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Rethinking the role of lipids in lager yeast cell during beer fermentation from a transcriptome and systems biology perspective

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Short title: Lipid metabolism and brewing

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Abstract

Brewing lager yeasts (*Saccharomyces pastorianus*) should deal with stressful conditions during beer fermentation, including ethanol toxicity. In response to ethanol toxicity, different biological mechanisms are modulated, including lipid biosynthesis. It is well known that during beer fermentation, the composition of yeast membranes change in response to ethanol toxicity, making it less fluid and permeable. Additionally, neutral lipids and lipid droplets (LDs) are produced in response to ethanol toxicity. LDs are membranous organelles that transport lipids and proteins, acting as a hub for inter-organellar communication and module the activity of mechanisms necessary for ethanol tolerance, like proteostasis and autophagy. Unfortunately, little is known about the interplay of lipid biosynthesis, proteostasis, and autophagy in lager cells during beer fermentation. Thus, a transcriptome single and meta-analysis using publically available DNA microarray data of lager yeast cells in conditions of beer fermentation and cell biomass propagation was used to select all the upregulated genes associated to autophagy, lipid biosynthesis, and proteostasis (ALP) during beer fermentation. Following transcriptome data collection, a top-down systems biology analyses was applied with the design of an ALP-associated shortest pathway protein-protein network (ALP network), selection of central nodes and communities within ALP network, and the observation of the overrepresented biological processes and cellular components by gene ontology (GO) analysis. The transcriptome results show the upregulation of 204 non redundant ALP genes in conditions of beer fermentation, whose respective proteins interact in a shortest pathway ALP network. Thirteen communities where selected from ALP network containing overrepresented processes and components like mitophagy, cytoplasm-to-vacuole transport, pieacemeal micrautophagy of the nucleus, endoplasmic reticulum (ER) stress, ergosterol and lipid biosynthesis, LDs, ER membrane, phagophore assembly, among others. These results indicated that ethanol tolerance in lager yeasts could be due to the modulation of proteostasis and different forms

of autophagy by lipid biosynthesis and LDs, and thus extending the importance of lipids for beer fermentation.

Keywords: Lager yeasts, Lipid biosynthesis, Lipid droplets, Proteostasis, Autophagy, Ethanol tolerance.

1. Introduction

Nowadays, there is a tendency of brewing industry in to adopt high gravity (HG) and very high gravity (VHG) fermentation technologies for beer production, allowing to reduce the consumption of water during brewing process and increase the ethanol yield, thus maximizing the brewing efficiency and reducing the costs of production and energy demand (Puligundla et al., 2019, 2011). However, the accumulation of high amounts of ethanol (> 5% v/v) due to the fermentation of HG/VHG worts drastically alter the yeast's physiology (Hallsworth, 1998) and promotes an ethanol stress response that recapitulate the molecular mechanisms associated to the heat shock response (Odumeru et al., 1992; Piper, 1995).

The ability of different yeast strains to couple the toxic effects of ethanol depends on the modulation of cell membrane fluidity by modifying the ratio of incorporated saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs), and 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 yeast *Saccharomyces cerevisiae* (ale yeast) and *Saccharomyces pastorianus* (lager yeast) (Ahvenainen, 1982; Mishra and Kaur, 1991). In wine yeast strains, high concentration of ergosterol in cell membrane promotes ethanol tolerance by diminishing its fluidity (Aguilera et al., 2006); however, increased levels of UFAs in cell membrane have an opposed effect of ergosterol by promoting its fluidity (Alexandre et al., 1994), and it was found that the more yeast ethanol-tolerant strains incorporates long-chain fatty acids ($C_{18:0}$ and $C_{18:1}$) compared with the less ethanol-tolerant strain (Chi and Arneborg, 1999). Additionally, high concentrations of ethanol induce the fluidification and thinning of membranes, and changes in the activity of membrane-associated proteins as well as its aggregation (Thibault et al., 2012). Unfortunately, little is known about how lipids can modulate different biological mechanisms in yeast cell during beer fermentation besides affecting membrane structure and/or permeability. For example, membrane fluidification by ethanol activates the ER-linked UPR mechanism (Navarro-

Tapia et al., 2018) and additional roles of lipids in proteostasis have been proposed, like the remotion of unfolded proteins from ER by lipid droplets (LDs) (Vevea et al., 2015), which are an important and highly dynamic cytoplasmic organelle that connect different parts of the cell, including ER (Jacquier et al., 2011), mitochondria (Pu et al., 2011), peroxisome (Kohlwein et al., 2013), and vacuole (Barbosa et al., 2015). Interestingly, ER stress induces the formation of LDs (Fei et al., 2009) and stimulate lipid biosynthesis that are associated with ER membrane expansion during UPR (Cox et al., 1997; Schuck et al., 2009). Additionally, lipid biosynthesis coordinate the proteotoxic response of both mitochondria and cytosol (Kim et al., 2016). Thus, it can be hypothesize that LDs and lipid biosynthesis are integrative processes necessary for proteostasis in different organelles. In fact, regulation of inter-organellar proteostasis is an important mechanism for stress tolerance and recently was shown that beer fermentation promotes in lager yeast cells a so called “inter-organellar/cross-organellar communication/response” (CORE mechanism), a series of signaling-associated protein networks that regulate the inter-organellar proteostasis, which includes the endoplasmic reticulum (ER) and mitochondria unfolded protein responses (UPRs), chaperone and co-chaperone activity, and *N*-glycosylation quality control pathway proteins. (Telini et al., 2020). One major aspect of inter-organellar proteostasis mechanism induced by ethanol stress is the coordination and/or activation of organellar-linked microautophagy responses, like mitophagy (Carmona-Gutierrez et al., 2012) and lipophagy of LDs (Vevea et al., 2015). Moreover, macroautophagy can also be induced by reactive oxigen species (ROS) generated from damaged mitochondria promoted by ethanol stress (Jing et al., 2018).

Thus, the purpose of this work is to evaluate how lipid metabolism interplay with different mechanisms linked to inter-organellar proteostasis and autophagy in lager brewing yeast during beer fermentation. In this sense, public available DNA microarray gene expression sets from lager yeast in different times of fermentation and propagation were selected and a transcriptome single- and meta-analysis was performed. The pan differentially expressed genes (Pan-DEGs) were used to

generate a protein-protein interactome (PPI) network followed by local and global topological analyses as well as a gene ontology (GO) evaluation of major clusters find within the PPI network. The data gathered from transcriptome and PPI network analyses indicated a cross-communication of different pathways linked to inter-organelles proteostasis, autophagy, and lipid metabolism during lager beer fermentation.

2. Experimental procedures

2.1. DNA microarray gene expression datasets selection and analysis

DNA microarray gene expression datasets (GSE9423, GSE10205, and GSE16376) containing the transcriptome data of lager yeast CB11 strain (*Saccharomyces pastorianus*) in conditions of industrial beer fermentation (F) and cell biomass propagation (P) in different times were obtained from Gene Expression Omnibus (GEO) database [http://www.ncbi.nlm.nih.gov/gds] (Table S1). The GSE9423 dataset contains the transcriptome data matrix of lager yeast CB11 strain in both conditions of cell biomass propagation and beer fermentation (Gibson et al., 2008) and was defined as transcriptome single analysis. By its turn, GSE10205 and GSE16376 data matrix, which individually describes the transcriptome of CB11 strain in fermentation and propagation conditions, respectively, were combined for a differentially expressed gene (DEG) meta-analysis (Figure 1).

The analysis of transcriptome datasets was performed in the R platform (https://www.r-project.org) with different packages (Figure 1). For data matrix importing, processing, and array quality analysis, the GEOquery, affy, and arrayQualityMetrics packages were respectively employed (Davis and Meltzer, 2007; Gautier et al., 2004; Kauffmann et al., 2009). Differentially expressed gene (DEG) analysis was performed with limma package (Ritchie et al., 2015). The False Discovery Rate (FDR) algorithm, implemented in limma package (Ritchie et al., 2015), was used to access DEGs significance level. DEGs from DNA microarray single- (GSE9423) and meta-analysis (GSE10205 versus GSE16376) with mean $|logFC| \geq 2.0$ and $FDR < 0.05$ were selected and were specifically filtered for annotated autophagy, lipid metabolism, and proteostasis (ALP) associated

genes using the annotated data for *Saccharomyces cerevisiae* from *Saccharomyces* Genome Database (https://www.yeastgenome.org). The ALP files containing the curated data used in this work are also available in https://github.com/bonattod/Lipid_stress_data_analysis.git under the names "Autophagy_Table.txt", "Lipid_metabolism_Table.txt", and "Proteostasis_Table.txt". For further analyses, only upregulated DEGs in beer fermentation compared to yeast biomass propagation observed for both DNA microarray single- and meta-analysis were selected.

Common ALP-associated DEGs present in all DNA microarray single- and meta-analysis were sort out (Pan-DEGs) and a meta-log2FC ± standard deviation (SD) was calculated. Pan-DEGs associated with lipid droplets in yeast were also selected using the data of (Grillitsch et al., 2011). Finally, ALP-associated Pan-DEGs were used for ALP network design and analyses (Figure 1).

2.2. Network design and topology analyses

Initially, a protein-protein interactome network for *Saccharomyces cerevisiae* was designed from interactome data downloaded from STRING 11.0 (https://string-db.org) and processed in R environment (Figure 1). This yeast interactome was filtered by selecting the subscore information channels named "experiments" and "curated databases", following by the generation of a combined score from the two channels using the equation described by von Mering et al. (2005). The resulting "String_data_2019_11.txt" file used for network design and topology analysis containing the source nodes ("Feature"), the target nodes ("Feature_2") and the edge information ("combined_score) can be downloaded from the GitHub repository (https://github.com/bonattod/Lipid_stress_data_analysis.git). From this major yeast interactome network, an ALP network was obtained from the analysis and selection of the shortest pathway among the Pan-DEGs using the R package igraph (Csardi and Nepusz, 2006) and visualized in Cytoscape 3.7.2 (Shannon et al., 2003) by means of RCyc3 package (Gustavsen et al., 2019) (Figure 1). Once generated, the ALP network was evaluated considering node's centrality and the presence of communities/clusters.

For centrality analysis (Figure 1), the node degree and betweenness, both implemented in the R package igraph (Csardi and Nepusz, 2006), where calculated. Node degree indicates the number of connections that a specific node have, while betweenness indicates the number of the shortest paths that pass through each node. All nodes that have a degree value above the mean node degree value for that network were called "hubs". On the other hand, all nodes that have betweenness values above the mean betweenness value of the network were named as "bottleneck" (Yu et al., 2007). Finally, the combination of node degree and betweenness scores allows 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 those nodes that potentially have control of the flow of information through the network topology and display key regulatory functions in the cell (Yu et al., 2007).

Cluster analysis was performed in R environment using the walktrap community finding algorithm described by Pons and Latapy (2005) and fully implemented in igraph package (Csardi and Nepusz, 2006). Specific cluster were selected on 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).

2.3. Gene ontology analysis

The biological processes and cellular component associated with selected clusters from proteostasis-lipid metabolism network were obtained using the R package clusterProfile (Yu et al., 2012) (Figure 1). The degree of functional enrichment for a given biological process category was quantitatively assessed (p-value < 0.01) using a hypergeometric distribution. Multiple test correction was also assessed by applying FDR algorithm (Benjamini and Hochberg, 1995) at a significance level of $p < 0.05$. Semantic comparison among biological processes and cellular component associated to the nodes' clusters were made using R package GOSemSim (Yu et al., 2010) (Figure 1) using FDR < 0.01 and q-value < 0.05. Heatmaps combining GOs and selected

clusters from autophagy-lipid metabolism-proteostasis network were designed with R package ComplexHeatmap (Gu et al., 2016) (Figure 1), where rows and columns were grouped using *k*-means distance method.

2.4. Data sharing repository

All files and figures generated for this work can be freely downloaded from https://github.com/bonattod/Lipid_stress_data_analysis.git.

3. Results

3.1. Transcriptome single and meta-analysis of genes associated to autophagy, lipid metabolism, and proteostasis in lager yeast CB11 strain during beer fermentation

The initial comparison of DNA microarray transcriptome analyses of GSE9423 dataset (single analysis; Figure 1) and GSE10205 versus GSE16376 datasets (meta-analysis; Figure 1) indicated a similar pattern of up- and downregulated differentially expressed genes (DEGs) for lager yeast CB11 strain in condition of industrial beer fermentation compared to yeast biomass propagation (Figures 2A and B). The transcriptome single analysis showed a total of 5,134 upregulated and 4,954 downregulated DEGs (Figure 2A; Table S2), while a total of 10,258 upregulated and 9,342 downregulated DEGs was observed for transcriptome meta-analysis (Figure 2A; Table S2). It should be pointed that the high frequency of total down- and upregulated DEGs observed is due to gene redundancy found in different contrasts for both single and meta-analysis. After removing the gene data redundancy in both transcriptome single and meta-analysis, it was observed 1,315 upregulated and 1,209 downregulated non-redudant (unique) DEGs for single analysis, and 1,727 upregulated and 1,502 downregulated non-redudant DEGs for meta-analysis (Figure 2B).

Once the transcriptome single- and meta-analysis were generated (Table S2), the next step was evaluate the expression profile of genes annotated for autophagy, lipid metabolism, and proteostasis (ALP) mechanisms. For this purpose, the curated gene information for ALP

mechanisms available in the *Saccharomyces* Genome Database (Figure 1) was used as a filter to select the associated under- and overexpressed DEGs. The filtered transcriptome data showed that the total (redundant) and non-redundant frequency of overexpressed ALP DEGs were higher than underexpressed ALP DEGs in both single- and meta-analysis (Figures 2C and D). Considering overexpressed ALP DEGs, a high frequency of upregulated genes associated with lipid metabolism was observed in both transcriptome data analyses, followed by proteostasis- and autophagy-associated genes, respectively (Figures 2C and D). Interestingly, the number of redundant and unique underexpressed ALP DEGs was similar in all transcriptome datasets evaluated (Figures 2C and D). Then, this first transcriptome data evaluation was followed by a specific analysis of the absolute and average frequencies of ALP DEGs present in each pairwise beer fermentation and yeast cell propagation timepoints contrasts (Figure 3; Tables S3 and S4). It was observed that the absolute and average frequencies of down- and upregulated ALP DEGs in different beer fermentation and early cell propagation timepoints contrasts was low (0 hours for single- and meta-analysis; 4 hours for meta-analysis) (Figure 3; Tables S3 and S4). On the other hand, the absolute and average numbers of up- and downregulated ALP DEGs increased in all contrasts made between different fermentation and advanced propagation timepoints (from 8 to 30 hours of propagation) for both transcriptome single and meta-analysis (Figure 3; Tables S3 and S4). For the subsequent transcriptome and systems biology analyses, only the overexpressed non redundant (unique) ALP DEGs observed in all pairwise contrasts were considered.

3.2. Differentially expressed pan genes (Pan-DEGs) linked to autophagy, lipid metabolism, and proteostasis in lager yeast CB11 strain during beer fermentation

When both transcriptome single and meta-analyses where compared, the frequency of non redundant overexpressed ALP DEGs for each of three mechanism evaluated was similar (Figure 4A). In this sense, autophagy display the lowest frequency of overexpressed DEGs for each transcriptome analysis made (27 DEGs for single analysis and 42 for meta-analysis; Figure 4A). By

its turn, proteostasis have 76 overexpressed DEGs for single analysis and 88 for meta-analysis (Figure 4A), while lipid metabolism have the highest frequency of overexpressed DEGs (121 DEGs for single analysis and 138 for meta-analysis; Figure 4A).

The next point of this analysis was to evaluate the frequency of common ALP DEGs shared by both transcriptome data (Figure 4B). It could be observed that for lipid metabolism there is 117 non redundant DEGs commonly observed in both transcriptome analyses, while proteostasis share 71 DEGs, and autophagy display 26 common DEGs (Figure 4B and C). These overlapping ALP DEGs were selected as ALP-associated common (Pan)-DEGs (Table S6) and their expression patterns was evaluated.

Thus, considering the expression pattern of ALP Pan-DEGs (Figure 4D and Table S6), it was observed that the median value of meta-log_2FC was similar for autophagy (meta-log_2FC = 2.39), lipid metabolism (meta-log_2FC = 2.57), and proteostasis (meta-log_2FC = 2.68). Additionally, the minimal and maximal values of meta-log_2FC among ALP Pan-DEGs groups was also similar (Figure 4D), where autophagy-associated Pan-DEGs display minimal and maximal values of meta-log_2FC from 2.13 to 4.39, while proteostasis Pan-DEGs display a meta-log_2FC from 2.09 to 4.45, and lipid metabolism Pan-DEGs from 2.04 to 4.17 (Figure 4D).

The similar expression pattern of ALP Pan-DEGs prompt to evaluate how these elements are potentially connected each other in terms of protein-protein interactions (PPI) by means of a top-down systems biology approach using *S. cerevisiae* interactome data (Figure 1). Moreover, the importance of these ALP Pan-DEGs for PPI network local and global topologies was also measured, as well as the overrepresented biological processes and cellular components within the PPI network.

3.3. Top-down systems biology analysis of autophagy, proteostasis, and lipid metabolism-associated pan-DEGs

The resulting ALP Pan-DEGs obtained from transcriptome analyses were selected as seeds to generate a shortest pathway PPI network using the publically available *S. cerevisiae* interactome

data (Figure 1). In this sense, the yeast interactome data was previously filtered using only databases and experiments as evidences of interaction among proteins (as described previously in this work), and then allowing to get an interactome network composed by 5,063 nodes and 185,404 edges (please refer to the Supplementary Files "graph_string_db_exp.txt" and "Cytoscape_data.cys" available at https://github.com/bonattod/Lipid_stress_data_analysis for more details). Then, a subnetwork containing all the shortest pathways among the proteins coded by ALP DEGs was generated from the yeast interactome network (Figure 1). This subnetwork named as "ALP network" containing 1,705 nodes and 22,806 edges (Figure 5A) and included almost all ALP Pan-DEGs with exception of the genes *FAT3, IZH2, IZH4, MZM1, OPI10, PPX1,* and *TMA17* that could be not be mapped using the currently available interactome data from *S. cerevisiae.*

Following the generation of ALP network, a node centrality analysis was performed in order to identify all those nodes that exert a local influence in the network's topology and, consequently, have relevant roles in the ethanol stress tolerance of lager yeast cells during beer fermentation. For this purpose, two centralities commonly used for PPI networks were selected, the node degree and betweenness. The node degree evaluate the potential of a protein to connected with different other proteins and thus composing functional complexes (Yu et al., 2007). In this sense, all proteins with values of node degree above the mean value of node degree of the network were defined as hubs (Yu et al., 2007). By its turn, the betweenness allow to evaluate the ability of a node to connect different cluster/communities of nodes, thus serving as a "bottleneck" for where the biological information can traverse from a community to another (Yu et al., 2007). By combining both node degree and betweenness centralities analyses, it is possible to select nodes that display a high value of these two parameters, defining then as the hub-bottleneck (HB) nodes (Figure 5B). The HB nodes are critical elements within a network since they concentrate the highest number of shortest pathways and connections with other nodes, being important components for signal transduction among protein clusters/communities (Yu et al., 2007). Thus, the centrality analysis of ALP network

indicated the presence of 423 HB nodes, 221 hub-nonbottleneck (HNB) nodes, 59 nonhub-bottlenenck (NHB) nodes, and 1,002 nonhub-nonbottleneck (NHNB) nodes (Figure 5B and Supplementary File "topologies_lipid_stress_data.txt" available at https://github.com/bonattod/Lipid_stress_data_analysis). Once the central nodes in ALP network were defined, it is necessary to know if they are organized in communities within the network. In a general sense, a graph community can be defined as a specific network topology that contain nodes highly connected betweenn then but have low degree values with other nodes outside the community. Moreover, node communities can be potentially associated with specific biological processes (Pons and Latapy, 2005; Ravasz et al., 2002). In order to identify the nodes communities in the ALP network, the walktrap community (WTC) algorithm was applied (Pons and Latapy, 2005), which allows to efficiently find nodes communities by using the random walks technique. By using the WTC algorithm, it were identified 36 communities associated to ALP network (see Supplementary File "topologies_lipid_stress_data.txt" available at https://github.com/bonattod/Lipid_stress_data_analysis). For further analysis, it was necessary to select the major ALP network-associated communities by considering the presence of HB nodes and ALP Pan-DEGs within these communities. Thus, 13 communities were selected (Figure 5C and D; Table S7), being Cluster 4 the largest community with 521 nodes and 4,424 edges and Cluster 31 the smallest community with 16 nodes and 69 edges (see Supplementary File "Cytoscape_data.cys" available at https://github.com/bonattod/Lipid_stress_data_analysis). Additionally, Cluster 4 also contains the largest number of ALP Pan-DEGs, while Cluster 24 contains only one ALP Pan-DEG (Figure 6 and Table S7).

Following the community detection, a gene ontology (GO) analysis was applied for each one of the selected 13 clusters in order to identify the major overrepresented biological processes (Figure 7 and Table S8) and the cellular components categories (Figure 8 and Table S9). The GO analysis of clusters-associated overrepresented biological processes indicated that three groups of

clusters and seven groups of biological processes could be categorized by applying *k*-means distance (Figure 7). In this sense, the cluster group 1 (composed by clusters 3, 23, and 24) contain major biological processes associated to mitochondria structure and organization, while cluster group 2 (composed by clusters 2, 9, and 31) is associated to lipid, ergosterol, and alcohol metabolism (Figure 7). Both clusters groups 1 and 2 also contains proteins involved in oxidation-reduction process (Figure 7). On the other hand, the cluster group 3 (composed by clusters 1, 4, 5, 6, 12, 10, and 14) contains different processes linked to autophagy and autophagosome assembly, response to ER stress and protein folding, piecemeal microautophagy of the nucleus and mitochondria autophagy, vesicle-mediated transport, among different other biological processes (Figure 7). These data lead to the evaluation of the major cellular components associates with these cluster. Again, three clusters groups were observed (Figure 8). Cluster group 1 (clusters 1, 4, and 31) mainly contain nodes associated with ER membrane and lipid droplet, where cluster group 2 (clusters 2, 3, 5, 9, and 23) is constituted by proteins found in mitochondria envelope and matrix, Golgi apparatus, membrane protein complexes and organelles like peroxisome (Figure 8). Finally, cluster group 3 (clusters 8 and 12) is made by proteins that are mainly found in cytoplasmic vesicles and phagophore assembly site (Figure 8).

3.4. Evaluation of ALP Pan-DEGs linked to lipid droplet structure and function

Using the data available about the different proteins found associated to LD (Grillitsch et al., 2011) and the ALP Pan-DEGs obtained in this work, it was possible to identify 17 overexpressed ALP Pan-DEGs linked to LDs structure in lager yeast cells during beer fermentation in comparison to propagated yeast cells (Figure 9A and B; Table S11). From these 17 LD-associated ALP Pan-DEGs, 11 genes are directly involved with lipid metabolism and 6 genes are related to proteostasis mechanisms (Table S11).

4. Discussion

The transcriptome and the systems biology data obtained in this work suggest that, during lager beer fermentation, different genes coding for autophagy, proteostasis, and lipid metabolism are positively modulated and their products cooperate in a shortest pathway PPI network, composing different clusters/communities of proteins (Figure 5A and D). The GO analysis of these communities showed that they are associated to a large number of processes related to autophagy, mitochondria organization and activity, ER stress and Golgi organization, lipid/ergosterol metabolism, cytoplasmic vesicles, lipid droplets (LDs), and phagophore assembly site (Figures 7 and 8). All these processes are known to be modulated by the stressful conditions that yeast cells suffer 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 is well characterized in yeast strains during wine fermentation (Piggott et al., 2011), the autophagy studies in lager yeasts are virtually absent. Interestingly, malt-derived wort used for beer fermentation contains different types of nitrogen sources that could prevent the activation of autophagy (Gibson et al., 2007); however, it was demonstrated that during wine fermentations many autophagy-associated genes are upregulated even in the presence of nitrogen sources (Piggott et al., 2011). In this work, the transcriptome data allow to observe the expression of different *ATG* genes during lager beer fermentation (Table S10), many of these genes involved in the regulation of macroautophagy and formation of phagosome, but also related to microautophagy of organelles like mitochondria and nucleus (Table S10). From all autophagy-associated genes observed upregulated in beer fermentation, three *ATG* genes (*ATG1, ATG8,* and *ATG18*) were characterized as a major HB node in the ALP network (Table S10). *ATG1, ATG8* and *ATG18* are part of the so called "autophagy core machinery", which are important for both micro- and macroautophagy (Lynch-Day and Klionsky, 2010). In this sense, the transcriptome and systems biology data points that during beer fermentation the cytoplasm-to-vacuole targeting (Cvt) mechanism, mitophagy, and the

piecemeal microautophagy of the nucleus (PMN) are potentially activated (Figure 7), all of three depending on the activity of Atg1p, Atg8p, and Atg18p (Kanki et al., 2015; Krick et al., 2008; Lynch-Day and Klionsky, 2010). While the importance of Cvt, mitophagy, and PMN to brewing yeasts strains are unknown in the context of beer fermentation, it has been reported that these all three microautophagy processes are important for yeast cell adaptation in a fermentative environment (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 different cargo components for degradation in the vacuole (Lamb et al., 2013). The formation of autophagosomes strongly depends on the lipid biosynthesis that includes triacylglycerols (TGs) and sterol esters (SEs) originated from ER membranes in the form of LDs (Velázquez et al., 2016a). In fact, it was demonstrated that low levels of nitrogen sources and the presence of glucose stimulate lipidogenesis in yeast and increases the number of LDs that, by its turn, are required for efficient autophagy (Li et al., 2015).

The transcriptome and systems biology data showed the upregulation of several genes linked to neutral lipid biosynthesis as well as ergosterol in lager yeast cels during beer fermentation (Table S10). Moreover, the GO analysis of cellular compartments linked to clusters 1, 4, and 31 indicate the participation of the proteins coded by that genes in ER membrane structure and its association with nuclear outer membrane and LDs (Figures 7 and 8).

Besides the importance of lipid biosynthesis for autophagy, it is known that lipids could be key molecules regulating proteostasis in addition to the well known modulation of cell membrane permeability to ethanol (Aguilera et al., 2006; Chi and Arneborg, 1999; Ma and Liu, 2010). In fact, lipid metabolism and LDs are important components of proteostasis, since it has been showed that yeast cells defective in the biosynthesis of neutral lipids and LDs display a chronic ER stress (Graef, 2018; Velázquez et al., 2016a). Additionally to proteostasis, LDs are essential components

to macroautophagy (Velázquez et al., 2016b; Velázquez and Graef, 2016), having a role on the assemble of autophagosomes and also in the induction of mitophagy (Carmona-Gutierrez et al., 2012) and microlipophagy (Vevea et al., 2015). Noteworthy, six upregulated proteostasis genes described in this work that are linked to the inter-organellar proteostasis mechanism (*CPR5, KAR2, PDI1*, *PMT1*, *RPL5,* and *SSA1*) (Telini et al., 2020) are also found in LDs structure (Figure 9B and Table S10). Interestingly, ER stress can induce the formation of LDs (Fei et al., 2009) and it has been reported that LDs interact with mitochondria and peroxisome by means of Erg6p (Pu et al., 2011), a protein codified by *ERG6* that was found upregulated in this work (Figure 9B and Table S10)

Finally, despite the importance of LDs and lipid biosynthesis for different mechanisms and organelles (Barbosa et al., 2015), little is unknown how they regulate cell viability and ethanol tolerance in different brewing yeast strains in conditions of HG/VHG beer production. Considering LDs and its central role in inter-organellar communication as well as by promoting the activity of autophagy and proteostasis, it become essential to understand the regulation and function of this organelle in a beer fermentation environment. Moreover, how LDs are regulated and produced in a context of a hybrid species, like the lager yeast *Saccharomyces pastorianus* (Gorter de Vries et al., 2019), in comparison to its parental counterparts (*Saccharomyces eubayanus* and *Saccharomyces cerevisiae*) is an essential question in order to design new resistant lager strains to high ethanol concentrations during HG/VHG beer production.

Compliance with ethical standards

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Figure legends

Figure 1. Experimental design used in DNA microarray single and meta-analysis and systems biology. Abbreviation: differentially expressed genes (DEGs); Saccharomyces Genome Database (SGD); gene ontologies (GO).

Figure 2. Frequency of total (A) and non-redundant or unique (B) differentially expressed genes (DEGs) in transcriptome single- (GSE9423) and meta-analysis (GSE10205 versus GSE16376). Specific DEGs associated to autophagy, proteostasis, and lipid metabolism (ALP) were filtered from transcriptome single- (GSE9423) and meta-analysis (GSE10205 versus GSE16376) and the frequency of total (C) and non-redundant or unique (D) ALP DEGs were determined. The numbers inside the squares shown the total of underexpressed and overexpressed DEGs observed in a specific contrast.

Figure 3. Frequency of total under- and overexpressed genes associated to autophagy, proteostasis, and lipid metabolism in different contrasts from transcriptome single- (GSE9423) and meta-analysis (GSE10205 versus GSE16376).

Figure 4. Frequency of non-redundant or unique overexpressed genes associated to autophagy, proteostasis, and lipid metabolism (ALP DEGs) in transcriptome single- (GSE9423) and meta-analysis (GSE10205 versus GSE16376) (A). The numbers inside the squares shown the frequency of overexpressed DEGs observed in a specific mechanism. The frequency of overlapping ALP DEGs for both transcriptome analyses is indicated in the Venn diagram (B) and barplot (C). The overlapping ALP DEGs were denominated as ALP Pan-DEGs and its expression values in $\log_2$ fold are shown in (D).

Figure 5. Shortest pathway protein-protein interaction (PPI) network obtained from ALP Pan-DEGs (ALP network; A). From this ALP network, a centrality analysis was applied and the nodes were classified in hub-bottleneck (HB), hub-nonbottleneck (HNB), nonhub-bottleneck (NHB), and nonhub-nonbottleneck (NHNB) considering its nodes degree and betweenness values in

comparison to the mean values of node degree and betweenness of the network (dashed lines; B). Additionally, communities/clusters of proteins were determined within the ALP network and selected considering the presence of ALP Pan-DEGs (C) and HB nodes (D). In (C), the diameter of dots is proportional to the number of ALP Pan-DEGs found in the community/cluster. In (D), HB nodes are represented by square elements.

Figure 6. Expression data for ALP Pan-DEGs found in the selected communities/clusters derived from the ALP network. The mean expression values are indicated by log2 fold change ± standard deviation (SD) on the x-axis. Gene names are indicated on the y-axis.

Figure 7. Heatmap plot showing the clustered biological processes obtained from gene ontology analysis of ALP network-associated communities/clusters. Heatmap rows and columns were grouped using the *k*-means distance method. Horizontal and vertical dotted lines indicate the cut-off point used to define the numbered rows and column groups.

Figure 8. Heatmap plot showing the clustered cellular components obtained from gene ontology analysis of ALP network-associated communities/clusters. Heatmap rows and columns were grouped using the *k*-means distance method. Horizontal and vertical dotted lines indicate the cut-off point used to define the numbered rows and column groups.

Figure 9. Venn diagram showing the intersection of ALP Pan-DEGs and lipid droplet associated proteins (A). In (B), mean expression values of ALP Pan-DEGs whose products are also found in lipid droplets. The expression values are indicated by log2 fold change ± standard deviation (SD) on the y-axis and the gene names are indicated on the x-axis.

Figure 1.

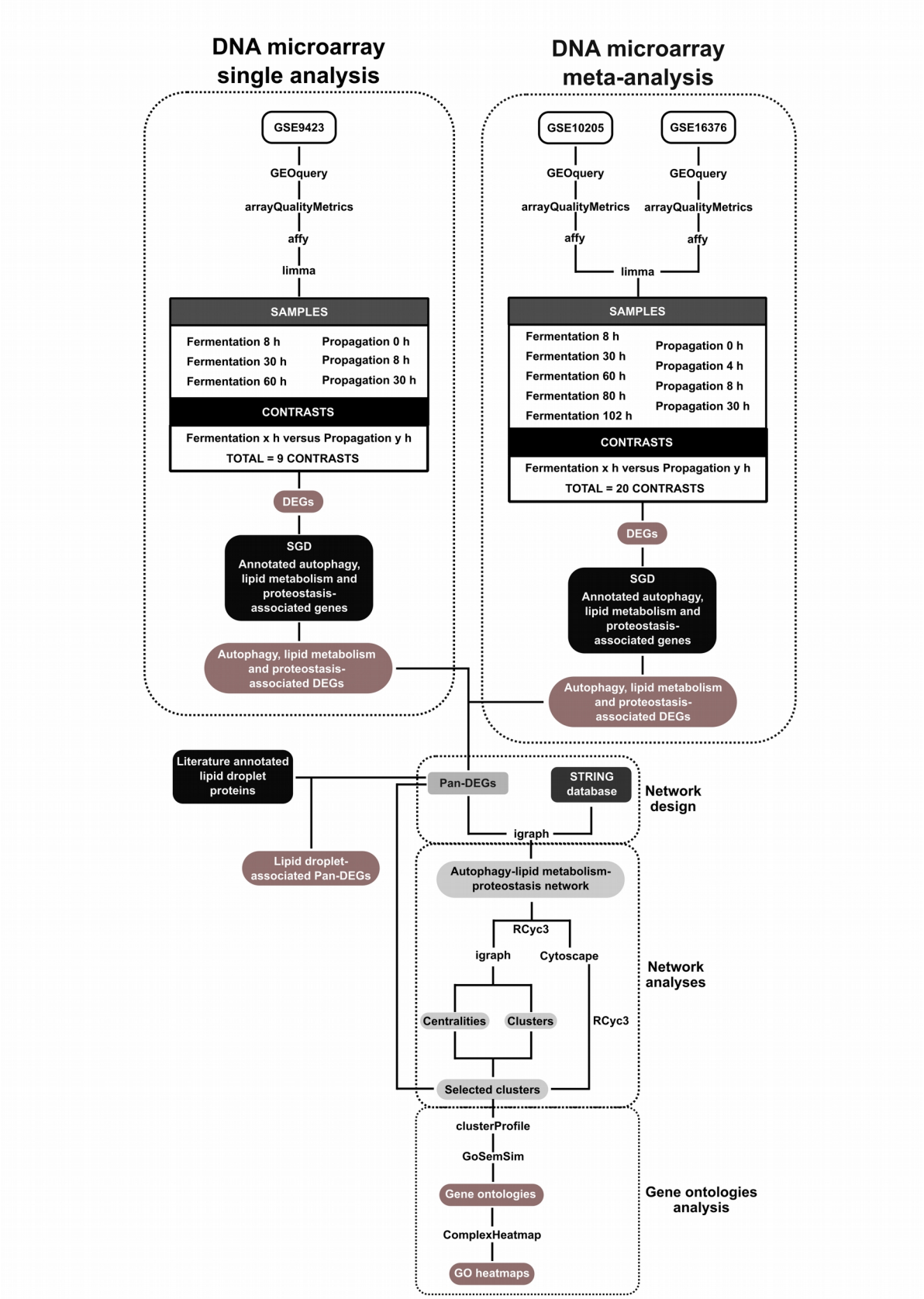
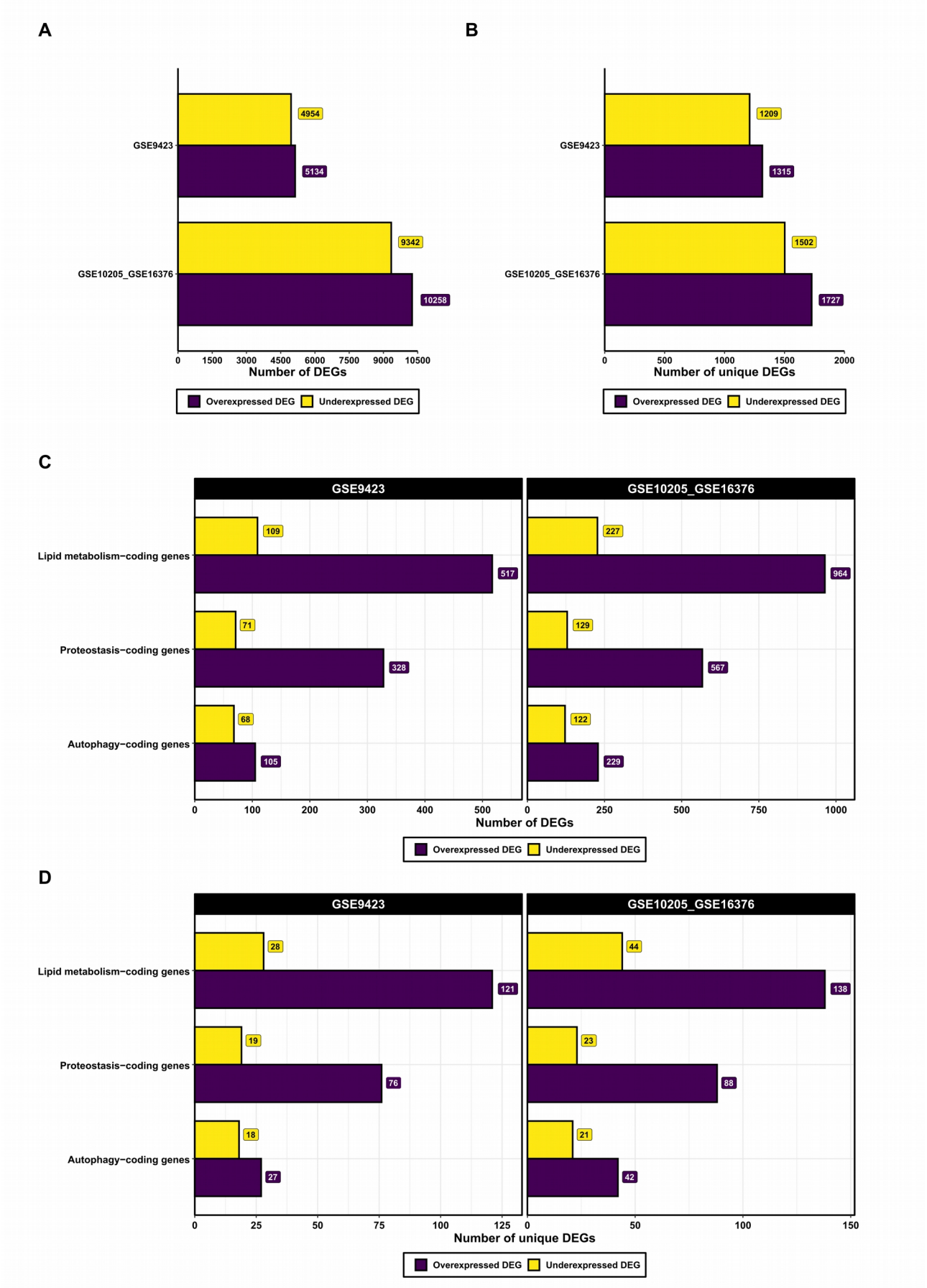


Figure 2.



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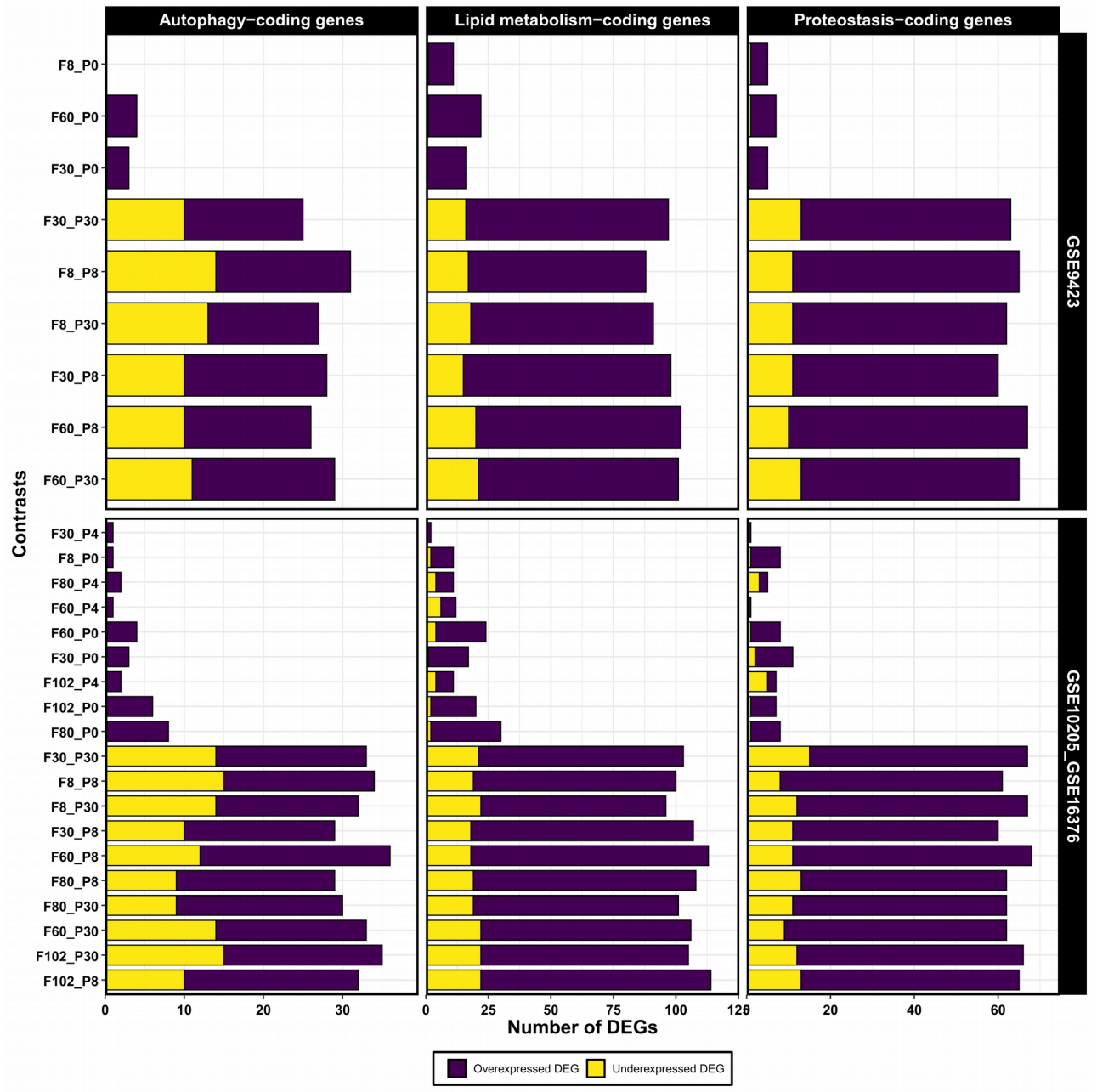

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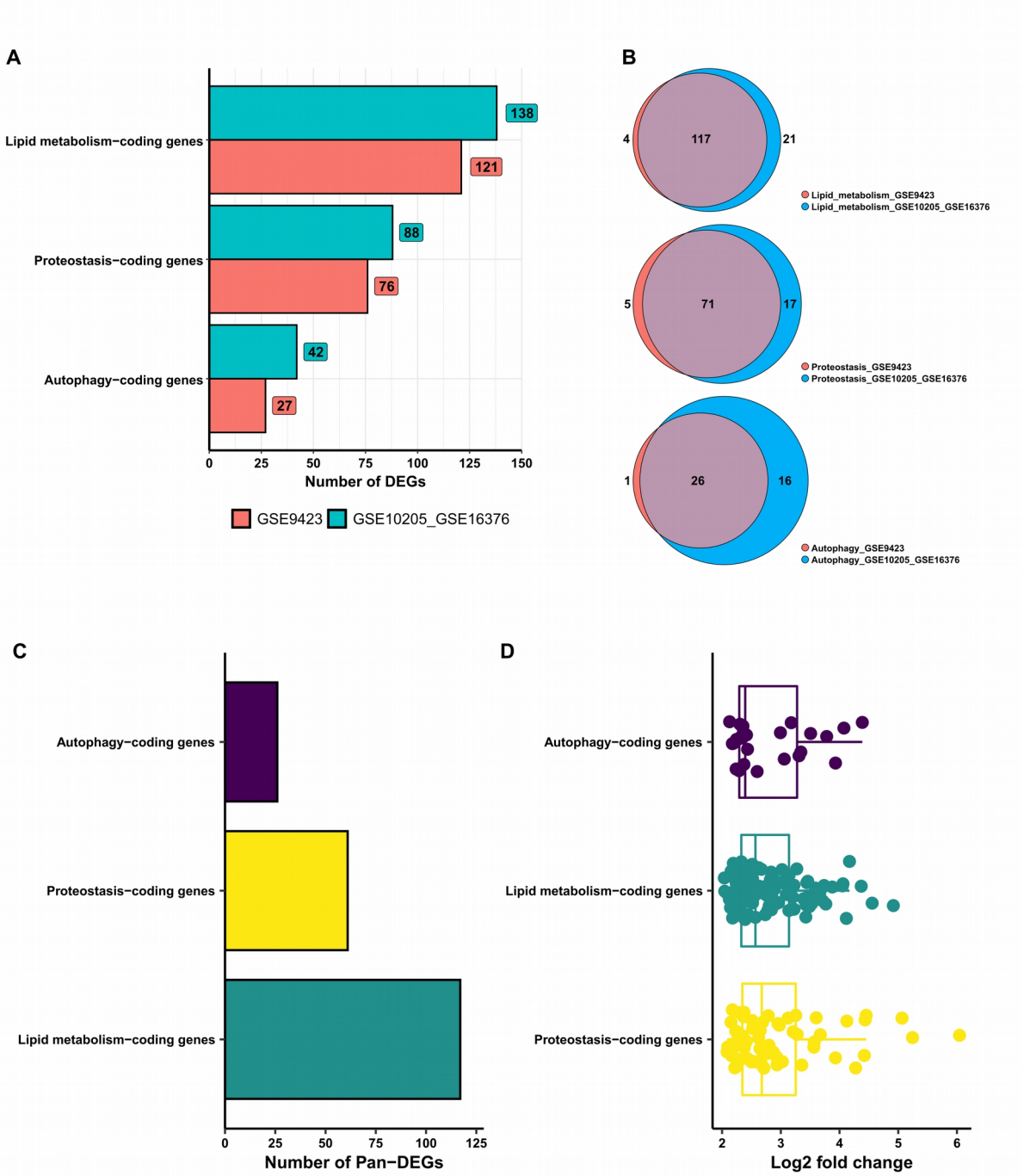

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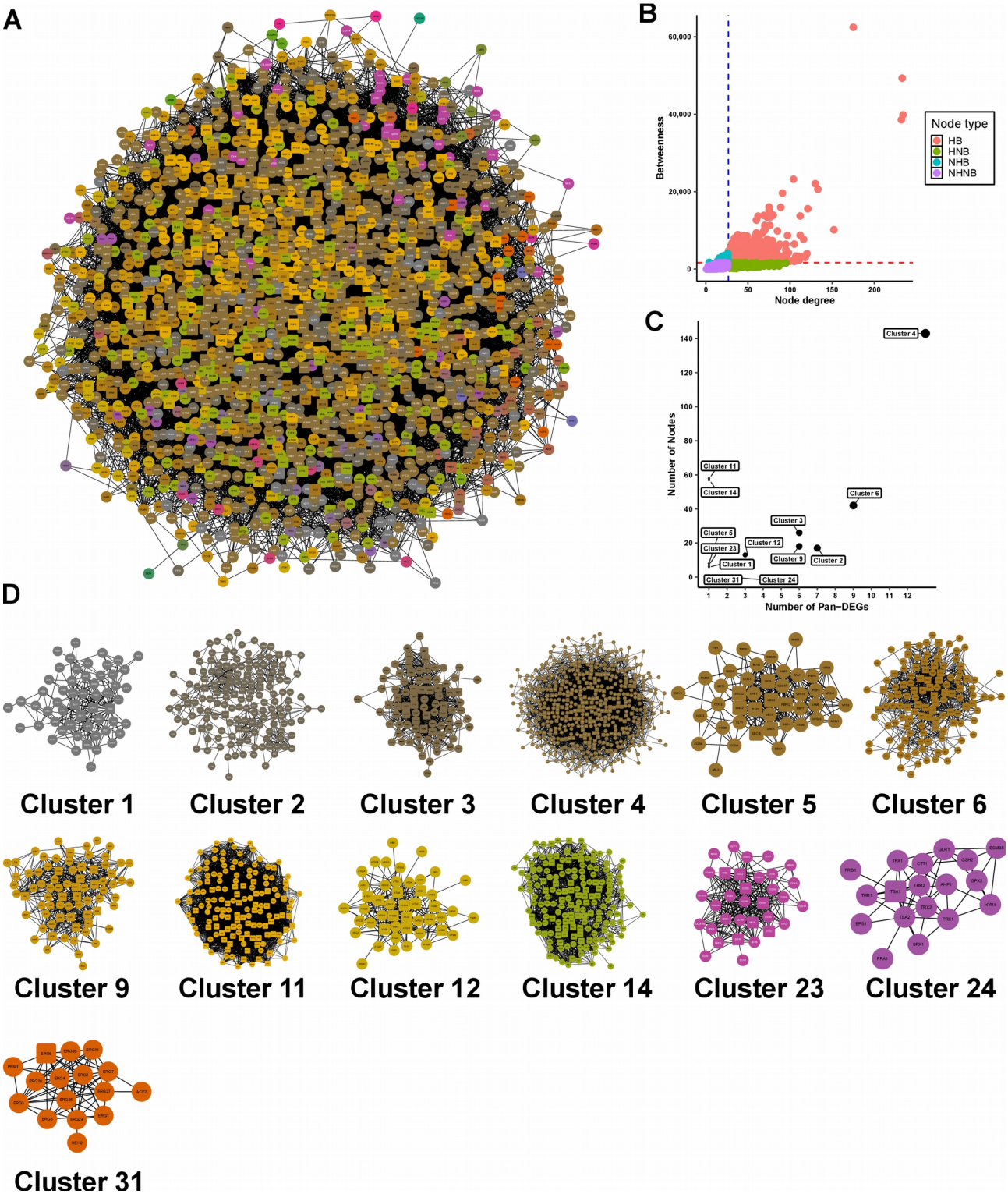

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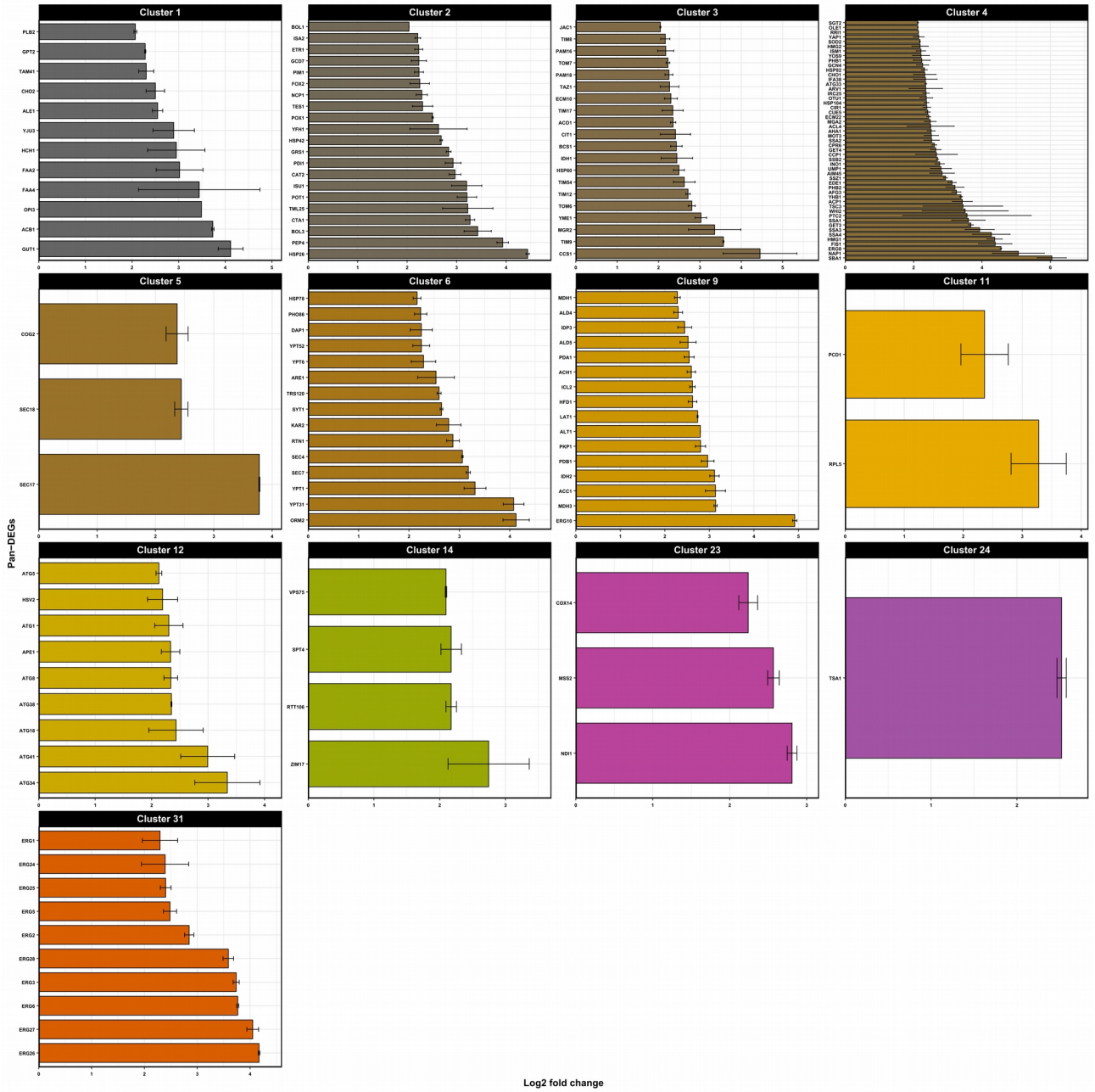

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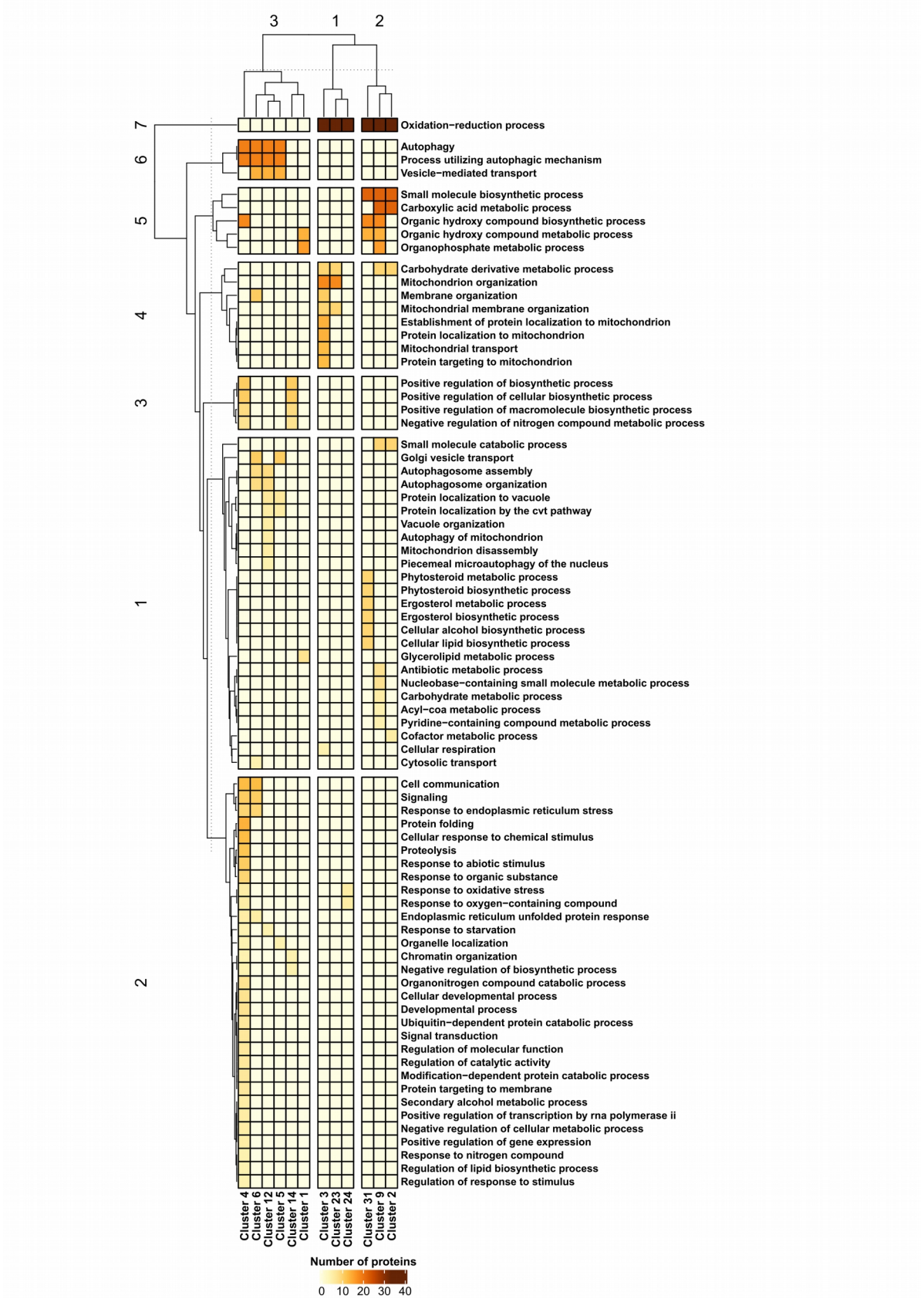

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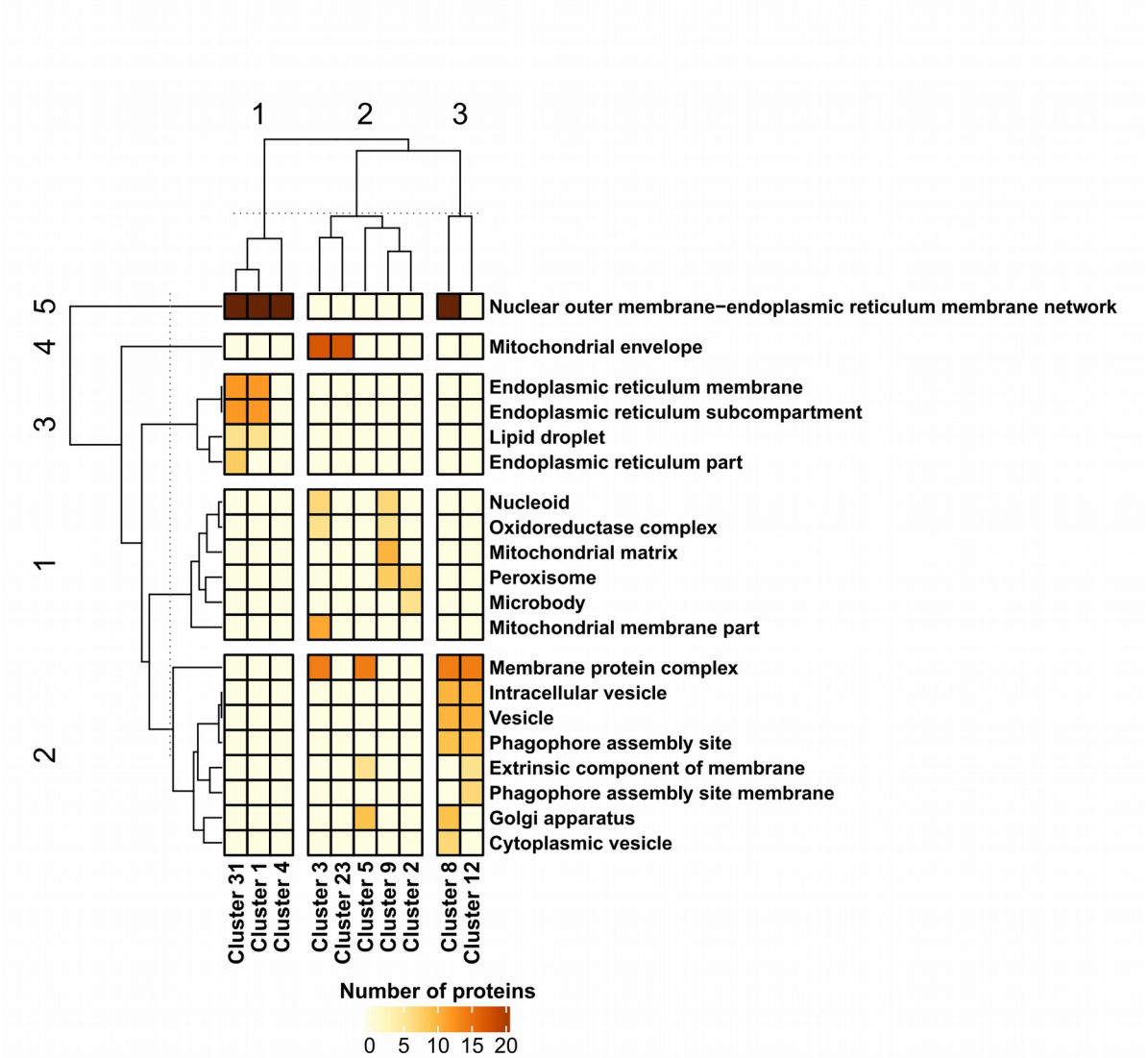

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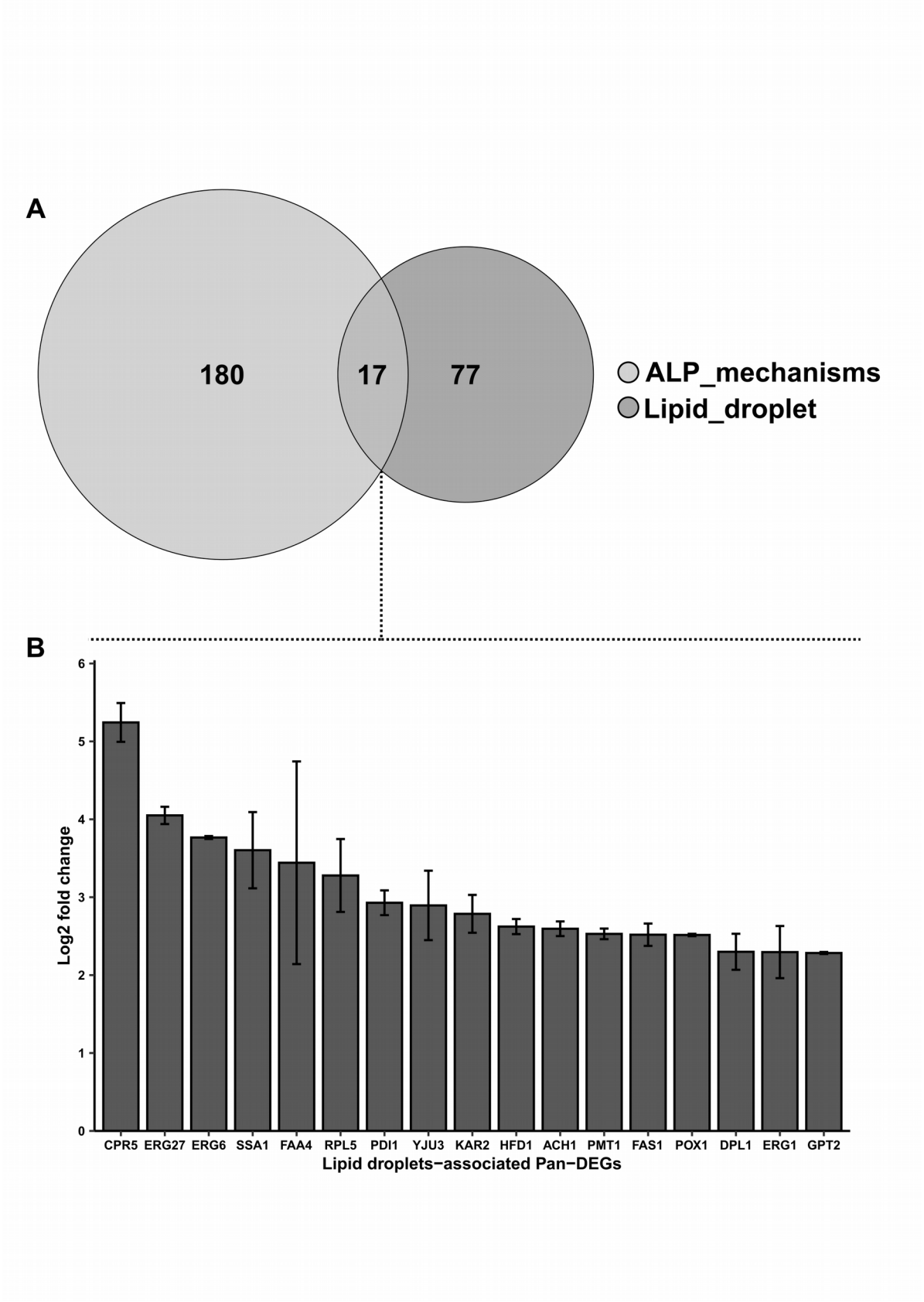

Highlights for review

1. Transcriptome data of lager yeast show the importance of lipids in beer fermentation.

2. Systems biology show the interplay of lipid metabolism, autophagy, and proteostasis.

3. Lipid droplets coordinate autophagy and proteostasis during beer fermentation.