration of ergosterol in the cell membrane promotes ethanol tolerance by decreasing membrane fluidity (Aguilera *et al.*, 2006); however, the increased levels of unsaturated fatty acids in the cell

membrane increase the membrane fluidity (Alexandre *et al.*, 1994). It was found that yeast strains that are more ethanol tolerant incorporate long-chain fatty acids (C_{18:0} and C_{18:1}) compared with strains that are less ethanol tolerant (Chi and Arneborg, 1999). Additionally, high concentrations of ethanol induce the fluidification and thinning of membranes, along with changing the activity and aggregation of membrane-associated proteins (Thibault *et al.*, 2012).

Unfortunately, little is known about how lipids modulate various biological mechanisms in yeast cells during beer fermentation besides affecting membrane structure and/or permeability. However, it is known that membrane fluidification by ethanol can activate the endoplasmic reticulum (ER)-linked unfolded protein response (UPR) (Navarro-Tapia *et al.*, 2018) and lipids may have other roles in proteostasis, such as the removal of unfolded proteins from the ER by lipid droplets (LDs) (Vevea *et al.*, 2015). LDs are important and highly dynamic cytoplasmic organelles that connect different parts of the cell, including the ER (Jacquier *et al.*, 2011), mitochondria (Pu *et al.* 2011), peroxisomes (Kohlwein *et al.*, 2013), and vacuoles (Barbosa *et al.*, 2015). Interestingly, ER stress induces the formation of LDs (Fei *et al.*, 2009) and stimulates lipid biosynthesis, which are associated with ER membrane expansion during UPR (Cox *et al.*, 1997; Schuck *et al.*, 2009). Additionally, lipid biosynthesis coordinates the proteotoxic response of both mitochondria and the cytosol (Kim *et al.*, 2016). Thus, it can be hypothesized that LD-associated processes and lipid biosynthesis are integrative processes necessary for proteostasis in different organelles. In fact, regulation of inter-organellar proteostasis is an important mechanism underlying stress tolerance and it was recently shown that beer fermentation in *lager* yeast cells promotes a so-called “inter-organellar/cross-organellar

Send correspondence to Diego Bonatto. Universidade Federal do Rio Grande do Sul, Centro de Biotecnologia da UFRGS, Laboratório de Biologia Molecular e Computacional, Departamento de Biologia Molecular e Biotecnologia, Avenida Bento Gonçalves 9500 - Prédio 43431, 15005, Porto Alegre, RS, Brazil.
E-mail: diego.bonatto@ufrgs.br

communication/response” (CORE mechanism). This involves a series of signaling-associated protein networks that regulate inter-organellar proteostasis, which includes the ER and mitochondria UPRs, chaperone and co-chaperone activity, and *N*-glycosylation quality control pathway proteins (Telini *et al.*, 2020). A major aspect of the inter-organellar proteostasis mechanism induced by ethanol stress is the coordination and/or activation of organellar-linked microautophagy responses, such as mitophagy (Carmona-Gutierrez *et al.*, 2012) and lipophagy of LDs (Vevea *et al.*, 2015). Moreover, macroautophagy can also be induced by reactive oxygen species (ROS) generated by damaged mitochondria due to ethanol stress (Jing *et al.*, 2018).

Thus, the purpose of this study was to evaluate how lipid metabolism interacts with different mechanisms linked to inter-organellar proteostasis and autophagy in *lager* beer yeast during beer fermentation. Publicly available DNA microarray gene expression datasets obtained from *lager* beer yeast at various time points during industrial beer fermentation and yeast biomass propagation were selected and two transcriptome analyses were performed. The differentially expressed genes that were upregulated in both analyses (Pan-DEGs) were used to generate a protein–protein interaction (PPI) network. This was followed by local and global topological analyses as well as a gene ontology (GO) analysis of major clusters within the PPI network. The results of the transcriptome and PPI network analyses indicated cross-communication between various pathways linked to inter-organellar autophagy, lipid metabolism, and proteostasis (ALP) during *lager* beer fermentation.

Material and Methods

DNA microarray gene expression dataset selection and analysis

DNA microarray gene expression public datasets (GSE9423, GSE10205, and GSE16376) containing transcriptome data obtained from the *lager* yeast strain CB11 (*Saccharomyces pastorianus*) at various time points during industrial beer fermentation and yeast biomass propagation were selected from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/gds>) (Table S1). The GSE9423 dataset contains the transcriptome data of strain CB11 during both beer fermentation (time points: 8, 30, and 60 h) and yeast biomass propagation (time points: 0, 8, and 30 h) (Gibson *et al.*, 2008), and the analysis based on these data was designated the “single-analysis”. The GSE10205 and GSE16376 datasets contain the transcriptome data of strain CB11 during beer fermentation (time points: 8, 30, 60, 80, and 102 h) and yeast biomass propagation (time points: 0, 4, 8, and 30 h), respectively. They were combined for an analysis designated the “meta-analysis” (Figure 1). It is important to note that both the GSE10205 and GSE16376 datasets evaluated the same *lager* yeast strain (CB11) in identical fermentation and propagation conditions used in GSE9423 dataset. Moreover, the number of high-throughput (RNA-seq) studies involving *S. pastorianus* in conditions of yeast biomass propagation and beer fermentation are virtually nonexistent until now. In this sense, the presence of a high number of orthologous sequences and the closest phylogeny

of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus*, which composes the hybrid genome of *S. pastorianus*, allows to apply DNA microarray platform available for *S. cerevisiae* for *S. pastorianus* transcriptome analysis. Horinouchi *et al.* (2010) discuss the use of a tailor-made DNA microarray for *S. pastorianus* and show a strong correlation between the expression levels of *S. cerevisiae* and *S. bayanus* orthologous genes during fermentation (Horinouchi *et al.*, 2010).

The transcriptome analyses were performed using the R platform (<https://www.r-project.org>) with various packages downloaded from Bioconductor 3.13 (<https://www.bioconductor.org>) (Figure 1). For data matrix importing, processing, and array quality analysis, the GEOquery, affy, and arrayQualityMetrics packages, respectively, were employed (Gautier *et al.*, 2004; Davis and Meltzer, 2007; Kauffmann *et al.*, 2009). DEG analysis was performed using the limma package (Ritchie *et al.*, 2015). The false discovery rate (FDR) algorithm, implemented in the limma package (Ritchie *et al.*, 2015), was used to assess the significance level of the DEGs. DEGs from the single-analysis (GSE9423) and meta-analysis (GSE10205 versus GSE16376) with mean $|\log(\text{fold change [FC]})| \geq 2.0$ and $\text{FDR} < 0.05$ were selected and the ALP-associated genes were identified using *Saccharomyces cerevisiae* annotation data from the *Saccharomyces* Genome Database (<https://www.yeastgenome.org>). For the further analyses, only upregulated DEGs (i.e., DEGs that were upregulated during beer fermentation compared to yeast biomass propagation) that were common to both the single-analysis and meta-analysis were selected. For each Pan-DEG, meta- $\log_2\text{FC} \pm$ standard deviation (SD) were calculated, and the ALP Pan-DEGs were used for ALP network design and analyses (Figure 1). Pan-DEGs associated with LDs in yeast were also selected using data reported by Grillitsch *et al.* (2011).

Network design and topology analyses

Initially, PPI and chemical-protein interaction (CPI) networks for *Saccharomyces cerevisiae* were designed using *S. cerevisiae* interactome data downloaded from STRING 11.0 (<https://string-db.org>) and STITCH 5.0 (<http://stitch.embl.de>), and processed in the R environment (Figure 1). The *S. cerevisiae* interactome data were filtered by selecting the subscore information variables labeled “experiments” and “curated databases”, followed by the generation of a combined score from the two channels using the equation described by (von Mering *et al.*, 2005). From PPI interactome network, an ALP network was obtained by selecting the shortest pathways among the Pan-DEGs using the R package igraph (Csardi and Nepusz, 2006). By its turn, the CPI network was applied to select the shortest pathways among LDs-associated proteins and lipid molecules in yeast (Figure 1) and to generate a multilayered network containing both transcriptome and proteome data (LDP network). In this sense, the proteome data was obtained from Casanovas *et al.* (2015) and used to evaluate the expression of LDs-associated proteins during the fermentation-to-respiration transition (Figure 1). Chemical data of interacting lipid molecules was obtained from Lipid Maps® Lipidomics Gateway (<https://lipidmaps.org>). ALP and LDP network were visualized in Cytoscape 3.7.2 (Shannon *et al.*, 2003) using the RCyc3 package (Gustavsen *et al.*, 2019) (Figure 1).

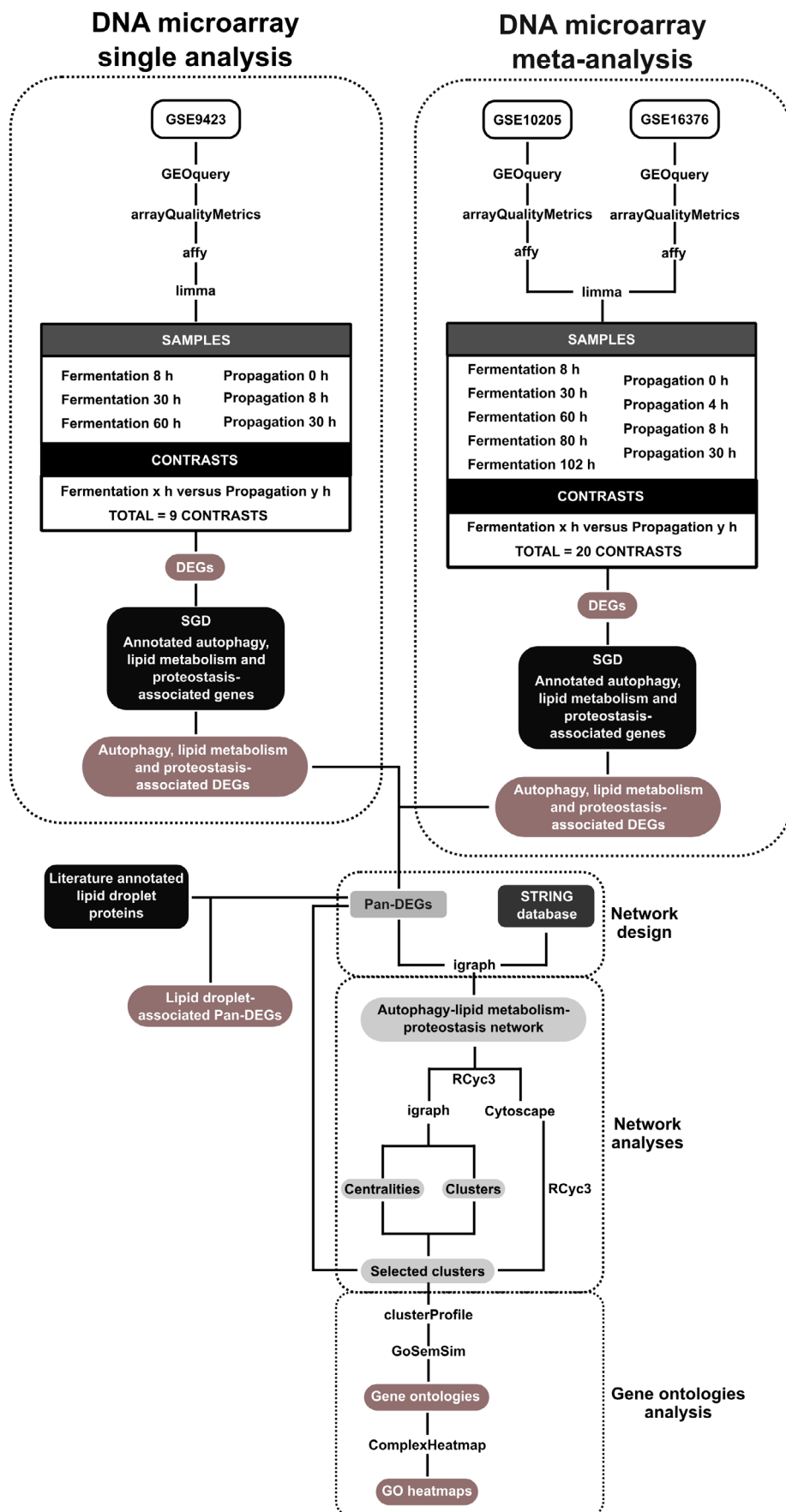


Figure 1 - Experimental design used in DNA microarray single- and meta-analyses and systems biology analysis. DEGs: differentially expressed genes; GO: Gene Ontologies; SGD: Saccharomyces Genome Database.

Regarding the centrality analysis of the ALP network (Figure 1), the node degree and betweenness values were calculated using the R package *igraph* (Csardi and Nepusz, 2006). Node degree indicates the number of connections that a specific node has, while betweenness indicates the number of shortest paths that pass through a specific node. All nodes that had a degree value above the mean for the network were designated “hubs”, while all nodes that had a betweenness value above the mean of the network were designated “bottlenecks” (Yu *et al.*, 2007). Finally, the node degree and betweenness values were used to group the nodes into four major groups: (i) hub-bottleneck (HB), (ii) nonhub-bottleneck (NHB), (iii) hub-nonbottleneck (HNB), and (iv) nonhub-nonbottleneck (NHNB). The HB group represents all nodes that potentially control the flow of information through the network and act as key regulators in the cell (Yu *et al.*, 2007).

Community analysis of ALP network was performed in the R environment using the *walktrap* community (WTC) finding algorithm described by (Pons and Latapy, 2005), and the analysis was fully implemented using the *igraph* package (Csardi and Nepusz, 2006). Communities were selected on the basis of two criteria: (i) presence of HB nodes and (ii) presence of Pan-DEGs (Figure 1). The selected clusters were visualized in *Cytoscape* 3.7.2 (Shannon *et al.*, 2003) using the R package *RCyc3* (Gustavsen *et al.*, 2019) (Figure 1).

GO analysis

The GO biological process and cellular component categories associated with the selected communities from the ALP network were determined using the R package *clusterProfile* (Yu *et al.*, 2012) (Figure 1). The degree of functional enrichment for each biological process and cellular component category was quantitatively assessed ($p < 0.01$) using a hypergeometric distribution. Multiple testing correction was also implemented using the FDR algorithm (Benjamini and Hochberg, 1995) with a significance level of $p < 0.05$. Semantic comparison of the biological processes and cellular components associated with the node clusters was conducted using the R package *GOSemSim* (Yu *et al.*, 2010) (Figure 1) using $FDR < 0.01$ and $q < 0.05$. Heatmaps combining GO categories (columns) and selected clusters from the ALP network (rows) were designed using the R package *ComplexHeatmap* (Gu *et al.*, 2016) (Figure 1), with columns and rows both being grouped using the *k*-means distance-based method.

Data sharing repository

All files, tables and figures generated in this study can be freely downloaded from https://github.com/bonattod/Lipid_stress_data_analysis.git.

Results

Transcriptome single- and meta-analyses of ALP-associated genes in lager yeast strain CB11 during beer fermentation

The initial comparison of upregulated and downregulated DEGs in strain CB11 (during industrial beer fermentation

compared to yeast biomass propagation) identified in the GSE9423 dataset analysis (single-analysis; Figure 1) and the GSE10205 versus GSE16376 dataset analysis (meta-analysis; Figure 1) indicated similar patterns of upregulated and downregulated DEGs. There were a total of 5,134 upregulated and 4,954 downregulated DEGs in the single-analysis, while there were a total of 10,258 upregulated and 9,342 downregulated DEGs in the meta-analysis. The high frequency of total upregulated and downregulated DEGs was due to gene redundancy across the various comparisons in both the single- and meta-analyses. After removing the redundant genes from both analyses, there were 1,315 upregulated and 1,209 downregulated non-redundant (unique) DEGs in the single-analysis, and 1,727 upregulated and 1,502 downregulated non-redundant DEGs in the meta-analysis. The next step was to evaluate the expression profile of ALP-associated genes. For this purpose, the gene information in the *Saccharomyces* Genome Database regarding ALP mechanisms (Figure 1) was used to select the ALP-associated DEGs.

This initial transcriptome data evaluation was followed by a specific analysis of the absolute frequencies of ALP DEGs in each pairwise comparison of beer fermentation and yeast biomass propagation at various time points (Figure S1). The absolute frequencies were low when beer fermentation was compared to early propagation time points (0 h in the single-analysis; 0 and 4 h in the meta-analysis) (Figure S1). On the other hand, the absolute frequencies increased when beer fermentation was compared to advanced propagation time points (8 and 30 h in both the single- and meta-analyses) (Figure S1). For the subsequent transcriptome and systems biology analyses, only the upregulated non-redundant (unique) ALP DEGs observed in all pairwise comparisons were considered.

ALP Pan-DEGs in lager yeast strain CB11 during beer fermentation

The frequency of non-redundant upregulated ALP DEGs for each of the three ALP processes was similar between the single-analysis and meta-analysis (Figure 2A). Regarding autophagy, there were 27 upregulated DEGs for the single-analysis and 42 for the meta-analysis (Figure 2A). Regarding proteostasis, there were 76 upregulated DEGs for the single-analysis and 88 for the meta-analysis (Figure 2A). Regarding lipid metabolism, there were 121 upregulated DEGs for the single-analysis and 138 for the meta-analysis (Figure 2A).

Next, the frequency of non-redundant upregulated ALP DEGs that were common to both transcriptome analyses was evaluated (Figure 2B). There were 117, 71, and 26 common DEGs for lipid metabolism, proteostasis, and autophagy, respectively (Figure 2B and C). These common ALP DEGs were designated ALP Pan-DEGs and their expression patterns were evaluated.

Regarding the expression patterns of the ALP Pan-DEGs (Figure 2D), the median meta- \log_2FC value was similar for autophagy (2.39), lipid metabolism (2.57), and proteostasis (2.68). Additionally, the minimum and maximum meta- \log_2FC values were similar, from 2.13 to 4.39 for autophagy, 2.09 to 4.45 for proteostasis, and 2.04 to 4.17 for lipid metabolism (Figure 2D).

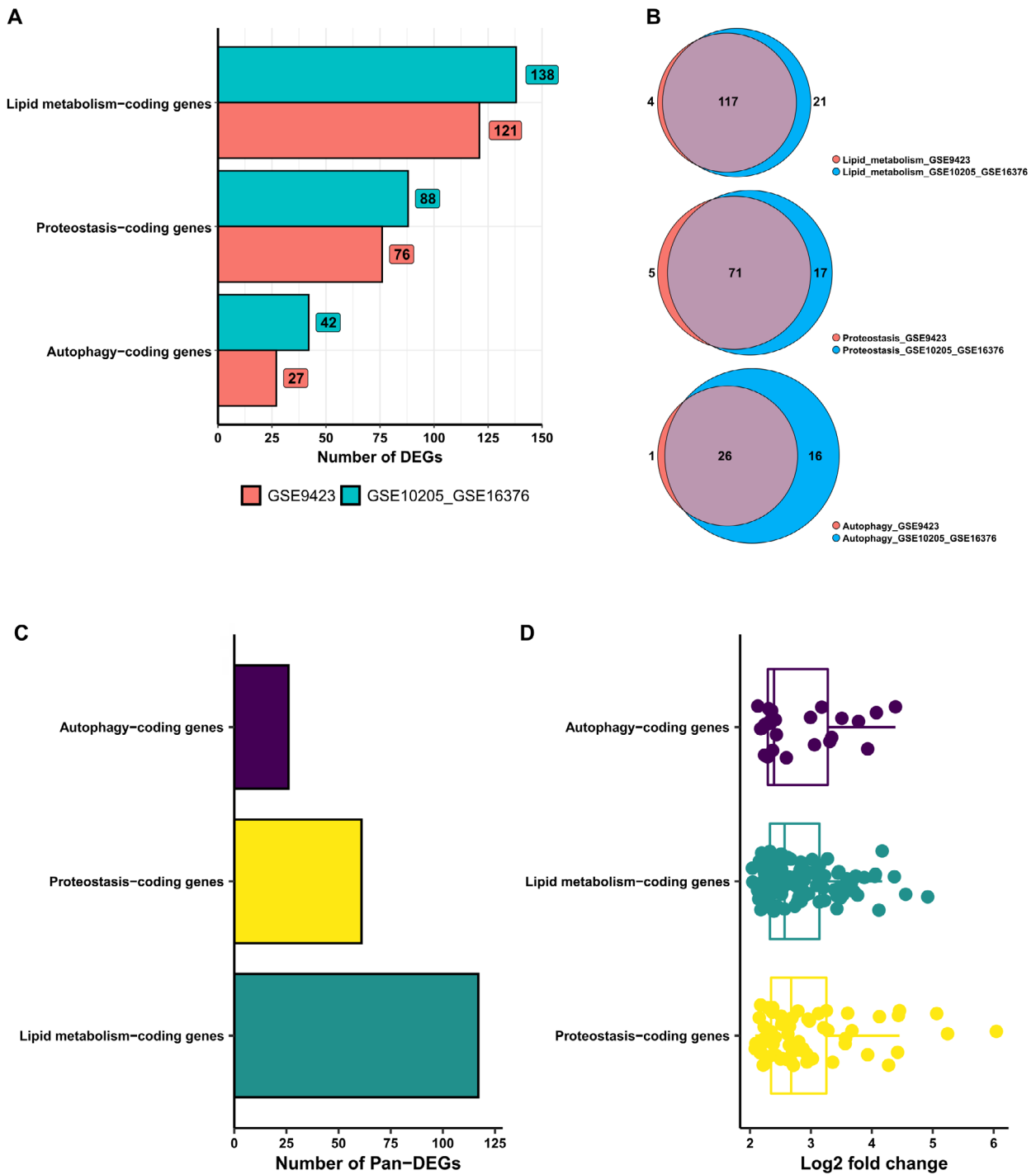


Figure 2 - In (A), Frequency of non-redundant (unique) upregulated differentially expressed genes associated with autophagy, lipid metabolism, and proteostasis (ALP DEGs) in the transcriptome single-analysis (GSE9423) and meta-analysis (GSE10205 versus GSE16376). The numbers inside the squares show the frequency of upregulated DEGs associated with a specific ALP process. The frequencies of ALP DEGs common to both transcriptome analyses (designated ALP Pan-DEGs) are indicated in the (B) Venn diagram and (C) bar chart. In (D) Expression values of ALP Pan-DEGs in log₂(fold change).

The similar expression patterns of the ALP Pan-DEGs lead to evaluate how the ALP Pan-DEGs are connected to each other in terms of PPIs using a top-down systems biology approach involving *S. cerevisiae* interactome data (Figure 1). Moreover, the importance of the ALP Pan-DEGs in the local and global PPI network topologies was also assessed, and the overrepresented biological processes and cellular components associated with the PPI network were identified.

Top-down systems biology analysis of ALP Pan-DEGs

The ALP Pan-DEGs obtained from the transcriptome analyses were selected as seeds to generate a shortest-pathway PPI network using publicly available *S. cerevisiae* interactome data (Figure 1). The subnetwork, designated the “ALP network”, contained 1,705 nodes and 22,806 edges and included almost all ALP Pan-DEGs with the exception of *FAT3*, *IZH2*, *IZH4*, *MZMI*, *OPI10*, *PPX1*, and *TMA17*, which could not be mapped using the currently available *S. cerevisiae* interactome data. Following the generation of the ALP network, a node centrality analysis was performed to identify all nodes that exert a local influence in the network and, consequently, may have roles in ethanol stress tolerance in *lager* yeast cells during beer fermentation. For this purpose, two centrality parameters commonly used for PPI network analyses were selected: node degree and betweenness. The node degree evaluates the potential of a protein to connect with other proteins, thus forming functional complexes (Yu *et al.*, 2007). All proteins with node degree values above the mean value of the network were defined as hubs (Yu *et al.*, 2007). Betweenness evaluates the ability of a node to connect to nodes in different clusters/communities, thus serving as a “bottleneck” for biological information to traverse from one community to another (Yu *et al.*, 2007). By combining the node degree and betweenness results, nodes that display high values for both parameters (HB nodes) can be selected. HB nodes are critical elements within a network as they concentrate the highest numbers of shortest pathways and connections with other nodes, so they are important components for signal transduction among protein clusters/communities (Yu *et al.*, 2007). The centrality analysis of the ALP network indicated the presence of 423 HB nodes, 221 HNB nodes, 59 NHB nodes, and 1,002 NHNB nodes. Once the centralities in the ALP network were defined, whether they were organized into communities was determined. In general, a community can be defined as a specific network topology that contains highly connected nodes that have low degree values with respect to nodes outside the community. Moreover, communities can potentially be associated with specific biological processes (Ravasz *et al.*, 2002; Pons and Latapy, 2005). To identify the communities in the ALP network, the WTC algorithm was applied (Pons and Latapy, 2005), which allows communities to be efficiently identified by using the random walk technique. By using the WTC algorithm, 36 communities were identified in the ALP network. For further analysis, it was necessary to select the major communities in the ALP network by

determining the presence of HB nodes and ALP Pan-DEGs within the communities. Thus, 13 communities were selected by applying the criteria above indicated.

Following the community identification, a GO analysis was applied to each of the 13 selected clusters in order to identify the major overrepresented biological process categories (Figure 3) and cellular component categories (Figure 4). Regarding biological processes, three superclusters and seven biological process groups were identified by applying a *k*-means distance-based method (Figure 3). Supercluster 1 (clusters 3, 23, and 24) was associated with major biological processes concerning mitochondria structure and organization (Figure 3), while supercluster 2 (clusters 2, 9, and 31) was associated with lipid, ergosterol, and alcohol metabolism (Figure 3). Both superclusters 1 and 2 also contained proteins involved in the oxidation-reduction process (Figure 3). On the other hand, supercluster 3 (clusters 1, 4, 5, 6, 12, 10, and 14) was associated with autophagy and autophagosome assembly, response to ER stress and protein folding, piecemeal microautophagy of the nucleus (PMN) and mitochondria autophagy, vesicle-mediated transport, and other biological processes (Figure 3). Next, the major cellular components associated with the clusters were identified. Again, three superclusters were observed (Figure 4). Supercluster 1 (clusters 1, 4, and 31) mainly contained proteins associated with ER membrane and LDs, while supercluster 2 (clusters 2, 3, 5, 9, and 23) contained proteins found in the mitochondria envelope and matrix, Golgi apparatus, membrane protein complexes, and organelles such as peroxisomes (Figure 4). Finally, supercluster 3 (clusters 8 and 12) contained proteins that are mainly found in cytoplasmic vesicles and at phagophore assembly sites (Figure 4).

Evaluation of ALP Pan-DEGs linked to LD structure and function

Using the available data on the various proteins associated with LD (Grillitsch *et al.*, 2011) and the ALP Pan-DEGs identified in this study, 17 ALP Pan-DEGs linked to LD structure were identified as being upregulated in *lager* yeast cells during beer fermentation compared to during yeast biomass propagation (Table S2). Of these 17 LD-associated ALP Pan-DEGs, 11 are directly involved with lipid metabolism and six are related to proteostasis (Table S2). This result was supported by a proteome and transcriptome multilayered chemical-protein interaction network of the 17 LD-associated ALP Pan-DEGs (LDP network; Figure 5), where it could be observed that *ERG6*, *ERG27*, *POX1*, *FAA4*, *YJU3*, *GPT2*, *ERG1*, *DPL1*, and *PDII* were found to be overexpressed in transcriptome and also in publicly available yeast proteolipidome data from Casanovas *et al.* (2015) (Figure 5 and Table S3). Additionally, the LDP network also contains lipid metabolism-associated nodes that were exclusively found to be expressed in proteolipidome data, like *TGL4*, *FAA1*, *FAS1*, *ERG4*, *ERG7*, and *ERG9*, which are connected with LD-associated ALP Pan-DEGs (Figure 5 and Table S3).

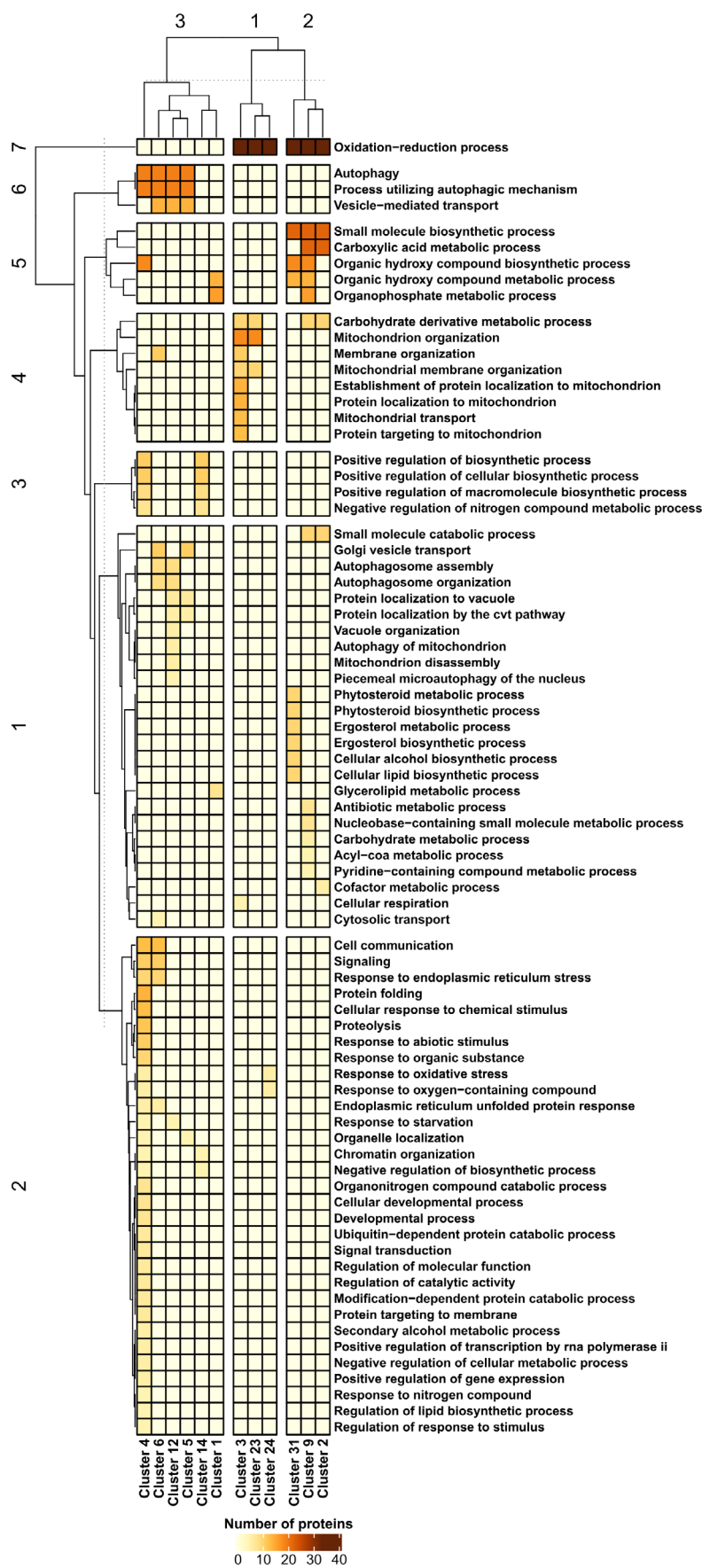


Figure 3 - Heatmap showing the clustered biological processes based on the gene ontology (GO) analysis of autophagy, lipid metabolism, and proteostasis (ALP) network-associated communities/clusters. Heatmap rows and columns were grouped using the k-means distance-based method. Horizontal and vertical dotted lines indicate the cut-off point used to define the numbered rows and column groups.

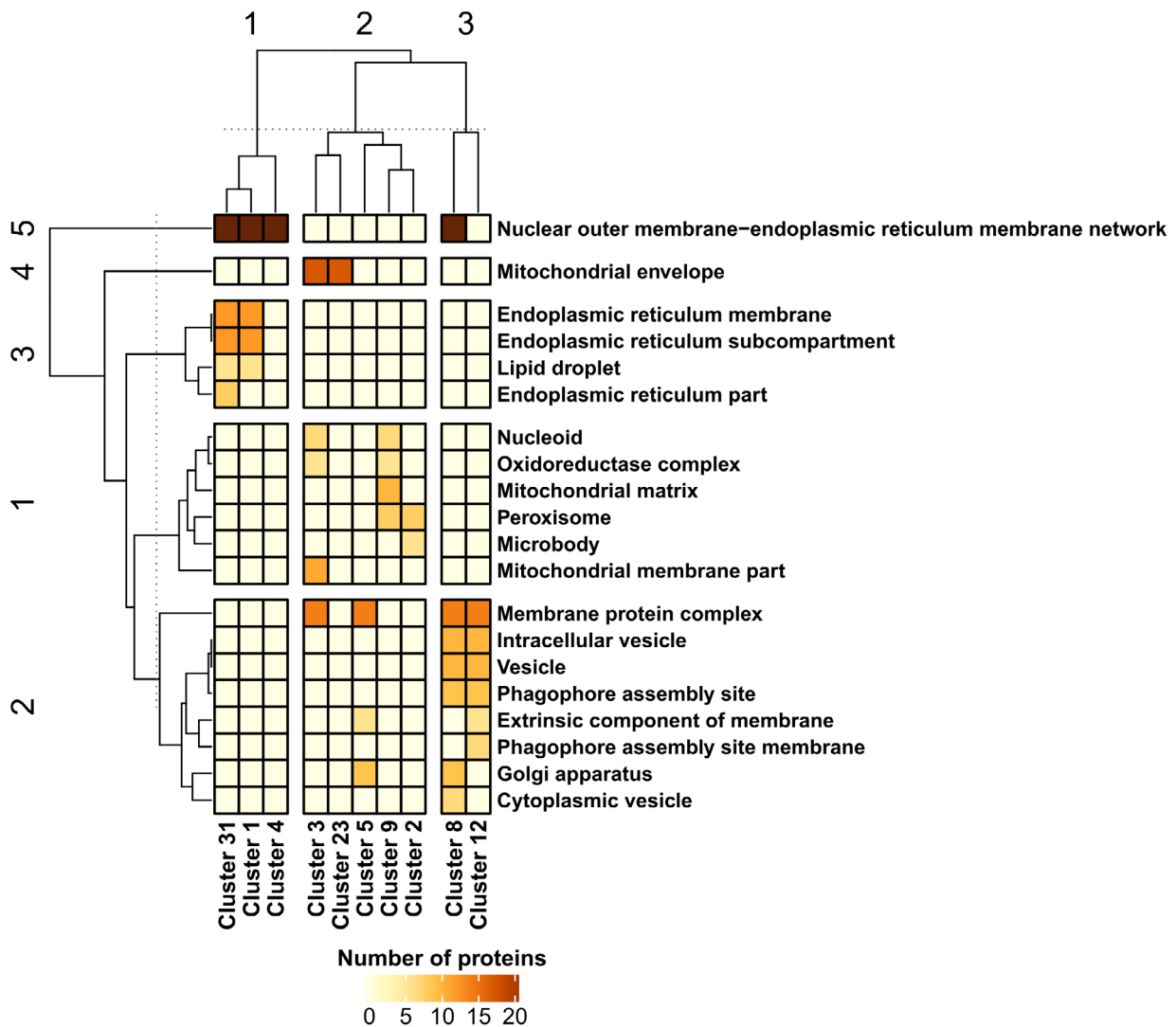


Figure 4 - Heatmap showing the clustered cellular components based on the Gene Ontology (GO) analysis of autophagy, lipid metabolism, and proteostasis (ALP) network-associated communities/clusters. Heatmap rows and columns were grouped using the k -means distance-based method. Horizontal and vertical dotted lines indicate the cut-off point used to define the numbered rows and column groups.

Discussion

The transcriptome and systems biology results obtained in this study suggest that, during *lager* beer fermentation, various ALP-associated genes are upregulated and their products cooperate in a shortest-pathway PPI network that is composed of multiple communities of proteins in order to deal with the nutritional and ethanol stress observed during beer fermentation (Gibson *et al.*, 2007; 2008). The GO analysis of these clusters showed that they are associated with a large number of processes related to autophagy, mitochondria organization and activity, ER stress and Golgi organization, lipid/ergosterol metabolism, cytoplasmic vesicles, LDs, and phagophore assembly sites (Figure 3). All these processes are known to be modulated by the stressful conditions that yeast cells are exposed to during beer fermentation, including ethanol toxicity (Telini *et al.*, 2020) and nitrogen and carbohydrate starvation (Gibson *et al.*, 2007). Nitrogen starvation is a key condition that promotes autophagy in yeast cells (Cebollero

and Reggiori, 2009), and despite this mechanism being well characterized in yeast strains during wine fermentation (Piggott *et al.*, 2011), autophagy studies in *lager* yeasts are virtually absent. Interestingly, malt-derived wort used for beer fermentation contains different types of nitrogen sources that can prevent the activation of autophagy (Gibson *et al.*, 2007); however, many autophagy-associated genes are upregulated during wine fermentation even in the presence of nitrogen sources (Piggott *et al.*, 2011). In this study, the transcriptome data indicated the upregulation of various autophagy-related (*ATG*) genes during *lager* beer fermentation (Table S3). Among the *ATG* genes that were upregulated during beer fermentation, three (*ATG1*, *ATG8*, and *ATG18*) are part of the so-called “autophagy core machinery”, which is important for both micro- and macroautophagy (Lynch-Day and Klionsky, 2010). The transcriptome and systems biology data indicate that, during beer fermentation, the cytoplasm-to-vacuole targeting (Cvt) mechanism, mitophagy,

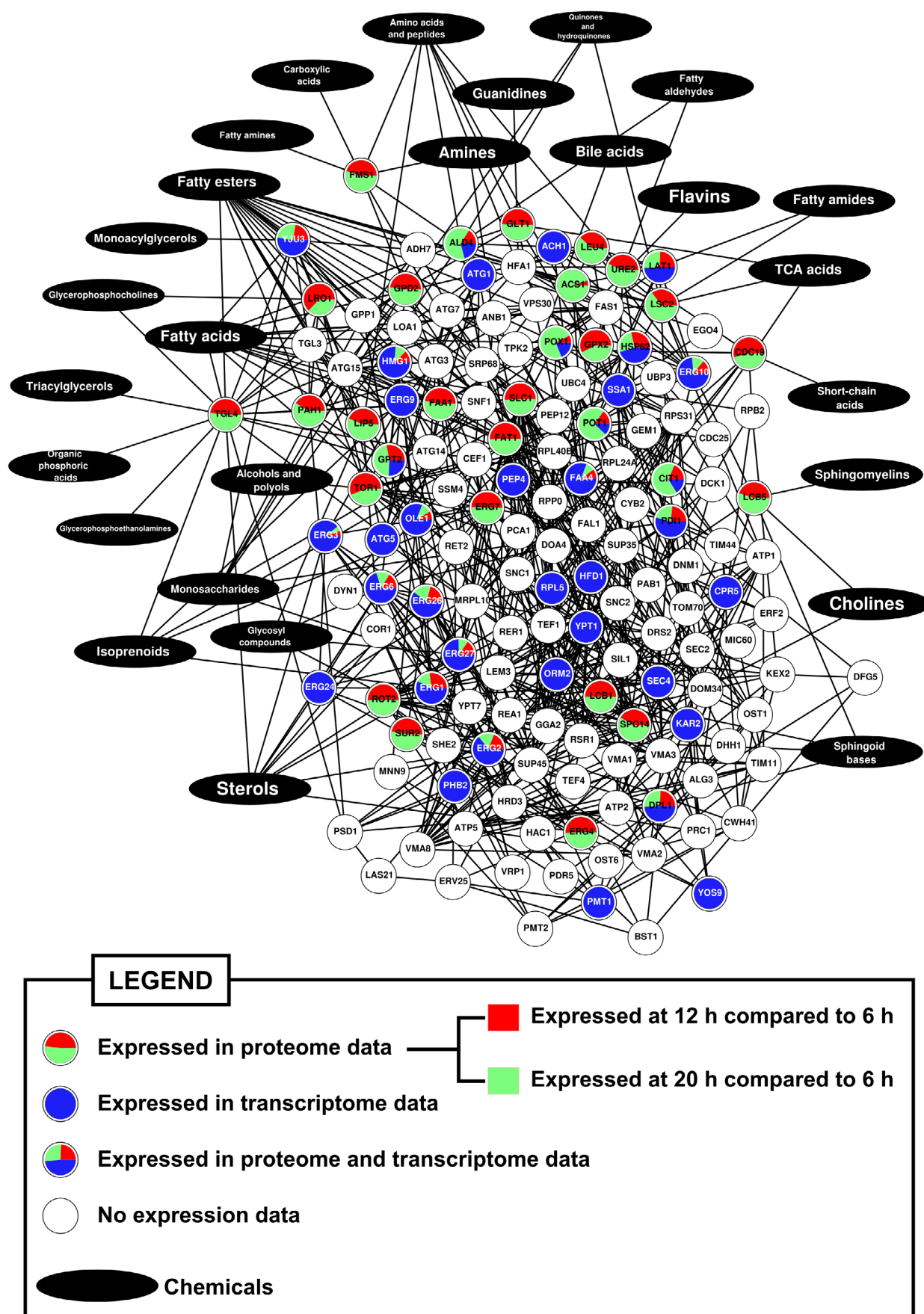


Figure 5 - Shortest-pathway chemical-protein interaction (CPI) network of lipid droplets-associated proteins and molecules linked to lipid metabolism (LDP network). Expression data from proteome (Casanovas *et al.*, 2015) and transcriptome (this study) analyses were added to network and are indicated as pie charts inside the nodes in different colors. Each pie slice is proportional to proteome and/or transcriptome expression values for a given node.

and PMN are potentially activated (Figure 3), all three of which depend on the activity of Atg1p, Atg8p, and Atg18p (Krick *et al.*, 2008; Lynch-Day and Klionsky, 2010; Kanki *et al.*, 2015). Although the importance of Cvt, mitophagy, and PMN to brewing yeasts strains is unknown in the context of beer fermentation, it has been reported that all three of these microautophagy processes are important for yeast cell adaptation in fermentative environments (Cebollero and Gonzalez, 2006; Kurihara *et al.*, 2012).

Another fundamental aspect of micro- and macroautophagy mechanisms that should be considered is the formation of autophagosomes, which are membranous vesicular structures that deliver various cargo components for degradation in vacuoles (Lamb *et al.*, 2013). The formation of autophagosomes strongly depends on the biosynthesis of lipids such as triacylglycerols and sterol esters, which originate from ER membranes in the form of LDs (Velázquez *et al.*, 2016). In fact, low levels of nitrogen sources and the presence of glucose in yeast stimulate lipogenesis and increase the number of LDs, which, in turn, are required for efficient autophagy (Li *et al.*, 2015).

The transcriptome and systems biology data demonstrated the upregulation of several genes linked to neutral lipid and ergosterol biosynthesis in *lager* yeast cells during beer fermentation (Table S3). Moreover, the GO analysis of cellular components linked to clusters 1, 4, and 31 indicated associations with ER membrane structure, the ER association with the nuclear outer membrane, and LDs (Figure 4).

Besides the importance of lipid biosynthesis for autophagy, lipids may be key regulators of proteostasis and modulators of the permeability to ethanol of the cell membrane (Chi and Arneborg, 1999; Aguilera *et al.*, 2006; Ma and Liu, 2010). In fact, lipid metabolism and LDs are important factors in proteostasis, as it has been shown that yeast cells exhibiting defective biosynthesis of neutral lipids and LDs have chronic ER stress (Velázquez *et al.*, 2016; Graef, 2018). In addition to being important during proteostasis, LDs are essential for macroautophagy (Velázquez and Graef, 2016; Velázquez *et al.*, 2016), helping to assemble autophagosomes and induce mitophagy (Carmona-Gutierrez *et al.*, 2012) and microlipophagy (Vevea *et al.*, 2015). Notably, six upregulated proteostasis-associated genes identified in this study that are linked to the inter-organellar proteostasis mechanism (*CPR5*, *KAR2*, *PDII*, *PMT1*, *RPL5*, and *SSA1*) (Telini *et al.*, 2020) are also found in LD structures (Table S3). Interestingly, ER stress can induce the formation of LDs (Fei *et al.*, 2009) and LDs interact with mitochondria and peroxisomes via Erg6p (Pu *et al.*, 2011), a protein encoded by *ERG6*, which was found upregulated in this study (Table S3). A supporting proteome and transcriptome multilayered chemical-protein interaction network (LDP network) using the proteolipidome data of Casanovas *et al.* (2015) pointed that many of the overexpressed LD-associated genes found from meta-transcriptome analysis are proteomically expressed during the transition of fermentation to respiration in yeast cells, including genes linked to synthesis of LD-associated fatty acids and triacylglycerols, like *FAA4* (Figure 5 and Table S3). The fermentation-to-respiration transition is also observed in yeast cells during brewing, with the exception that the

anaerobiosis and nutrient depletion in beer avoid the use of ethanol as carbon source by yeast cells (Gibson *et al.*, 2007). Interestingly, Erg6p, Erg27p, Pox1, Faa4p, Yju3, Gpt2p, Erg1p, Dpl1p, and Pdi1p were also found to be differentially expressed in the proteome data of industrial wine yeast strains during wine fermentation (Rossouw *et al.*, 2010). Additionally, Erg6p, Erg27p, Faa4p, and Yju3p were found in high resolution proteome analysis of purified LD fraction from yeast (Currie *et al.*, 2014), also including Fat1p, Tgl3p, and Tgl4p, which are present in the LDP network (Figure 5). Thus, the systems biology data gathered in this work support the idea that beer fermentation promotes lipid biosynthesis in yeast cells, specially unsaturated fatty acids (UFAs) and ergosterol, which can be related to the composition of LD's membrane. In fact, it has been reported that the levels of UFAs and ergosterol increased during fermentation in order to antagonize the membrane fluidity induced by ethanol (Ding *et al.*, 2009) and LD-associated triacylglycerols and sterol esters can differentially change during the fermentation-to-respiration transition (Casanovas *et al.*, 2015).

Finally, lipid metabolism and LDs have a central role in inter-organellar communication and the promotion of autophagy and proteostasis; however, comparative proteome and transcriptome studies that focuses on lipid and LD metabolism from different *ale* and *lager* brewing strains are virtually non-existent until now. Thus, it is essential to determine how lipids and LDs are produced and regulated in the context of different *ale* and *lager* strains in order to design new yeast strains resistant to high ethanol concentrations during HG/VHG beer production.

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [grant number 314558/2020-9]. The sponsor had no role in the study design; collection, analysis, and interpretation of data; writing of the report; or decision to submit the article for publication.

Conflicts of interest

Not applicable.

Author Contributions

DB contributed to the design, acquisition, analysis, and interpretation of data for the work; DB prepared the figures, drafting the work, and approved the final manuscript.

References

- Aguilera F, Peinado R, Millan C, Ortega J and Mauricio J (2006) Relationship between ethanol tolerance, H⁺-ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. *Int J Food Microbiol* 110:34-42.
- Ahvenainen J (1982) Lipid composition of aerobically and anaerobically propagated brewer's bottom yeast. *J Inst Brew* 88:367-370.
- Alexandre H, Rousseaux I and Charpentier C (1994) Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and *Kloeckera apiculata*. *FEMS Microbiol Lett* 124:17-22.
- Barbosa AD, Savage DB and Sinioglou S (2015) Lipid droplet-organelle interactions: Emerging roles in lipid metabolism. *Curr Opin Cell Biol* 35:91-97.

- Benjamini Y and Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 57:289-300.
- Carmona-Gutierrez D, Sommer C, Andryushkova A, Kroemer G and Madeo F (2012) A higher spirit: Avoiding yeast suicide during alcoholic fermentation. *Cell Death Differ* 19:913-914.
- Casanovas A, Sprenger RR, Tarasov K, Ruckerbauer DE, Hannibal-Bach HK, Zanghellini J, Jensen ON and Ejsing CS (2015) Quantitative analysis of proteome and lipidome dynamics reveals functional regulation of global lipid metabolism. *Chem Biol* 22:412-425.
- Cebollero E and Gonzalez R (2006) Induction of autophagy by second-fermentation yeasts during elaboration of sparkling wines. *Appl Environ Microbiol* 72:4121-4127.
- Cebollero E and Reggiori F (2009) Regulation of autophagy in yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1793:1413-1421.
- Chi Z and Arneborg N (1999) Relationship between lipid composition, frequency of ethanol-induced respiratory deficient mutants, and ethanol tolerance in *Saccharomyces cerevisiae*. *J Appl Microbiol* 86:1047-1052.
- Cox JS, Chapman RE and Walter P (1997) The unfolded protein response coordinates the production of endoplasmic reticulum protein and endoplasmic reticulum membrane. *Mol Biol Cell* 8:1805-1814.
- Csardi G and Nepusz T (2006) The igraph software package for complex network research. *Int J Complex Syst* 1695:1-9.
- Currie E, Guo X, Christiano R, Chitraju C, Kory N, Harrison K, Haas J, Walther TC and Farese RV Jr (2014) High confidence proteomic analysis of yeast LDs identifies additional droplet proteins and reveals connections to dolichol synthesis and sterol acetylation. *J Lipid Res* 55:1465-1477.
- Davis S and Meltzer PS (2007) GEOquery: A bridge between the Gene Expression Omnibus (GEO) and BioConductor. *Bioinformatics* 23:1846-1847.
- Ding J, Huang X, Zhang L, Zhao N, Yang D and Zhang K (2009) Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 85:253-263.
- Fei W, Wang H, Fu X, Bielby C and Yang H (2009) Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in *Saccharomyces cerevisiae*. *Biochem J* 424:61-67.
- Gautier L, Cope L, Bolstad BM and Irizarry RA (2004) affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 20:307-315.
- Gibson BR, Lawrence SJ, Boulton CA, Box WG, Graham NS, Linforth RST and Smart KA (2008) The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation: Oxidative stress response of lager brewing yeast. *FEMS Yeast Res* 8:574-585.
- Gibson BR, Lawrence SJ, Leclaire JPR, Powell CD and Smart KA (2007) Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiol Rev* 31:535-569.
- Graef M (2018) Lipid droplet-mediated lipid and protein homeostasis in budding yeast. *FEBS Lett* 592:1291-1303.
- Grillitsch K, Connerth M, Köfeler H, Arrey TN, Rietschel B, Wagner B, Karas M and Daum G (2011) Lipid particles/droplets of the yeast *Saccharomyces cerevisiae* revisited: lipidome meets proteome. *Biochim Biophys Acta* 1811:1165-1176.
- Gu Z, Eils R and Schlesner M (2016) Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* 32:2847-2849.
- Gustavsen JA, Pai S, Isserlin R, Demchak B and Pico AR (2019) RCy3: Network biology using Cytoscape from within R. *F1000Research* 8:1774.
- Hallsworth JE (1998) Ethanol-induced water stress in yeast. *J Ferment Bioeng* 85:125-137.
- Horinouchi T, Yoshikawa K, Kawaide R, Furusawa C, Nakao Y, Hirasawa T and Shimizu H (2010) Genome-wide expression analysis of *Saccharomyces pastorianus* orthologous genes using oligonucleotide microarrays. *J Biosci Bioeng* 110:602-607.
- Jacquier N, Choudhary V, Mari M, Toulmay A, Reggiori F and Schneider R (2011) Lipid droplets are functionally connected to the endoplasmic reticulum in *Saccharomyces cerevisiae*. *J Cell Sci* 124:2424-2437.
- Jing H, Liu H, Zhang L, Gao J, Song H and Tan X (2018) Ethanol induces autophagy regulated by mitochondrial ROS in *Saccharomyces cerevisiae*. *J Microbiol Biotechnol* 28:1982-1991.
- Kanki T, Furukawa K and Yamashita S (2015) Mitophagy in yeast: Molecular mechanisms and physiological role. *Biochim Biophys Acta* 1853:2756-2765.
- Kauffmann A, Gentleman R and Huber W (2009) arrayQualityMetrics—a bioconductor package for quality assessment of microarray data. *Bioinformatics* 25:415-416.
- Kim H-E, Grant AR, Simic MS, Kohnz RA, Nomura DK, Durieux J, Riera CE, Sanchez M, Kapernick E, Wolff S *et al.* (2016) Lipid biosynthesis coordinates a mitochondrial-to-cytosolic stress response. *Cell* 166:1539-1552.e16.
- Kohlwein SD, Veenhuis M and van der Klei IJ (2013) Lipid droplets and peroxisomes: Key players in cellular lipid homeostasis or a matter of fat-store 'em up or burn 'em down. *Genetics* 193:1-50.
- Krick R, Muehe Y, Prick T, Bremer S, Schlotterhose P, Eskelinen E-L, Millen J, Goldfarb DS and Thumm M (2008) Piecemeal microautophagy of the nucleus requires the core macroautophagy genes. *Mol Biol Cell* 19:4492-4505.
- Kurihara Y, Kanki T, Aoki Y, Hirota Y, Saigusa T, Uchiumi T and Kang D (2012) Mitophagy plays an essential role in reducing mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast. *J Biol Chem* 287:3265-3272.
- Lamb CA, Yoshimori T and Tooze SA (2013) The autophagosome: Origins unknown, biogenesis complex. *Nat Rev Mol Cell Biol* 14:759-774.
- Li D, Song J-Z, Li H, Shan M-H, Liang Y, Zhu J and Xie Z (2015) Storage lipid synthesis is necessary for autophagy induced by nitrogen starvation. *FEBS Lett* 589:269-276.
- Lynch-Day MA and Klionsky DJ (2010) The Cvt pathway as a model for selective autophagy. *FEBS Lett* 584:1359-1366.
- Ma M and Liu ZL (2010) Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 87:829-845.
- Mishra P and Kaur S (1991) Lipids as modulators of ethanol tolerance in yeast. *Appl Microbiol Biotechnol* 34:697-702.
- Navarro-Tapia E, Querol A and Pérez-Torrado R (2018) Membrane fluidification by ethanol stress activates unfolded protein response in yeasts. *Microb Biotechnol* 11:465-475.
- Odumeru JA, D'Amore T, Russell I and Stewart GG (1992) Effects of heat shock and ethanol stress on the viability of a *Saccharomyces uvarum* (*carlsbergensis*) brewing yeast strain during fermentation of high gravity wort. *J Ind Microbiol* 10:111-116.
- Piggott N, Cook MA, Tyers M and Measday V (2011) Genome-wide fitness profiles reveal a requirement for autophagy during yeast fermentation. *G3 (Bethesda)* 1:353-367.
- Piper PW (1995) The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiol Lett* 134:121-127.

- Pons P and Latapy M (2005) Computing communities in large networks using random walks. In: Yolum P, Güngör T, Gürgeç F and Özturan C (eds) Computer and Information Sciences - ISCIS 2005. Springer Berlin Heidelberg, Berlin, pp 284-293.
- Pu J, Ha CW, Zhang S, Jung JP, Huh W-K and Liu P (2011) Interactomic study on interaction between lipid droplets and mitochondria. *Protein Cell* 2:487-496.
- Puligundla P, Smogrovicova D, Mok C and Obulam VSR (2019) A review of recent advances in high gravity ethanol fermentation. *Renew Energy* 133:1366-1379.
- Puligundla P, Smogrovicova D, Obulam VSR and Ko S (2011) Very high gravity (VHG) ethanolic brewing and fermentation: a research update. *J Ind Microbiol Biotechnol* 38:1133-1144.
- Ravasz E, Somera AL, Mongru DA, Oltvai ZN and Barabási A-L (2002) Hierarchical organization of modularity in metabolic networks. *Science* 297:1551-1555.
- Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W and Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43:e47-e47.
- Rossouw D, Dool AH van den, Jacobson D and Bauer FF (2010) Comparative transcriptomic and proteomic profiling of industrial wine yeast strains. *Appl Environ Microbiol* 76:3911-3923.
- Schuck S, Prinz WA, Thorn KS, Voss C and Walter P (2009) Membrane expansion alleviates endoplasmic reticulum stress independently of the unfolded protein response. *J Cell Biol* 187:525-536.
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T (2003) Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res* 13:2498-2504.
- Telini B de P, Menoncin M and Bonatto D (2020) Does inter-organellar proteostasis impact yeast quality and performance during beer fermentation? *Front Genet* 11:2.
- Thibault G, Shui G, Kim W, McAlister GC, Ismail N, Gygi SP, Wenk MR and Ng DTW (2012) The membrane stress response buffers lethal effects of lipid disequilibrium by reprogramming the protein homeostasis network. *Mol Cell* 48:16-27.
- Velázquez AP and Graef M (2016) Autophagy regulation depends on ER homeostasis controlled by lipid droplets. *Autophagy* 12:1409-1410.
- Velázquez AP, Tatsuta T, Ghillebert R, Drescher I and Graef M (2016) Lipid droplet-mediated ER homeostasis regulates autophagy and cell survival during starvation. *J Cell Biol* 212:621-631.
- Vevea JD, Garcia EJ, Chan RB, Zhou B, Schultz M, Di Paolo G, McCaffery JM and Pon LA (2015) Role for lipid droplet biogenesis and microlipophagy in adaptation to lipid imbalance in yeast. *Dev Cell* 35:584-599.
- von Mering C, Jensen LJ, Snel B, Hooper SD, Krupp M, Foglierini M, Jouffre N, Huynen MA and Bork P (2005) STRING: Known and predicted protein-protein associations, integrated and transferred across organisms. *Nucleic Acids Res* 33:D433-D437.
- Yu G, Li F, Qin Y, Bo X, Wu Y and Wang S (2010) GOSemSim: An R package for measuring semantic similarity among GO terms and gene products. *Bioinformatics* 26:976-978.
- Yu G, Wang L-G, Han Y and He Q-Y (2012) clusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS* 16:284-287.
- Yu H, Kim PM, Sprecher E, Trifonov V and Gerstein M (2007) The importance of bottlenecks in protein networks: Correlation with gene essentiality and expression dynamics. *PLoS Comput Biol* 3:e59.

Supplementary material

- The following online material is available for this article:
- Figure S1 – Frequency of total downregulated and upregulated genes associated with autophagy, lipid metabolism, and proteostasis (ALP).
- Table S1 – DNA microarray gene expression datasets used in this work.
- Table S2 – Upregulated ALP Pan-DEGs associated with lipid droplets.
- Table S3 – Transcriptome and proteolipidome data of nodes associated with lipid droplet-linked protein (LDP) network.

Associate Editor: Guilherme Correa de Oliveira

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License (type CC-BY), which permits unrestricted use, distribution and reproduction in any medium, provided the original article is properly cited.