How do engineered *Yarrowia lipolytica* strains secrete free fatty acids: hints from comparative transcriptomics

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Abstract

Yarrowia lipolytica has been considered one of the most promising platforms for the microbial production of fatty acids and derived products. The deletion of the *faa1* gene coding for an acyl-CoA synthetase leads to the accumulation and secretion of free fatty acids (FFAs) into the extracellular space. The secretion of products is beneficial for the development of microbial cell factories to avoid intracellular inhibitory effects and reduce downstream processing costs. However, the mechanism behind the secretion of fatty acids is not well known. As a starting point, we compared the transcriptome of this mutant showing FFA secretion to a wildtype-like strain not showing this phenotype. The 12 most upregulated genes were evaluated for involvement in FFA secretion by the creation of deletion and overexpression mutants, among them *MCH2, YMOH*, three cell wall proteins *CWP3*, *CWP4* and *CWP11*, *M12B*, and three proteins with unknown functions *YUP1*, *YUP2,* and *YUP3*. None of these proteins take a clear or isolated role in FFA export. As the transcriptomic data revealed an overrepresentation of cell wall-related proteins, some of them were further examined on a theoretical and experimental way. Surprisingly, overexpression of Ygpi led to the production of FFAs in the wildtype-like genetic background. Finally, some of the evaluated genes showed involvement in resistance to FFA toxicity.

Introduction

Microorganisms are a promising source of sustainable oleochemicals, and many efforts are dedicated to finding algae, fungi and yeasts accumulating lipids, as well as engineering them to improve and diversify their lipid production. Among the different lipids that cells can produce, free fatty acids (FFAs) and their derivatives offer a wide range of applications in different fields. FFAs can be used to produce detergents or texturizing products used in the cosmetic industry (Vasconcelos *et al.* 2019). Polyunsaturated fatty acids (PUFAs) have applications in the nutraceutical sector and the feeding sector for fisheries. Special fatty acids, such as ricinoleic acid, can also be used for the synthesis of aromas (Waché *et al.* 2001, 2003; Aguedo *et al.* 2005; Ledesma-Amaro and Nicaud 2016a). Finally, many FFA derivatives, such as fatty alcohols, methyl esters or alkanes, are common components of biodiesel (Lennen and Pfleger 2013). Among the different microorganisms that can be used as production hosts, oleaginous yeasts offer some advantages compared to the other alternatives. Oleaginous yeasts can produce lipids at more than 20% of their cell dry weight (CDW), and they can reach high cell densities resulting in increased productivity compared to bacteria and microalgae (Sitepu *et al.* 2014).

Yarrowia lipolytica W29 is the best studied oleaginous yeast due to its genetic accessibility, which has allowed not only to study the function of individual genes, but also to obtain engineered strains with increased lipid productivity or strains able to produce different lipids such as PUFAs or β-carotenes (Larroude *et al.* 2018; Gemperlein *et al.* 2019; Abeln and Chuck 2021). Indeed, *Yarrowia lipolytica* was the first yeast to be genetically engineered for industrial oil production (Xie et al., 2015).

Y. lipolytica strains are often isolated from lipid-rich environments (Barth and Gaillardin 1996; Fickers *et al.* 2005; Madzak 2021) and as a result, they are able to secrete large amounts of lipases. While *Y. lipolytica* can grow on a wide range of hydrophobic substrates as well as glycerol (Ledesma-Amaro and Nicaud 2016b), the diversity of sugars in which it can grow is limited (glucose, fructose and mannose). Since *Y. lipolytica* cannot grow on sugar polymers, as well as xylose and other five-carbon sugars, its application for the revalorization of lignocellulosic waste is limited (Niehus *et al.* 2018). When growing exclusively on soluble substrates, such as glycerol or glucose, this yeast only produces lipids during a transient accumulation phase, after which it starts degrading them to obtain energy (Makri, Fakas and Aggelis 2010). Nitrogen limitation is one of the most common ways to stimulate lipid accumulation, and under these conditions, wildtype strains of *Y. lipolytica* have been observed to intracellularly accumulate up to 45% CDW when growing on glucose as a carbon source (Carsanba *et al.* 2020), and up to 70% when growing on oil as carbon source (Bati, Hammond and Glatz 1984). Further engineering resulted in 80-90% CDW of intracellular lipid accumulation (Blazeck *et al.* 2014; Friedlander *et al.* 2016).

Under normal conditions, microorganisms do not accumulate FFAs due to their cytotoxic effects, and therefore they are found esterified to other molecules such as glycerol or coenzyme A (Desbois and Smith 2010). Nevertheless, different genetic modifications can lead to an accumulation of FFAs. In *Saccharomyces cerevisiae*, the deletion of two acyl-CoA synthetases (*ScFAA1* and *ScFAA4*) leads to FFA buildup and subsequent export to the medium to reach up to 220 µM extracellular FFAs (eFFAs) (Scharnewski *et al.* 2008). *S. cerevisiae* possesses five acyl-CoA synthetases (*ScFAA1-4* and *ScFAT1*), which seem to be involved in different processes and present distinct fatty acid specificities. The acyl-CoA synthetase activities have been linked to fatty acid uptake, as well as the mobilization of

intracellular lipid storages (Black and DiRusso 2007). In contrast to *S. cerevisiae, Y. lipolytica* has only one homologue to the soluble acyl-CoA synthetases, *FAA1* (YALI0_D17864g). Deleting the gene affects the utilization of eFFAs to produce neutral lipids and membrane lipids, but not their uptake or catabolism (Tenagy *et al.* 2015), as well as leading to an increase in the intracellular levels of saturated fatty acids (Wang, Zhang and Chen 2011). Further studies showed the involvement of this enzyme in the incorporation of FFAs into lipid particles (Dulermo, Gamboa-meléndez and Ledesma-amaro 2015).

When grown in a medium with a high carbon-to-nitrogen (C/N) ratio (lipogenic conditions) the deletion of *FAA1* not only leads to intracellular accumulation of FFAs, but also to the export of these lipids to the extracellular space (Ledesma-Amaro *et al.* 2016). The amount of eFFAs can be improved by deleting the *MFE2* gene coding for a hydratase-dehydrogenase-epimerase involved in β-oxidation, which decreases the consumption of both acyl-CoA and FFAs. To transform the acyl-CoA molecules into FFAs, the gene *DGA2* encoding for a diacylglycerol acyltransferase was overexpressed to increase the deposit of fatty acid-moieties as triacylglycerides in lipid bodies *(Δfaa1 Δmfe1 DGA2)*. This, in combination with the overexpression of the genes *TGL4* and *klTGL3* encoding for lipases to liberate FFAs from the lipid bodies, led to eFAA levels as high as 4.3 g/L, and total lipid production of 92% of the CDW (Ledesma-Amaro *et al.* 2016). These high values indicate the advantages of exporting the compound of interest outside the cell factories, allowing reduced inhibitory and toxic effects associated to high intracellular concentrations of the compound of interest (Kell *et al.* 2015). Furthermore, secreted lipids are also desirable because they decrease the downstream costs by skipping cell disruption steps, which can be further used for other processes or to produce biomass (Jezierska and van Bogaert 2017; Borodina 2019). These advantages show the benefits of identifying proteins influencing export of the product of interest, whose overexpression or engineering could lead to improvements in the bioproduction process. However, while the import of exogenous fatty acids in yeast has been more studied and it is most likely happening through a process known as vectorial acylation (Black and Dirusso 2003), the mechanism behind fatty acid export in yeast is not clear yet. Although traditionally it was thought that lipids may diffuse passively through membranes, now it is accepted that these processes are controlled by specific proteins in cells (Claus, Jezierska and van Bogaert 2019). For example, in *S. cerevisiae,* Pry proteins have an impact on fatty acid export (Darwiche *et al.* 2017). These are extracellular fatty acid-binding proteins from the CAP superfamily that have also been associated to sterol export (Darwiche *et al.* 2018).

The goal of the presented research is to address the knowledge gap in fatty acid export mechanisms in yeast, in order to identify potential strategies to improve cell factories. As a starting point, we used a comparative transcriptomics approach to study the response of *Y. lipolytica* to the FFA accumulation caused by the *FAA1* deletion. We expected to identify genes directly or indirectly involved in FFA export or other genes involved in the prevention of lipotoxicity.

Methodology

Strains and media

All cloning procedures were performed in *Escherichia coli* Top10 cells, grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). When necessary, LB medium was supplemented with 100 mg/L ampicillin. *E. coli* cells were cultivated at 37°C and a rotatory shaker at 200 rpm was used for liquid media. Selective plates were prepared by addition of 2% agar to LB medium.

The W29 auxotrophic derivative strain JMY195 (ura-, leu-), its Ura+ derivative JMY330 (leu-) and the prototrophe derivative JMY2900 (called WT in the rest of the article due to its wildtype-like phenotype) were obtained from Dr. Jean-Marc Nicaud (INRAE, Micalis, BIMLIp group). The *Y. lipolytica* Δ*faa1* strain (JMY3234) used in the transcriptomic analysis was described in Dulermo et al. 2015. All mutants generated in this work are summarized in Supplementary Table 1. All strains were maintained in YPD medium (1% yeast extract, 2% peptone, 2% glucose monohydrate). YPD medium was supplemented with 200 mg/L hygromycin (Corning) for selection, when necessary. Transformants containing an auxotrophic marker were selected on SD medium lacking the necessary nutrient or nutrients (2% glucose monohydrate, 0.67% yeast nitrogen base (YNB) without amino acids (DIFCO) and 0.077% of CSM-Ura, CSM-Leu or CSM-Ura-Leu (MP Biomedicals Europe). For fatty acid production during shake-flask experiments, 3C medium was used (10% glucose monohydrate, 1% yeast extract, 0.1% urea). This medium contains a higher concentration of glucose than usual to achieve a high C/N ratio, which stimulates lipid production (Beopoulos, Nicaud and Gaillardin 2011). *Y. lipolytica* strains were incubated at 28°C and on a rotatory shaker at 200 rpm for liquid media. Plates were prepared by adding 2% agar to the respective media.

Strain engineering

For gene deletion, *Y. lipolytica* strains were modified through homologous recombination using transformation cassettes flanked by 1000 bp homologous regions to the target sequence (Supplementary Figure 1A). For overexpression experiments, *Y. lipolytica* strains were modified through random integration (Supplementary Figure 1B). The different fragments, including the marker genes, homology regions, coding sequences, and the backbone, were amplified with PrimeSTAR HS polymerase (TakaraBio) and assembled with Circular Polymerase Extension Cloning (CPEC, (Quan and Tian 2009)). The assembly mix was transformed into *E. coli* Top10 cells through a standard electroporation protocol (Warren 2011). Correct assembly was verified through colony PCR and sequencing.

The linear cassette to be transformed into *Y. lipolytica* was derived from the cloned plasmid either by PCR amplification or by restriction enzyme digest. The obtained linear DNA was transformed into *Y. lipolytica* through a protocol based on the lithium-acetate (LiAc) method. (Barth & Gaillardin, 1996) and adapted from (Leplat, Nicaud and Rossignol 2015). To verify correct genomic integration of the disruption cassette, transformants were verified by colony PCR. All primers used in this study can be found in Supplementary Table 2, while the genetic sequences of the target genes can be found in Supplementary Table 3.

RNA-sequencing

RNA extraction

The WT and Δ*faa1 Y. lipolytica* strains were inoculated overnight in 5 ml of 3C media. The grown cultures were reinoculated in 5 ml of 3C in quintuplicates with a starting OD_{600} of 0.1. After 48 hours, when fatty acids are already present in the extracellular medium, cells were harvested by centrifugation and lysed using liquid nitrogen. Then, RNA was extracted using the RNeasy Midi kit (Qiagen) following the recommended protocol. The RNA samples were measured with a NanoDrop and their quality was checked on an agarose gel electrophoresis.

RNA-Sequencing Analysis

The extracted RNA was sent to NXTGNT (Ghent University, Faculty of Pharmaceutical Sciences) for sequencing. The sequencing library was constructed using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina from Lexogen and was sequenced as SE50 on an Illumina Hiseq. Raw sequencing reads were inspected using FastQC (v0.11.8) for their quality and length. A summary report was generated using MultiQC (v1.8). Adaptor trimming was done using cutadapt (v1.18) with added filtering of reads containing ambiguities, bases not passing the phred score threshold of 20, or reads shorter than 15 after trimming. Putative contaminations were checked using fastq_screen (v0.12.0). The quality of the remaining read pairs was checked using FastQC. *Yarrowia_lipolytica* GCA_000002525.1.45 (from strain CLIB122) was used as the reference genome for the differential gene expression analysis. For all samples, the reads were mapped using the splice-aware STAR (v2.6.0c) mapper. Features were counted at the gene and transcript isoform level using rsem_calc_expression (RSEM v1.3.1). All further statistics were done using counts at the gene level. Differential expression was analyzed in R with the edgeR package comparing *Δfaa1* versus WT. The quasi-likelihood model was used for a more reliable error rate. The model considers statistically significant genes as those with a logarithmic fold-change with base 2 (logFC) higher or equal to 1, and with a p-value lower or equal to 0.05. Blast2GO (OmicsBox) and KEGG Mapper Color were used for the functional analysis of the sets of identified up-regulated and down-regulated genes. The node score obtained from Blast2GO (OmicsBox) was used to compare the abundance of different gene ontology terms (Götz et al. 2008). Only genes whose expression was statistically significant were included. The node score is calculated using the following equation:

$$nodescore = \sum_{GOs} seq \times \alpha^{dist}$$

In this equation *GOs* are the different gene ontology terms, *seq* the number of different sequences annotated at a child GO term, α is a preset parameter that acts as a filter and *dist* is the distance to the node child.

Shake-flask experiment and sample analysis

Y. lipolytica colonies picked from YPD plates were inoculated in 5 mL of 3C medium. After 48 h of incubation, the appropriate volume was transferred to a fresh 25 mL of 3C medium in 100 mL shake flask to obtain an initial OD_{600} of 0.1. The cultures were incubated at 28°C for 4 days at 200 rpm. Samples for FFA, glucose, and OD_{600} were collected at the end of the experiment. Every experiment was performed in triplicate. Glucose was analyzed as described before (Jezierska et al. 2019). Extracellular FFAs were extracted from the extracellular medium by mixing equal volumes of culture

sample (containing cells and supernatant) and ethyl acetate. To check for fatty acids presence in the extracellular medium, 10 µl of the ethyl acetate fraction were analyzed through thin layer chromatography (TLC), using hexane:ethyl ether:formic acid (80:20:2) as mobile phase. The fatty acid bands were revealed by soaking the TLC sheet in 10% H_2SO_4 and scorching with heat (Supplementary Figure 2).

The ethyl acetate samples containing fatty acids were evaporated and the samples were resuspended in an equal volume of tetrahydrofuran. FFAs were analyzed through Gel Permeation Chromatography, by using an Agilent Technologies 1960 Infinity System. An isocratic method using tetrahydrofuran as eluent and a flow of 1 mL/min was used to separate the fatty acids from other components. This was achieved by the use of two sequential columns: the first one was a Phenogel™ 5 µm 100 Å column and the second one was a Phenogel™ 5 µm 50 Å column. Fatty acids were analyzed by an RID detector at 30°C. The results are expressed as mean values ± standard error (SE). The significance of differences between samples and the reference was analyzed with two-tailed unpaired T-test of Student. A P-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Growth and toxicity assay

The growth of *Y. lipolytica* on different fatty acids, methyl esters and ethyl esters as carbon source, as well as the toxicity of different fatty acids, methyl esters and ethyl esters was studied through drop-tests on agar medium. In order to prepare agar media containing fatty acids, methyl esters or ethyl esters, these were first diluted 10 times in autoclaved Tween40 0.5% and emulsified through sonication. The mix was then added to autoclaved SD medium containing 2% agar, 0.67% YNB without amino acids (DIFCO) and 0.077% of CSM (MP Biomedicals Europe) and poured into square petri dishes. Additionally, if the medium was used for toxicity studies, 0.2% glucose was added. The final concentrations of the fatty acids, methyl esters and ethyl esters were 0.2% for growth studies, and 1% for toxicity studies.

The different strains to be tested were inoculated in 5 ml of SD medium overnight. After measuring OD_{600}, samples were transferred to sterile microcentrifuge tubes and centrifuged at maximum speed for 3 min. The supernatant was removed and the appropriate volume of buffer (0.17% YNB, 0.5% NH_4CL and 0.05M phosphate buffer at pH 6.8) was added to reach an OD_{600} of 1. Samples were subject to four serial decimal dilutions and 5 µl of each dilution were spotted on each plate. Plates were incubated at 28°C for 3 days. Pictures of plates were taking with a GelDoc Go using the colorimetric method.

Results and Discussion

Comparative transcriptomics as a starting point to identify mechanisms involved in FFA export

RNA-Seq data analysis

Dulermo *et al.* (2015) have shown that the *faa1* deletion triggers FFA overproduction and secretion. We therefore hypothesized that genes involved in eFFA transport may be induced during growth in lipogenic conditions. To identify genes involved in the response to overproduction of FFAs, RNA-Seq

was performed on five replicate samples from both the Δ*faa1* and WT strains growing in high C/N ratio. For these 10 samples, 21±1 million reads mapped to the reference genome, with a mapping efficiencies between 95 and 98% (Table 1). Whole transcriptome differences between the two tested strains were explored both through Principal Component Analysis (Supplementary Figure 3) and through cluster plotting (Supplementary Figure 4), where it can be observed that the different strains cluster together based on the gene expression data. The volcano plot of the transcriptome study shows that 339 genes are significantly up-regulated (logFC≤1, p-value≥0.05) and 205 genes are significantly down-regulated when *Y. lipolytica* overproduces FFAs (Figure 1). The full data was deposited in the GEO database under the N° GSE217432.

Gene Ontology Analysis

To further elucidate the cellular effects caused by the overproduction and accumulation of FFAs in *Y. lipolytica Δfaa1*, the down-regulated and up-regulated genes were submitted to Blast2GO for a gene ontology enrichment analysis (Figure 2). This analysis allowed to observe the functional divergence of the differentially expressed genes (DEGs) in terms of molecular functions, biological processes and cellular components. The GO terms shown in Figure 2 are from the level 3 in classification, and therefore they encompass many more specific GO terms from lower levels in the hierarchy of GO terms. The distance of all GO terms analyzed to the level 3 is taken into account when calculating the Corrected Node Score.

An analysis of the molecular function terms shows that there are DEGs associated with both binding and catalytic activities, although these seem to be unevenly distributed. Binding activity seems to be more prominent among up-regulated genes except for cofactor binding, while oxidoreductase and transmembrane transport activities seem to be more represented in the down-regulated gene pool. However, both transferase and hydrolase activities can be found in both groups of genes. Concerning biological processes, cellular component biogenesis, regulation, cell cycle, and cell wall related processes are more represented among the up-regulated genes. When looking specifically at metabolic processes, it can be observed that biosynthetic, oxidation-reduction and organic processes are more represented among down-regulated genes while up-regulated genes are more associated with catabolic and nitrogen metabolic processes. Finally, the differences in the cellular components are mainly due to up-regulated genes being more linked to organelles, endomembrane systems, and cell periphery.

Analysis of up-regulated genes of the FFA secretion phenotype

The deletion of acyl-CoA synthetases has been associated with toxic effects in yeast associated with the accumulation of FFAs. One of the different mechanisms organisms employ to combat toxicity associated with hydrophobic compounds is to pump out the toxic compound. This has been widely observed in the case of multidrug resistance. Since the deletion of the gene *FAA1* leads both to eFFAs as well as slightly reduced growth in *Y. lipolytica* (Wang, Zhang and Chen 2011; Ledesma-Amaro *et al.* 2016), we hypothesized that this yeast responds to the accumulation of FFAs inside the cell by expressing specific proteins related to the removal of the molecules, in this way keeping the intracellular conditions within certain margins to minimize toxic effects. Currently, one can only speculate on what mechanism(s) play a key role here. Transport could be governed by typical membrane proteins known to promote transbilayer movement of hydrophobic compounds, small fatty acid-binding proteins could take part in the efflux process, extracellular proteins could extract FFA from the membrane or vesicle-mediated phenomena could be involved. Moreover, also changes

in the lipid bilayer and/or the cell wall could affect cell permeability and one cannot exclude synergetic effects of different adaptations. In an attempt to discover proteins related to FFA efflux, we analyzed the most up-regulated genes of the FFA phenotype, expecting that they would give information about how FFAs get secreted from the cell.

Table 2 shows the 12 genes that were most up-regulated in *Δfaa1 Y. lipolytica* when growing in a medium that stimulates production of lipids, leading to a FFA secretion phenotype. The most up-regulated gene codes for a transporter that shares 54% amino acid similarity to the Mch2 transporter protein from *S. cerevisiae*. This transporter is part of a family homologous to a family of monocarboxylates transporters present in the plasma membrane of mammalian cells. However, studies in yeast have shown that this activity is not conserved and that these transporters seem to be associated with intracellular membranes (Makuc *et al.* 2001), which is supported by the localization prediction of the Mch2 protein from *Y. lipolytica* (Table 2). Although the substrates of these transporters might be related to monocarboxylates, they still remain to be identified. The second most up-regulated gene codes for Ymoh, which is one out of nine Baeyer-Villiger monooxygenases in *Y. lipolytica* (Bordewick *et al.* 2018). These flavin-dependent enzymes can catalyze ketone oxidations, sulfoxidations and N-oxidations, and have been linked both to catabolic processes and the synthesis of secondary metabolites (Tolmie, Smit and Opperman 2019). Although the substrate specificity of Ymoh is not known, the up-regulation under these conditions might indicate its ability to metabolize related compounds to fatty acids, such as aliphatic ketones. Some of the other up-regulated genes include an acetyl-transferase, a chaperone-like protein and a putative nicotinic acid transporter, as well as three proteins that do now show homology with any studied protein. Most interestingly, three proteins have been associated with the cell wall, which coincidently are the highest expressed genes among the ones on this list (see RPKM values in Table 2). Of these, Cwp3 and Cwp4 are two out of six cell wall proteins sharing homology that have been used for cell-surface display (Yuzbasheva *et al.* 2011). Furthermore, Cwp4, also named Ywp1 in the literature, has been identified as a marker for hyphae growth in *Y. lipolytica* (Ramón *et al.* 1996; Morales-Vargas, Domínguez and Ruiz-Herrera 2012). On the other hand, Cwp11 has been described as an essential GPI-anchored protein involved in cell wall remodeling and protein secretion (Korpys-Woźniak, Kubiak and Celińska 2021). Finally, a metalloprotease predicted to be extracellular and inserted into the membrane by a transmembrane helix was also found to be up-regulated. Based on homology, M12b is part of the ADAM superfamily, which fulfill different activities, including the proteolysis of ectodomains of latent forms of cytokines or growth factors in animals, and are therefore linked to signaling roles (Takeda 2016). These proteins have been mainly studied in animals, being absent in plants and found only in some yeasts (Edwards, Handsley and Pennington 2009). Due to the wide range of activities found for these proteins, further experiments are needed to determine the role of M12b in *Y. lipolytica.*

Several deletion mutants were constructed to evaluate the possible implication of the up-regulated genes in the secretion of FFAs. It was expected that the extracellular levels of fatty acids would decrease in the knockout strain if the protein is linked to export, as it has been observed before in experiments in *S. cerevisiae* in the case of ScPry proteins (Darwiche *et al.* 2017). In total, eight out of the 12 most up-regulated genes were deleted, both in the WT genetic background, as well as in the *Δfaa1* background. CWP11, CWP4, HSP20 and ACT were not deleted due to technical difficulties with the cloning procedure. ACT was found to be just upstream of the URA3 locus, which had been modified to originate the auxotrophic parental strain, which not only explains the difficulties

encountered, but it may also have affected the expression levels. The results show that the *Δfaa1 Δyup1 Y. lipolytica* is the only knockout mutant whose eFFA production changed significantly, achieving 2.85±0.05 g/L, around 30% more production than *Δfaa1 Y. lipolytica* (2.20±0.17 g/L; Figure 3A), which remains significant after normalization to the OD600 values (Figure 3C). Furthermore, *Δfaa1 Δyup1 Y. lipolytica* displays a significantly lower consumption of glucose (Supplementary Figure 5) than *Δfaa1 Y. lipolytica,* allowing an 46% increase of the FFA yield ($Y_{X/G}$), from 0.026±0.002 to 0.038±0.002. Concerning the function of the Yup1 protein, it only shows homology to two other hypothetical proteins of *Y. lipolytica,* YALI0_D10945p and YALI0_E34441p. Both proteins, just as Yup1, are predicted to be soluble proteins with nuclear export signals. This might indicate their role in regulation pathways, and their interaction with other proteins should be studied to further elucidate their cellular function. This was a surprising finding since it was expected that genes up-regulated in *Δfaa1 Y. lipolytica,* would be helping to achieve the fatty acid secretion phenotype, but the fact that after deletion of the *YUP1* gene, *Y. lipolytica* can produce more FFAs indicates that the relation among these genes and the studied phenotype is more complex. Nevertheless, some of these genes have paralogues in the *Y. lipolytica* genome (Supplementary Table 4) and combinatorial deletions would be required to account for masking effects result of redundant functions.

Besides the knockout mutants, overexpression strains were constructed for the two most up-regulated genes, MCH2 and YMOH, whose transcription levels were increased by using a pTEF promotor, both in the wildtype-like and *Δfaa1* background. Although none of the overexpression strains led to any significant change compared to *Δfaa1 Y. lipolytica* (Figure 3B), after correcting the FFA values with the OD_{600} measurements, *Δfaa1 Δymoh pTEF-YMOH Y. lipolytica* showed a significant increase of 34% (Figure 3D). Nevertheless, neither the OD_{600} values nor the consumed glucose (Supplementary Figure 5) show any significant changes, so more research would be needed to verify the effect of the YMOH gene on eFFA production.

The putative role of cell wall modifications in the fatty acid secretion phenotype

GO analysis showed that cell wall proteins are overrepresented in the *Δfaa1* background (Figure 2). Moreover, several cell wall proteins appear among the most up-regulated genes (Table 2), displaying the highest RPKM values among the different up-regulated genes. Even though the deletion of CWP3 did not lead to any phenotypic change in the production of eFFAs, it cannot be excluded that cell wall proteins play a role in the fatty-acid secretion phenotype. As reported in the case of Cwp4 (Ramon *et al.* 1999), it is possible that potential phenotypic changes are being hidden due to redundant activities being performed by similar proteins. Indeed, both CWP3 and CWP4 belong to a family of cell wall proteins comprising six members that might share cellular functions, although they are so far unknown. When analyzing the expression levels of the other proteins from the same family, it can be observed that all of them show overexpression, with four out of six showing a significant overexpression (Table 3). Further combinatorial deletion studies should be performed to analyze the possible effects of these cell wall proteins on the FFA secretion phenotype.

Furthermore, analysis of the up-regulated metabolic pathways through the KEGG database showed several genes involved in the synthesis of chitin, the major component of the cell wall (Figure 4A, Supplementary Table 5). Although the first gene in this pathway (hexokinase) is common to the central metabolism, the other five up-regulated genes indicate that the genetic background and growth conditions specifically have an effect on the cell wall synthesis and remodeling. Considering that the FFA secretion phenotype leads to a slower growth than in the wildtype-like case, it could be

considered that this cell wall remodeling is due to hyphae formation. Indeed both cell wall proteins and enzymes related to cell wall synthesis have been detected in previous transcriptomic studies on dimorphism (Morales-Vargas, Domínguez and Ruiz-Herrera 2012). However, the cell wall synthesis genes found by Morales-Vargas et al. are not the same as the ones up-regulated in this study, indicating that a different kind of cell remodeling might be taking place. Indeed, as observed in Figure 4B, the hyphae formation is similar between the two conditions studied in this transcriptomics analysis, indicating that the proteins identified are involved in processes other than cell wall remodeling for hyphae formation.

Exploring extracellular proteins

No clear membrane transporter protein involved in FFA export came forward from the comparative transcriptomics analysis. Yet, cell wall proteins and processes associated to the cell wall are hinted to be involved. This led us search scientific literature for possible additional candidates that are not integral membrane proteins, but execute their putative activity extracellularly or associated to the cell surface. The first identified candidate is YALI0_D08140g (*PRY1*), one of the two *Y. lipolytica* homologues of the *S. cerevisiae* Pry proteins. The other homologue YALI0_D06149g (*PRY2*) was not detected in the RNA-seq data and therefore was not included in this experimental work. The Pry proteins of *S. cerevisiae* have been identified experimentally to be involved in fatty acid export, with ScPry1 being the most important one. ScPry1 and ScPry2 are secreted proteins, while ScPry3 is a GPI-anchored protein. The structure of ScPry1 harbors a binding pocket for palmitic acid (Darwiche *et al.* 2017). Darwiche *et al.* identified several residues involved in fatty acid binding and experimentally confirmed the effect of three of them. These residues, K173, V227, and V254, can be observed marked by green boxes in Figure 5. As noted in Figure 5, two out of three residues are conserved in the Pry1 protein of *Y. lipolytica,* indicating that the fatty acid binding activity of this protein might be conserved.

The second candidate is YALI0_F24255g (*YGPI*), a GPI-anchored protein that was first described to increase FFA production after being expressed in a FFA-accumulating *S. cerevisiae* strain (Shi *et al.* 2016). However, its mechanism is not clear although the authors notice that there are GPI-anchored proteins in plants that transfer fatty acid derivatives for their accumulation in the cuticle (Lee *et al.* 2009). The expression of these two candidates was checked in the RNA-seq experiment and both of them were found to be slightly down-regulated, although the expression difference between the two conditions is not significant. Moreover, their RPKM values are higher than those of any other gene covered in this study, which might indicate a high constitutive expression.

After choosing the candidates, both knockout and overexpression strains were obtained, and their eFFA production was measured (Figure 6). Concerning Pry1, no significant difference was observed in comparison to *Δfaa1 Y. lipolytica*, neither for the deletion mutant nor for the overexpression mutant. Despite the similarities to the *S. cerevisiae* Pry proteins, these results indicate that the Pry proteins from *Y. lipolytica* are not directly involved in fatty acid export. Nevertheless, like *S. cerevisiae* Pry proteins, they could still be involved in the transport of other lipid molecules such as sterols (Darwiche *et al.* 2016). Furthermore, recent studies have also shown that the function of Pry proteins does not seem to be conserved across yeast species, since the deletion of a gene coding for a Pry protein in oleaginous yeast *Starmerella bombicola* led to the opposite effect of that observed in *S. cerevisiae* (Salvador Lopez *et al.* 2022). Further research is needed to elucidate the divergent functions of Pry proteins in *Y. lipolytica* and in other yeasts. On the other hand, interesting results

were found when overexpressing *YGPI* (Figure 6). Although the knockout mutant did not lead to any eFFA significant change, the overexpression of *YGPI* in the wildtype-like background led to an eFFA production phenotype, reaching FFA levels similar to those of *Δfaa1 Y. lipolytica*. Surprisingly, in the *Δfaa1 Δygpi pTEF-YGPI Y. lipolytica* mutant, combining the three modifications did not significantly change the eFFA levels. When analyzing these data, it is important to notice that the results described by *Shi et al.* (2016) where obtained from experiments performed in a *S. cerevisiae* strain that, although accumulating FFAs, did not have any acyl-CoA synthetase gene knocked out. This *S. cerevisiae* strain was able to accumulate FFAs because it had different usage pathways knocked out, including the β-oxidation and the synthesis of neutral lipids (Valle-Rodríguez *et al.* 2014). This means that in both in this *S. cerevisiae* strain and in *Y. lipolytica* there was a significant intracellular pool of acyl-CoA, contrary to the situation in *Δfaa1 Y. lipolytica.* Therefore, these results could be explained if YGPI would code for an acyl-CoA transporter or an acyl-CoA binding protein. In this case, eFFA levels would not increase in *Δfaa1 Δygpi pTEF-YGPI Y. lipolytica* because there are no acyl-CoA molecules available to transport (Figure 7). Extracellular acyl-CoA binding proteins have been identified previously in plant cells, in which they are involved in the synthesis of the wax-containing cuticle (Guerrero *et al.* 2006; Lung and Chye 2016). In *Y. lipolytica,* the Ygpi protein might be involved in the transport of acyl-CoA molecules for the synthesis of some lipidic compounds of the cell wall, or it might have a role in the transport and regulation of fatty acid content in the plasma membrane and the cell wall, which might be an important feature considering that this yeast usually inhabits lipid-rich environments.

Some of the most up-regulated genes affect toxicity protection against linolenic acid

As explained in the Introduction section, we assume that yeast cells engineered to accumulate FFA find ways to avoid intracellular toxicity caused by these compounds. A straightforward strategy is the expulsion of the toxic compound to the extracellular environment. Yet, no clear candidate proteins involved in this process were found based on our comparative transcriptomics strategy and related investigations. However, export is one of the several ways in which cells can fight against the toxicity of different compounds, and other mechanisms exist, such as chemical detoxification, degradation or accumulation in vacuoles. In order to further elucidate the roles of the up-regulated genes in such processes, the knockout mutants were used for toxicity studies. The knockout mutants of *YGPI* and *PRY1* were also included to test their effects. We tested toxicity against different FFAs, methyl esters, and ethyl esters with a concentration of 1% supplemented with 0.2% glucose. Furthermore, growth experiments were performed without glucose and lower concentrations of the fatty acid or derivative to check for the effect of the different genes when import is essential for utilization of the exogenous fatty acids as the sole carbon source.

Oleic acid, methyl oleate, and ethyl oleate were used to verify that the use of methyl esters does not lead to toxicity due to the release of methanol after their extracellular hydrolysis by native lipases. No difference in growth was observed when these compounds were present in the media (Supplementary Figure 6). Decanoic acid, octanoic acid, methyl decanoate and methyl octanoate were used to test the effects of the knockout genes in the resistance against toxic medium-chain fatty acids. Both decanoic acid and octanoic acid were too toxic to observe any growth (data not shown), but their methyl esters showed less toxicity, allowing for some growth. However, no differences between the strains were observed using these compounds (Supplementary Figure 6). Finally, linolenic acid and methyl linolenate were used to test the toxic effects of polyunsaturated

fatty acids (PUFAs). Methyl linoleate showed less toxicity towards the cells and some differences among strains could be observed. However, these differences are more pronounced when using free linolenic acid. In the growth experiment without glucose in the medium (Figure 8A), it was observed that WT, *Δfaa1, Δmch2, Δcwp3* and *Δm12b Y. lipolytica* mutants were able to show some growth, but no growth was detected in the case of *Δymoh, Δyup1, Δyup2, Δyup3, Δymfs, Δpry1,* and *Δygpi Y. lipolytica.* When looking at the double knockouts in combination with *Δfaa1,* only *Δfaa1 Δmch2 Y. lipolytica* showed some growth. These results indicate that these genes have some role related to fatty acid uptake or metabolism, although this interaction might be indirect.

On the other hand, the toxicity experiment without glucose and 1% linolenic acid showed only growth in the case of *Δmch2 Y. lipolytica.* In order to study the effect of the different genes on the linolenic acid toxicity more in detail, other toxicity assays were performed with different concentrations of linolenic acid (0.5%, 0.2% and 0.1%). These results are depicted in Figure 8B and they show a wide range of responses depending on the mutant. As observed in Figure 8B, the single deletion of *MCH2* seems to confer resistance to linolenic acid. This is surprising considering that *MCH2* is the most up-regulated gene, and we expected this gene to be involved in cellular functions that alleviate toxicity. However, as the results show, this is not the case, at least with linolenic acid, and more studies will be needed to identify the exact function of this protein, the substates that it can transport or interact with, and what indirect effect it may have with fatty acids. While most of the other single knockouts show the same sensitivity to linolenic acid by growing at 0.5%, *Δyup2, Δpry1 and Δygpi* show toxicity at 0.5% linolenic acid. It is interesting to notice that all these three proteins are predicted to be localized extracellularly. Furthermore, these results support the role of YGPI as a protein involved in acyl-CoA export, which might be able to remove linolenoyl-CoA to keep the intracellular concentration under toxic levels. The role of the other two proteins might also be associated with this process, or they might act on another level, and more research might be necessary to understand their localization, mode of action and possible lipid-binding properties. It is also possible that these proteins interact among each other, forming an extracellular system to control lipids across the cell wall, so combinatorial mutants and physical interactions should also be studied. Finally, when looking at the knockouts in combination with *Δfaa1*, we can see that all these differences disappear. *MCH2* no longer confers resistance towards linolenic acid, and the absence of *YUP2, PRY1,* or *YGPI* no longer leads to increased susceptibility. These results indicate that the toxicity of linolenic acid might be related, although not exclusively, to the incorporation of these fatty acids into membrane lipids after their activation to acyl-CoA by *FAA1*, which leads to lipotoxicity (Richard *et al.* 2014). A previous study on the toxicity of linoleic acid in *S. cerevisiae* demonstrated that the lipotoxicity of this fatty acid was a result of its incorporation to the membrane, where it is more prone to be oxidized, producing peroxides eventually leading to the death of the cell (César Ferreira, Pepe de Moraes and Geralda Campos 2011).

Conclusions

The present research attempted to identify mechanisms involved in the export of FFAs in *Y. lipolytica* with comparative transcriptomic analysis as a starting point. An RNA-seq experiment was performed to identify differentially expressed genes in *Δfaa1 Y. lipolytica* eFFA-producing compared to the non-producing wildtype-like strain. This analysis revealed a number of differentially expressed genes, and the 12 most up-regulated ones were subjected to further investigation. Of these, the

most up-regulated gene *MCH2* codes for a putative transporter homologous to mammalian monocarboxylate transporters. The second most up-regulated gene was *YMOH*, coding for a putative monooxygenase. Moreover, three cell wall proteins, *CWP3*, *CWP4* and *CWP11*, were upregulated, in addition being the the genes with the highest RPKM values. Other genes found to be up-regulated were *M12B*, a putative membrane-bound extracellular protease, and three proteins with unknown functions, *YUP1*, *YUP2,* and *YUP3*.

Most up-regulated genes did not affect fatty acid export when deleted. However, the *Δyup1* strain displayed an increased FFA secretion, as well as a 48% increase in the glucose yield. Also, the overexpression of *YMOH* led to a rise in eFFA levels. Although single knockouts did not seem to have any effect, the overrepresentation of cell wall proteins as well as other extracellular proteins in the transcriptomics results, led us to screen scientific literature to select other candidate proteins that might influence fatty acid export. One of the tested proteins, Pry1, did not seem to have a direct effect on fatty acid export. However, the other tested protein, Ygpi, led to the production of eFFAs upon overexpression in the wildtype-like genetic background. However, its overexpression in the *Δfaa1* background did not lead to any significant change in the extracellular accumulation of FFAs. Besides, the deletion of this gene led to increased toxicity towards PUFAs, although this increase disappeared if the *FAA1* gene was also deleted. The interaction observed between *FAA1* and *YGPI* suggests that the latter might be an extracellular acyl-CoA binding protein that might be involved in the export of fatty acids. Both *YUP2* and *PRY1* showed the same effect on PUFA toxicity, increasing it upon single deletion, but having no effect in combination with the deletion of *FAA1*. Finally, the single deletion of *MCH2* led to a decrease in the toxic effect of PUFAs, indicating an indirect effect on lipid metabolism or transport that was not observed in the experiments on fatty acid export.

Taken together, the results of this study have shown the implication of several proteins of *Y. lipolytica* in the export of FFAs, as well as in the toxicity against PUFAs, although more research is needed to understand their function and involvement. Most importantly, this study has highlighted the importance of extracellular and cell wall proteins in relation to fatty acids, both for their production and protection against toxicity. Cell wall proteins have been targeted before to improve coumaric acid and β-carotene *S. cerevisiae* cell factories (Ramos-Viana *et al.* 2022). These proteins have also been found to be important for tolerance to solvents (Nishida *et al.* 2013). These studies show that they are also promising candidates to improve cell factories for the production of FFAs and their derivatives.

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Figure Legends:

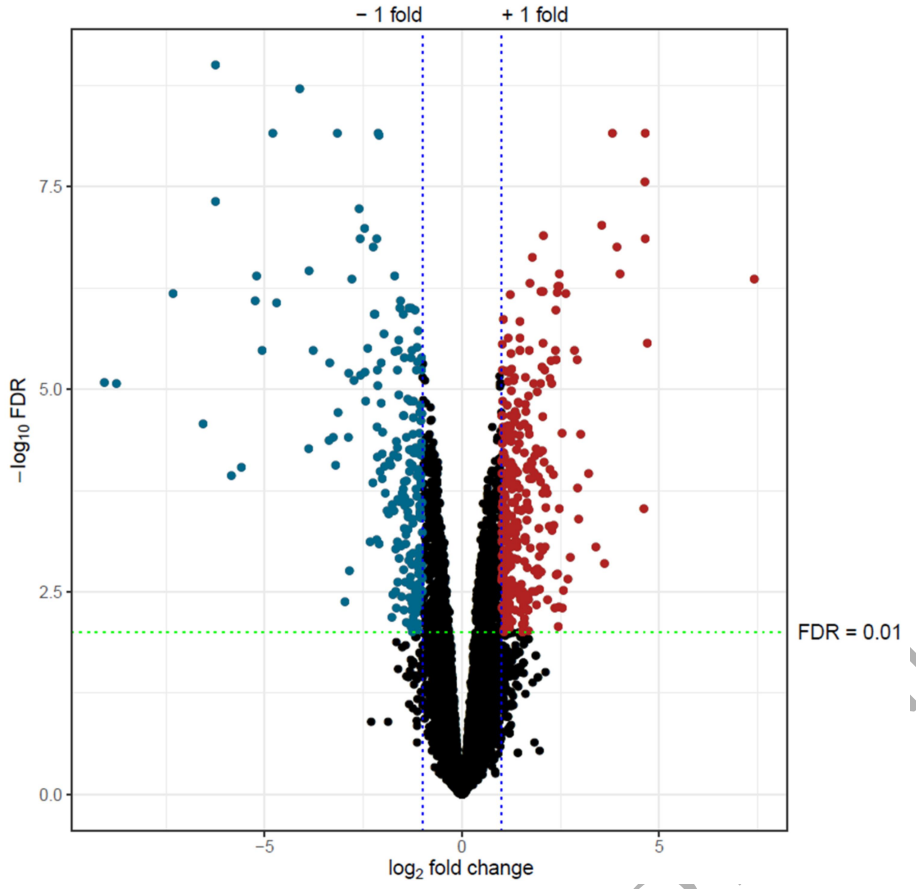

Figure 1: Volcano plot of the RNA-Seq data, representing the expressed genes in relation to their change in expression and the false discovery rate (FDR) associated to them. Dots in red represent genes up-regulated in the *Y. lipolytica Δfaa1* strain while dots in blue represent genes down-regulated in the *Y. lipolytica Δfaa1* strain.

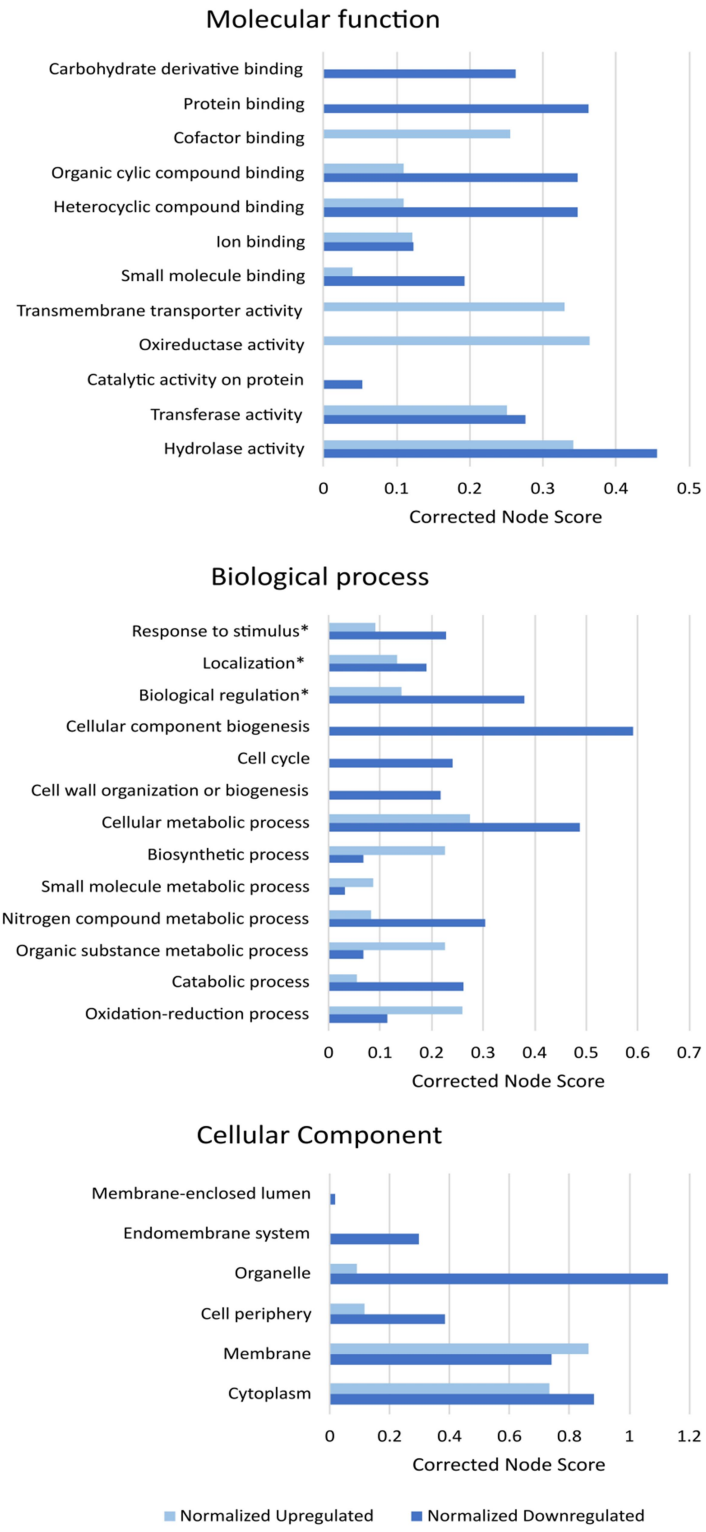

Figure 2: Gene ontology overrepresentation in the significantly DEGs from *Y. lipolytica Δfaa1,* divided by the three different categories of level 3 GO terms. The Corrected Node-Score is calculated by dividing the node score (which takes into account the number of genes associated to that term and their distance to it) by the total number of genes whose gene ontology terms fall in that category. *The GO terms "response to stimulus", "localization" and "biological regulation" belong to the GO level 2.

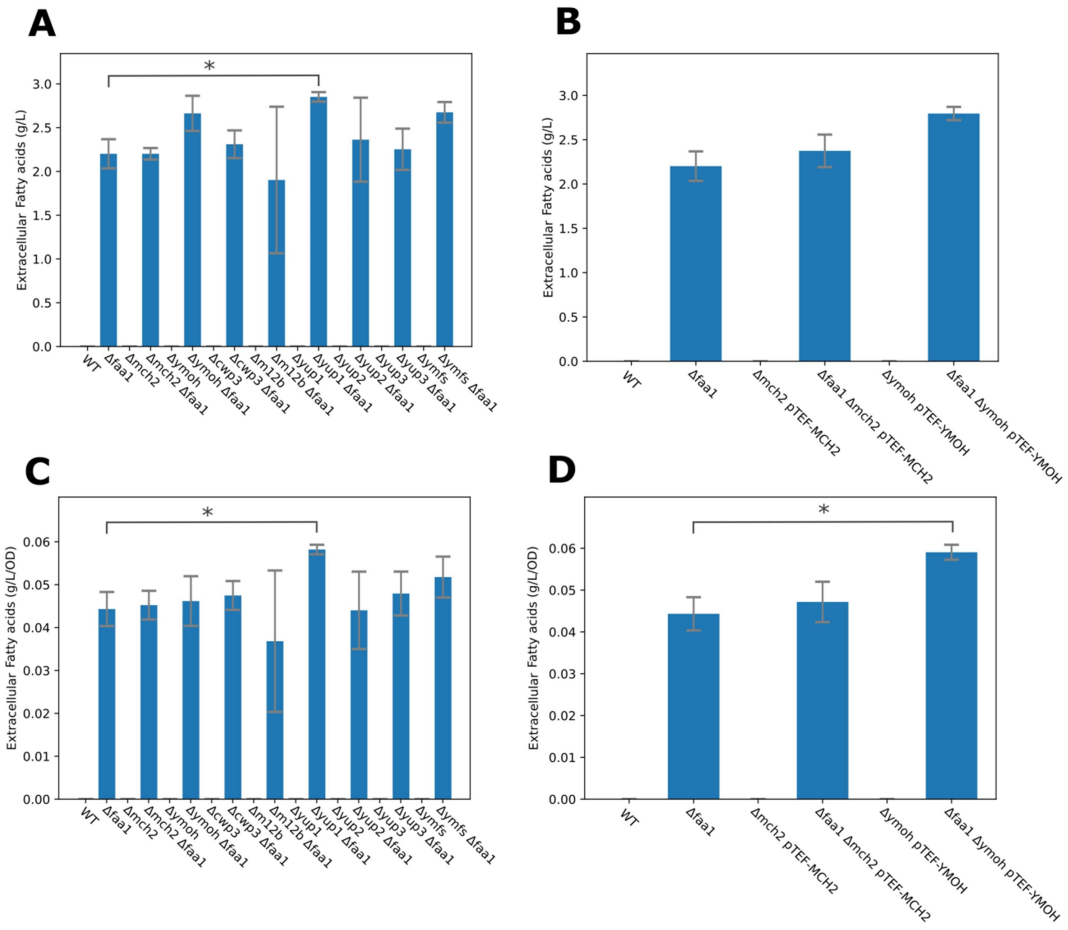

Figure 3: Extracellular fatty acids observed after growth of different *Y. lipolytica* knockout (A) and overexpression (B) mutants in medium with high C/N ratio. C, D) Extracellular fatty acid concentrations per OD_{600} values for knockout and overexpression mutants, respectively. The data represent means ± SE of at least three independent samples taken after 4 days of fermentation. Significant differences with a p-value lower than 0.05 are indicated with an asterisk (*).

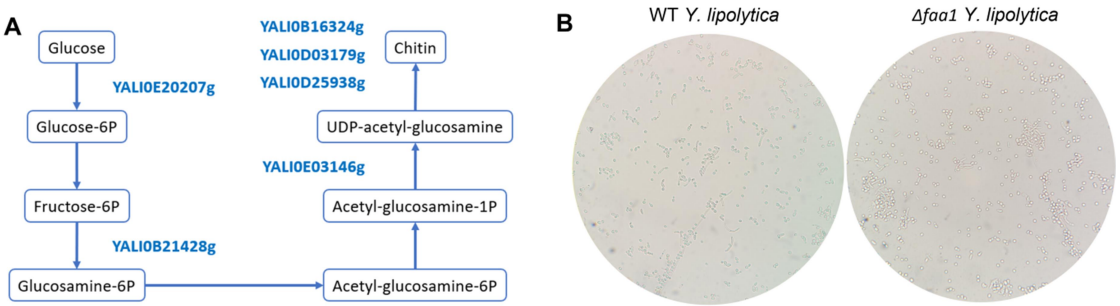

Figure 4: A) Chitin biosynthesis pathway for the biogenesis of the cell wall, with the up-regulated genes highlighted next to their catalyzed steps. The differential expression values of these genes can be found in Supplementary Table 5. B) Microscopic images of WT and *Δfaa1 Y. lipolytica* after growth for 2 days in medium containing high C/N. The same amount of hyphae formation can be observed in both of them using 400x total magnification in a brightfield microscope.

Figure 5: Sequence alignment of *Y. lipolytica* Pry1 and Pry2 proteins with *S. cerevisiae* ScPry1 and ScPry2 proteins made with Clustal Omega (EMBL). Key residues for palmitic acid binding in ScPry1 are highlighted in green squares. Common motifs for the α-β-α structure of the CAP domain are underlined in blue and red. The cysteine residues forming the two conserved disulfide bridges are boxed in yellow.

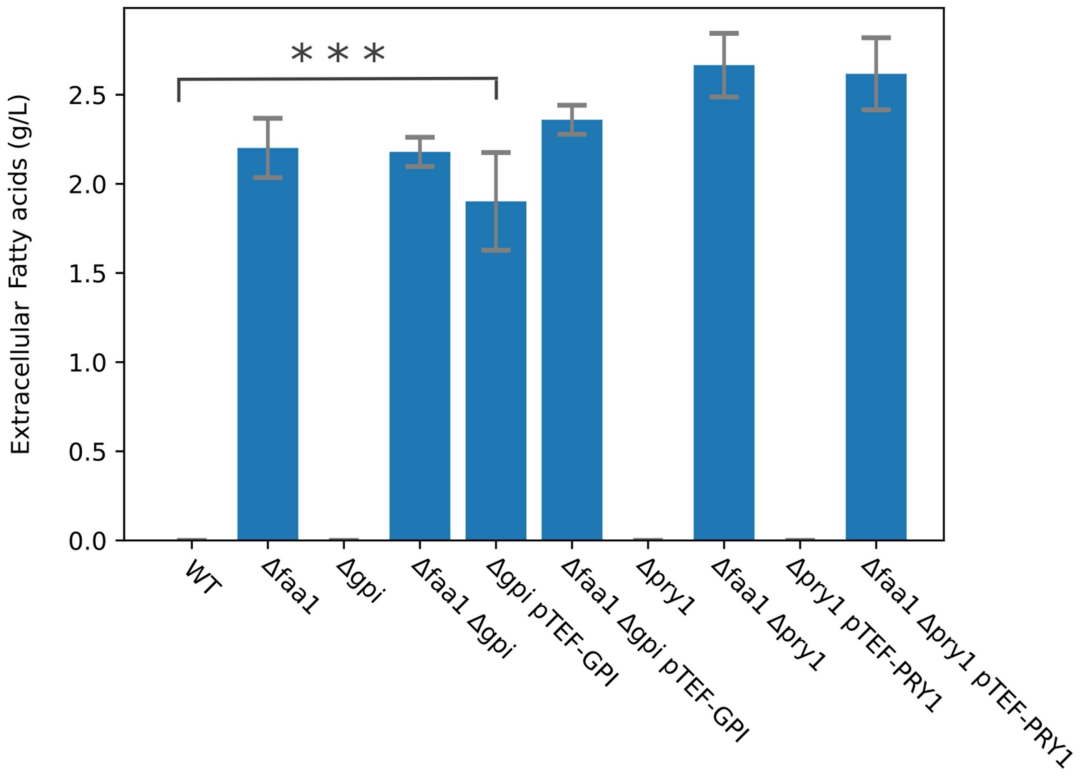

Figure 6: Extracellular fatty acids observed after growth of different *Y. lipolytica* knockout and overexpression mutants for gene PRY1 and YGPI in medium with high C/N ratio. The data represent means ± SE of at least three independent experiments. Significant differences with a p-value lower than 0.001 are indicated with a triple asterisk (***).

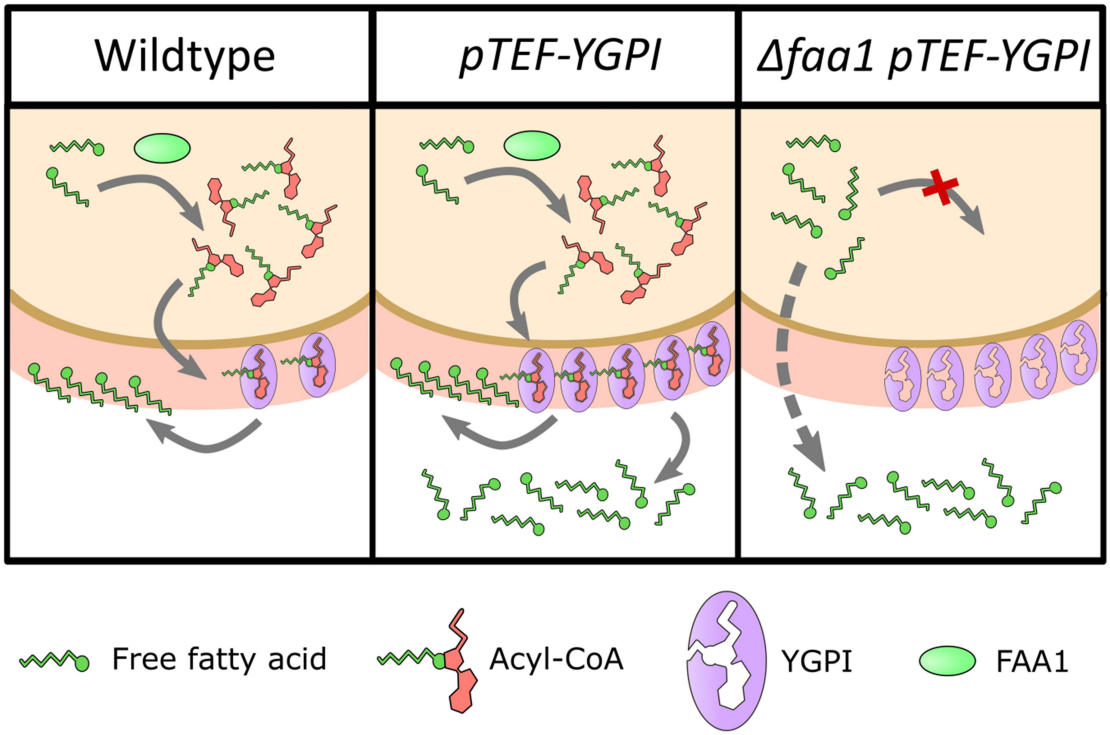

Figure 7: Proposed scenarios involving the hypothetical interaction between Ygpi proteins and acyl-CoA molecules. In the wildtype situation, these proteins assist in the export of acyl-CoA for the synthesis of lipidic components of the cell wall. If Ygpi proteins are overexpressed, an excess amount of acyl-CoA is exported, which gives place to extracellular free fatty acids after the hydrolysis by lipases. If the *FAA1* gene is knocked out, no acyl-CoA is formed and the overexpression of *YGPI* does not impact the export of free fatty acids.

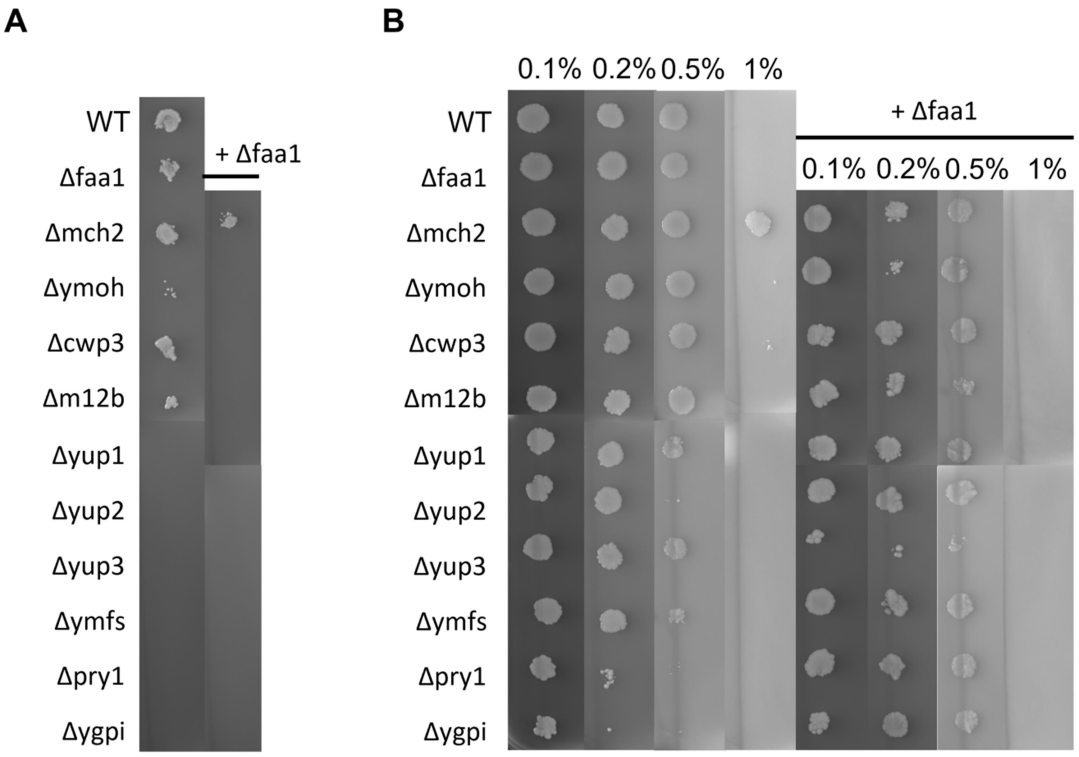

Figure 8: Growth and toxicity assays for linolenic acid. The colonies observed derive from a drop of cell culture of OD_{600} 1 which were incubated at 28°C for 3 days. To the right of the image there are the tests associated to the double knockouts performed in the *Δfaa1* background. A) Growth assay in linolenic acid 0.2% to assess the ability of the mutants to grow using this fatty acid as C-source B) Toxicity assay for different concentrations of linolenic acid to test the sensibility of different knockout mutants to polyunsaturated fatty acids.

Table 1: RNA-Seq statistical metrics of obtained and mapped reads

Strain	Sample	Number of raw reads	% Duplicate reads	Number of mapped reads	Percentage of correct mapping
WT	**1**	22608990	64.6%	21596946	95.52%
	2	28209390	67.8%	27410913	97.17%
	3	27382686	71.5%	26457958	96.62%
	4	20358234	66.6%	19895510	97.73%
	5	15443862	60.1%	15068180	97.57%
Δ*faa1*	**1**	23874372	64.7%	22841590	95.67%
	2	16332149	59.6%	15960795	97.73%
	3	21507051	64.8%	20971313	97.51%
	4	26623437	65.9%	25998063	97.65%
	5	19391919	61.6%	18821823	97.06%

Table 2: List of the 12 most up-regulated genes in the *Δfaa1* background with their predicted locations and functions based on homology and references where these genes have been either studied or mentioned. Predicted location was obtained through DeepLoc2.0, NES stands for nuclear export signal and NLS stands for nuclear localization signal. RPKM stands for "Reads per kilo base per million mapped reads" and represents the expression of the gene in the *Δfaa1* background in a comparable way to other genes.

Gene ID	Name used	logFC	RPKM	Predicted location	Predicted function	References
YALI0_F25619g	MCH2	7.41	188.29	Transmembrane in ER	Putative monocarboxylate transporter	No reference for *Y. lipolytica*
YALI0_F27005g	YMOH	4.69	47.45	Soluble in cytoplasm	Baeyer-Villiger monooxygenase	(Bordewick *et al.* 2018)
YALI0_D27214g	CWP3	4.64	246.18	Soluble extracellular	Cell wall protein	(Yuzbasheva *et al.* 2011)
YALI0_E26686g	YACT	4.64	449.35	Soluble in nucleus and cytoplasm (NES)	Acetyltransferase	No reference for *Y. lipolytica*
YALI0_E05423g	M12B	4.64	64.44	Transmembrane extracellular	Metalloprotease	No reference for *Y. lipolytica*
YALI0_E33385g	YUP1	4.60	8.11	Soluble cytoplasm (NES)	No hits	No reference for *Y. lipolytica*
YALI0_C06259g	YUP2	4.00	94.65	Soluble extracellular	No hits	No reference for *Y. lipolytica*
YALI0_E18546g	HSP20	3.93	187.90	Soluble in nucleus and cytoplasm (NLS)	Chaperone-like	No reference for *Y. lipolytica*
YALI0_F13761g	YUP3	3.81	40.97	Soluble in nucleus and cytoplasm (NLS)	No hits	No reference for *Y. lipolytica*
YALI0_E22286g	CWP11	3.61	634.02	Soluble extracellular	Cell wall protein	(Korpys-Woźniak, Kubiak and Celińska 2021)
YALI0_E11517g	YWP1/ CWP4	3.54	694.23	Soluble extracellular	Cell wall protein	(Ramón *et al.* 1996; Yuzbasheva *et al.* 2011)
YALI0_F21109g	YMFS	3.40	2.12	Transmembrane in cell membrane	Putative nicotinic acid transporter	(Li and Bao 2007)

Table 3: List with cell wall protein genes and their expression in the *Δfaa1* background, including the family of genes associated to up-regulated genes CWP3 and CWP6

Gene ID	Name used	logFC	RPKM
YALI0_E26125g	CWP7*	**2.93**	976.95
YALI0_F18282g	CWP6	0.82	405.98
YALI0_D27214g	CWP3	**4.64**	246.17
YALI0_E18788g	CWP1	**1.93**	195.82
YALI0_E31108g	CWP5	1.09	62.33
YALI0_E11517g	CWP4	**3.54**	694.23
YALI0_D08140g	PRY1	-0.76	2005.20
YALI0_D06149g	PRY2	0	Not detected
YALI0_F24255g	YGPI	-0.80	4203.43

* CWP7 has been named this way and not CWP2 to keep the names similar to the ones from (Yuzbasheva *et al.* 2011), in which CWP2 is a cell wall protein that is not a part of the same family as the other ones. The LogFC of genes showing significant overexpression are bolded.