Role of Pex11p in Lipid Homeostasis in *Yarrowia lipolytica*

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Peroxisomes are essential organelles in the cells of most eukaryotes, from yeasts to mammals. Their role in β-oxidation is particularly essential in yeasts; for example, in *Saccharomyces cerevisiae*, fatty acid oxidation takes place solely in peroxisomes. In this species, peroxisome biogenesis occurs when lipids are present in the culture medium, and it involves the Pex11p protein family: ScPex11p, ScPex25p, ScPex27p, and ScPex34p. *Yarrowia lipolytica* has three Pex11p homologues, which are YALI0C04092p (YLPex11lp), YALI0C04565p (YLpex11C), and YALI0D25498p (Pex11/25p). We found that these genes are regulated by oleic acid, and as has been observed in other organisms, *YPEX11* deletion generated giant peroxisomes when mutant yeast were grown in oleic acid medium. Moreover, Δ*Ylpex11* was unable to grow on fatty acid medium and showed extreme dose-dependent sensitivity to oleic acid. Indeed, when the strain was grown in minimum medium with 0.5% glucose and 3% oleic acid, lipid body lysis and cell death were observed. Cell death and lipid body lysis may be partially explained by an imbalance in the expression of the genes involved in lipid storage, namely, *DGA1*, *DGA2*, and *LRO1*, as well as that of *TGL4*, which is involved in lipid remobilization. *TGL4* deletion and *DGA2* overexpression resulted in decreased oleic acid sensitivity and delayed cell death of Δ*Ylpex11*, which probably stemmed from the release of free fatty acids into the cytoplasm. All these results show that YLPex11p plays an important role in lipid homeostasis in *Y. lipolytica*.

Peroxisomes are organelles that play an important role in different cellular processes and particularly in β-oxidation. In yeasts, β-oxidation (the process that breaks down fatty acids) takes place solely in this organelle (1). Peroxosome biogenesis involves more than 30 proteins (Pex proteins), and most of them (e.g., Pex1p, Pex2p, etc.) are crucial for the uptake of matrix proteins (2). Certain other Pex proteins, such as Pex11p, are involved in peroxisome fission and proliferation in higher and lower eukaryotes (3–8).

In *Saccharomyces cerevisiae*, Pex11lp (ScPex11p) is an inner membrane-associated homodimeric protein and is one of the most abundant peroxisomal proteins resulting from oleic acid induction (3, 4, 9). In the absence of oleic acid, Δ*Scpex11* peroxisomes demonstrate a normal morphology, although they are larger and less abundant than peroxisomes found in the parental strain in the presence of oleic acid (3, 4, 10). Moreover, overexpression of *ScPEX11* increases the number of normal-sized peroxisomes, an observation that indicates that ScPex11p is involved in peroxisome elongation and fission (4). It has been shown that *PEX11α* and *PEX11β* of *Homo sapiens* and *Pex11c*, *Pex11d*, and *Pex11e* of *Arabidopsis thaliana* can complement the lack of ScPex11lp function in Δ*Scpex11* mutants, which suggests that the role of Pex11p is conserved between *S. cerevisiae* and higher eukaryotes (8, 11).

The importance of ScPex11p remains unclear, as studies examining Δ*Scpex11* growth on fatty acid (FA) medium have yielded conflicting results. Indeed, Erdmann andBlobel (3) and Huber et al. (11) found that the strain did not grow on oleic acid. In contrast, Marshall et al. (4) and Rottenstein et al. (12) found that Δ*Scpex11* was still able to grow but grew more slowly than the wild-type strain. Erdmann andBlobel (3) reported that Δ*Scpex11* still forms buds but that these buds lack peroxisomes, which suggests that growth on oleic acid is reduced because of differences in peroxisome inheritance. Moreover, it was shown that ScPex11lp is involved in oxidation of medium-chain fatty acids (MCFA; C<sub>6</sub> or C<sub>12</sub>) (10, 12). Authors have suggested that Pex11lp is involved in transport of MCFA or cofactors needed to degrade MCFA (10).

ScPex11lp is activated by the phosphorylation of S165 and S167 via a Pho85p kinase-dependent pathway. The absence of these specific phosphorylation sites produces the same effect as that seen in the Δ*Scpex11* mutant (13). However, Pex11lp regulation differs in other organisms. In *Pichia pastoris*, for instance, PpPex11lp is activated by the phosphorylation of S174 by the fission machinery protein Fis1p (14), which recruits proteins, such as Mdv1p, Caf3p, and Dnm1lp, that are involved in mitochondrial and peroxisome division (15).

In *S. cerevisiae*, two other Pex11lp family proteins, Pex25p and Pex27p, also participate in peroxisome proliferation. Cells lacking one of these proteins contain fewer and enlarged peroxisomes, similar to the Δ*Scpex11* phenotype (12, 16–18). Interestingly, in contrast to the respective single mutants, the Δ*Scpex11* Δ*Scpex25* Δ*Scpex27* triple mutant is unable to grow on oleic acid and has impaired peroxisomal protein import. This result suggests that members of the Pex11lp family play a generalized role in peroxisome biogenesis, possibly assisting in protein uptake and thus facilitating growth on FA medium (12). A recent study showed that, although ScPex11lp keeps peroxisomes metabolically active and promotes the proliferation of preexisting peroxisomes,
ScPex25p is nonetheless needed to initiate membrane proliferation. Additionally, it seems that ScPex27p competes with ScPex25p, negatively affecting peroxisomal function (11). Recently, it was also found that another protein, ScPex34p, forms a homodimer and a heterodimer with ScPex11p, ScPex25p, and ScPex27p, and in fact, this protein appears to regulate peroxisome number in *S. cerevisiae* (17).

*Yarrowia lipolytica* is able to grow on different carbon sources, especially hydrophobic substrates such as oils, fatty acids, and alkanes (19). As peroxisomes play an essential role in FA oxidation in *S. cerevisiae* (2) and *P. pastoris* (20), we expect that Pex11p homologues may be essential for growth on fatty acids in *Y. lipolytica* and answer definitively questions about the fundamental role of PEX11 in lipid metabolism in eukaryotes. YAL1004092, YAL10040565, and YAL10D25498 all encode proteins containing a Pex11p domain. YAL1004092p, the closest homologue of ScPex11p, was named YlPEX11p, and its role in fatty acid oxidation and peroxisome fission is analyzed here. We found that YAL1004092p is essential for growth on FA medium and that its deletion results in enlarged peroxisomes in yeast grown in oleic acid medium. Surprisingly, the YlPEX11 knockout yeast shows severely impaired, dose-dependent growth in FA accumulation medium (yeast nitrogen base [YNB] with 0.5% glucose and 3% oleic acid), and its lipids bodies lyse into the cytoplasm. This phenomenon may be due, in part, to an imbalance between the expression of genes involved in lipid storage, DGA1, DGA2, and LRO1, and the expression of the TGL4 gene, which is involved in lipid remobilization. This imbalance probably increases free fatty acid (FFA) levels in the cytoplasm, leading to cell death, even if we cannot exclude the potential role of sphingolipids in this phenomenon.

**MATERIALS AND METHODS**

**Yeast growth and culture conditions.** The *Y. lipolytica* strains used in this study were derived from the wild-type *Y. lipolytica* W29 strain (ATCC 20460) (Table 1). The auxotrophic strain, PO1d (Leu− Ura−), has been described by Barth and Gaillardin (21). All the strains used in this study are listed in Table 1. Media and growth conditions for *Escherichia coli* have been previously described by Sambrook et al. (27), and those for *Y. lipolytica* have been described by Barth and Gaillardin (21). Rich medium (yeast extract-peptone-dextrose [YPD]) and minimal glucose medium (YNB) were prepared as previously described (28). The minimal medium (YNB) contained 0.17% (wt/vol) yeast nitrogen base (without amino acids and ammonium sulfate; YNBw); Difco, Paris, France), 0.5% (wt/vol) NH₄Cl, and 50 mM phosphate buffer (pH 6.8). As needed, this minimal medium was supplemented with uracil (0.1 g/liter) and/or leucine (0.1 g/liter). The YNBDₕₒ₂ medium contained 0.1% (wt/vol) yeast extract (Bacto-DB),...
0.5% glucose, and 3% oleic acid. Solid media were obtained by adding 1.6% agar. To add the fatty acids to liquid or solid media, a 50:50 emulsion of fatty acids–10% pluronic acid was first prepared and heated at 80°C for 10 min before being added to the media. The following fatty acids were used in our study: C6:0 (99%; Sigma-Aldrich), C10:0 (99%; Sigma-Aldrich), C14:0 (99%; Acros Organics), C16:0 (99%; Sigma-Aldrich), and C18:1 (70%; Sigma-Aldrich).

Plasmid construction. The deletion cassettes were largely generated by PCR amplification conducted in accordance with the procedure of Fickers and colleagues (29). First, the upstream (Up) and downstream (Dn) regions of a target gene were amplified using Y. lipolytica W29 genomic DNA as the template and the gene-specific Up and Dn oligonucleotides as primer pairs (Table 2). Primers UpIsceI and DnIsceI contained an extension that allowed the introduction of the I-SceI restriction site, making it possible to construct an UpDn fragment via PCR fusion (see above).

To disrupt YIPEX11, primer pairs C04092UpNotI/C04092UpIsceI and C04092DnNotI/C04092DnIsceIIceuI were employed (Table 2). The Up and Dn regions were purified and used in a PCR fusion. The resulting UpDn fragment was ligated into pCR4Blunt-TOPO, yielding the JMP1635 construct. The URA3ex marker (from JMP803) was then introduced at the I-SceI site, yielding the JMP1669 construct containing the Ylpex11::URA3ex cassette. This plasmid was then digested with NotI to obtain the deletion cassette.

To disrupt TGL4, the primer pair TGL4-P1/TGL4-T2 was used (Table 2) to directly amplify the tgl4::LEU2ex cassette from JMY2206 genomic DNA (24).

The complementation cassette employed for the Ylpex11::URA3ex strain was created using the primer pair C04092Start/C04092End. YlPEX11 was cloned into pCR4Blunt-TOPO and then digested with AvrII and BamHI and cloned into JMP1392 that had been previously digested with BamHI and AvrII; this generated JMP2023. The vector to localize YlPEX11 was digested with AvrII and BamHI and cloned into JMP1392 that had been previously digested with BamHI and AvrII; this generated JMP2023.

### Table 2 List of primers

| Primer            | Sequence                                      | Utilization                                           |
|-------------------|-----------------------------------------------|-------------------------------------------------------|
| C04092UpNotI      | GAATGCGGCCGCTAGCAGTTATGGAGATTGGC             | Upstream fragment of YIPEX11                          |
| C04092UpIsceI     | CGATTACCTGGTTATCCCTACCGGGCGAGGCGAAGCATC      |                                                       |
| C04092DnNotI      | GAATGCAGCGCGAGACACTGCTGGCTTCCTTAACG          | Downstream fragment of YIPEX11                        |
| C04092DnIsceIIceuI| GGTAGGGATAACAGGTTAATCGTAATACGTGTAAGAGAG     |                                                       |
| Ver1C04092        | GACTGAGATCTGTGTAAGGG                         | Verification of YIPEX11 disruption                    |
| Ver2C04092        | ACCGCATACCTGGATCCCTTG                       |                                                       |
| C04092Start       | ATCCTAGGGTAACGGTGATACGTGGCTGGCTGAGAGCG      | Complementation/overexpression of YIPEX11             |
| C04092End         | CATCCTAGGGTAACGGTGATACGTGGCTGGCTGAGAGCG      |                                                       |
| C04092End2        | CATCCTAGGGTAACGGTGATACGTGGCTGGCTGAGAGCG      |                                                       |
| TGL4-P1           | TGATTGTTCCACCTGCCCTCGACACC                   | Amplification of disruption cassette tgl4::LEU2ex using JMY2206 genomic DNA |
| TGL4-T2           | CAATGAGGCTGACCAAGCTGGGTCCAGGAGCTGGGACC      | Verification of TGL4 disruption                        |
| TGL4-Ver1         | TTAGATGAAATGCTCCAATACCTGCCGAGCTGGGGAGG      |                                                       |
| TGL4-Ver2         | CGTCTGAGGATTGATACCTCCGCTT                   |                                                       |
| pTEF-start        | GGGTATAAGAGGACCCAGCTGCCTG                 | Verification of pTEF-RedStar2SKL-LEU2ex or pTEF-YIPEX11-LEU2ex insertion into the Y. lipolytica genome |
| 61 stop           | GTGATGATGTTGAGGTAAGGG                      |                                                       |
| C04092F           | GAGAAGGAGAGGACACCA                          | YIPEX11 expression by RT-PCR                          |
| C04092R           | TTCTGGAGGACACCAATACCC                      |                                                       |
| C04565-F          | GAGAAGAAGGCTGAAAAGAC                      | YAL10C04565 expression by RT-PCR                        |
| C04565-R          | CAGGTTGTCACCAATAACAC                       |                                                       |
| D25498-F          | TGCTCTGAGAAATTTGGAG                       | YAL10D25498 expression by RT-PCR                        |
| D25498-R          | TGCTCTGAGAAATTTGGAG                       |                                                       |
| DGA1_F            | TGATACCGATCCAGCAGT                         | DGA1 expression by RT-PCR                              |
| DGA1_R            | GGTTGAGGAGATGGAAGCC                      |                                                       |
| DGA2_F            | TTCTCATCTTGAGCCTACTGCTCT                 | DGA2 expression by RT-PCR                              |
| DGA2_R            | GGAATAGATGAGACCGGTG                      |                                                       |
| LRO1_F            | CTCCGGCCACTTCCTTTATG                     | LRO1 expression by RT-PCR                              |
| LRO1_R            | GAAGTATGAGCTCTCGGCTG                      |                                                       |
| TGL4-A1           | GTTCGACAAGGAGCCTATT                     | TGL4 expression by RT-PCR                              |
| TGL4-A2           | GGTCGAGATGCGAGGATGAAAG                    |                                                       |
| ACT-A1            | TCCAGGGCGGCGCTCCCTCC                      | Actin expression by RT-PCR                             |
| ACT-A2            | GGCCAGCCATATCAGAGGCTGCA                   |                                                       |

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YLPEX1p was constructed with primer pair C04092Start/C04092End2. After digestion by BamHI and AvrII, the PCR fragment was cloned into JMPI427, carrying yellow fluorescent protein (YFP), to give JMP2616. YLPEX11 and YLPEX11/YFP were expressed under the constitutive and strong promoter pTET. The resulting plasmids were then digested with NotI to release the complementation cassette.

To acquire the DGA2 overexpression cassette, JMPI132 was digested with I-Scel to replace URA3ex with the LEU2ex marker, yielding JMPI1822. This plasmid was then digested with NotI to release the overexpression cassette.

Disruption or overexpression cassettes were used to transform the yeast via the lithium acetate method (30). Transformants were selected on YNB plus Leu medium or YNB medium, depending on the genotype. Genomic DNA from the transformants was prepared as described by Querol et al. (31). The corresponding ver1 and ver2 primers (Table 2) were used to verify YLPEX11 and TGL4 disruption, pTET-Start and 61 stop primers were used to check the insertion of the overexpression cassettes pTET-YLPEX11-LEU2ex and pTET-DGA2-LEU2ex.

Restriction enzymes were obtained from Ozyme (Saint-Quentin-en-Yvelines, France). PCR amplifications were performed in an Eppendorf 2720 thermal cycler using GoTaq DNA polymerases (Promega, Madison, WI) for PCR verification and PyroBest DNA polymerases (Takara, Saint-Germain-en-Laye, France) for cloning. PCR fragments were purified using a Qiagen purification kit (Hilden, Germany), and DNA fragments were recovered from agarose gels using a QIAquick gel extraction kit (Qiagen purification kit, Hilden, Germany) and quantification were achieved via comparisons to standards.

Postinoculation, frozen in liquid nitrogen, and stored at -80°C. RNA was treated with DNase (Ambion, Life Technologies, United States) for high-perfor-

Lipid determinations. Using 10- to 20-mg aliquots of freeze-dried cells, lipids were converted into their methyl esters by using the method described by Browse et al. (32). The esters produced were then used in gas chromatography (GC) analysis. The analysis was performed using a Varian 3900 gas chromatograph equipped with a flame ionization detector and equipped with Zeiss fluorescence microscopy filters 45 and 46. Axiovision 4.8 software (Zeiss, Le Pecq, France) was used to acquire images. Lipid bodies were stained by adding Bodipy lipid probe (2.5 mg/ml in ethanol; Invitrogen, Saint Aubin, France) to the cell suspension (A540 of 5) and letting the mixture incubate for 10 min at room temperature. The LIVE/DEAD BacLight bacterial viability kit (Life Technologies) was used as per the manufacturer’s instructions to count living and dead cells under the microscope.

**RESULTS AND DISCUSSION**

Pex11p is conserved in *Yarrowia lipolytica*. YAL1004C0922 (YLPEX11) encodes a protein of 234 amino acids that shares 31% identity with ScPex11p, 33% identity with the Pex11p found in *Pichia pastoris* (PpPex11p), and 39% identity with the Pex11p found in *Penicillium chrysogenum* (PcPex11pA) (Table 3). This protein is also distantly related to human and plant Pex11 proteins (20 to 27% shared identities) (Table 3). Alignment of YLPEX11, ScPex11p, PpPex11p, and PcPex11pA showed that the N-terminal amphipathic helix described for PcPex11p is relatively well conserved (34) (Fig. 1A). This domain promotes the association between Pcppex11p and liposomes in vitro and allows for membrane curvature (34); its presence in each of these four proteins suggests that the membrane-binding ability is also conserved. A Cys3 residue of ScPex11p involved in homodimerization (9) was found to be conserved in YLPEX11p (residue Cys4) but not in PcPex11p or PpPex11p (Fig. 1). ScPex11p has been described as a protein that binds tightly to peroxisomal membranes (9), and it was not predicted by TMHMM to have a transmembrane domain (TM) (Fig. 1). Likewise, no TM was predicted for PpPex11p; however, two TMs were predicted for YLPEX11p and PpPex11p.

| Pex11p protein (size) | % shared identity with*: |
|-----------------------|--------------------------|
| YLPEX11p (233 aa)    | YAL1004C0922p (305 aa)   |
| YAL1004C0922p (299 aa)|                          |

*aa, amino acids; NF, not found. *5, 55 to 60% coverage. E values are shown in parentheses.
FIG 1  Analysis of YlPex11p, PcPex11p, PpPex11p, and ScPex11p proteins. (A) Alignment of YlPex11p (YALI0C04092p), PcPex11p (Pc12g09400p), PpPex11p (PP7435_Chr2-0790p), and ScPex11p (YOL147C) using ClustalW. Yl, Y. lipolytica; Sc, S. cerevisiae; Pc, P. chrysogenum; Pp, P. pastoris. The amino acids in yellow are the transmembrane domains predicted by TMHMM. The black box encloses the pex11-Amph domain. The enlarged letters that are highlighted in gray indicate the location of the S165, S167, and C3 sites in ScPex11p, the putative phosphorylation sites S174 and C4 in YlPex11p, and the S173 site in PpPex11p. (B) TMHMM profiles of YlPex11p, ScPex11p, PpPex11p, and PcPex11p.
Mammalian Pex11β and Trypanosoma brucei Pex11 each also contain two transmembrane domains (5, 35), suggesting that the structures of YlPex11p and PpPex11p are more similar to that of higher-eukaryote Pex11ps than to ScPex11p. Additionally, the phosphorylated residues S165 and S167 of ScPex11p (13) are not conserved in YlPex11p and PcPex11p, a finding that further highlights the divergence between ScPex11p and PcPex11p/YlPex11p. In fact, it appears that YlPex11p has only one serine at the potential phosphorylation position S174, and PcPex11p has none (Fig. 1). Similarly, it is known that PpPex11p is activated via phosphorylation at a single position, S173, by the fission machinery protein Fis1p (14). Interestingly, the three mammalian Pex11p proteins also interact with Fis1p (36, 37). However, no phosphorylation of Pex11p/H9251 has been detected (38). All these data suggest that YlPex11p could be activated by the phosphorylation of Ser174, but it may also be the case that, as in mammals and P. pastoris (7), Y. lipolytica has multiple Pex11p proteins. As suggested by Kiel et al. (40), these two Pex11p-like proteins may play roles similar to those of ScPex25p and ScPex27p.

Interestingly, YALI0C04565p seems to have a dilysine motif at its C terminal (KKXX) (Fig. 2), just like AtPex11c, AtPex11d, and AtPex11e, and HsPex11α (7, 8, 46). The dilysine motif (KKXX or KXKXX) has been shown to facilitate binding between peroxisomes and COP1 (coat protein complex I) (47), a protein complex which regulates membrane traffic in eukaryotic cells (48). This finding suggests that some Pex11 proteins may mediate peroxisome division via a coatomer-dependent type of membrane vesiculation. Indeed, trypanosome Pex11p and rat Pex11α, which each have a C-terminal dilysine motif, bind to coatomers (49). The division system is not completely conserved among eukaryotes. This is in agreement with the absence of complementation of ΔScpex11 by YlPEX11 (data not shown).

**Yarrowia lipolytica contains two other putative Pex11p proteins.** More interestingly, Kiel et al. (40) described two Y. lipolytica proteins, YALI0C04565p and YALI0D25498p, as potential Pex11 proteins. *In silico* analysis of these proteins showed that they are not homologues of YlPex11p, ScPex11p, PpPex11p, or PcPex11; instead, YALI0C04565p seems to be distantly related to PcPex11C (Table 1). SMART (41, 42) indicated, at a low E value (9e−12), that YALI0C04565p contains a Pex11 domain (Fig. 2A, in gray). YALI0D25498p did not have any homologues in the nr database, but the conserved domain database (43) revealed that it does have a Pex11 domain (E value, 9.32e−6) (Fig. 2A, in gray). In contrast to its prediction for YlPex11, TMHMM did not predict any transmembrane domains for YALI0C04565p, YALI0D25498p did not have any homologues in the nr database, but the conserved domain database (43) revealed that it does have a Pex11 domain (E value, 9.32e−6) (Fig. 2A, in gray). In contrast to its prediction for YlPex11, TMHMM did not predict any transmembrane domains for YALI0C04565p or YALI0D25498p (Fig. 2B). It may be that, similar to humans and P. chrysogenum (7, 44, 45) (Table 3), Y. lipolytica has multiple Pex11p proteins. As suggested by Kiel et al. (40), these two Pex11p-like proteins may play roles similar to those of ScPex25p and ScPex27p.

![FIG 2 YALI0C04565p and YALI0D25498p sequences. (A) YALI0C04565p and YALI0D25498p sequences with the predicted Pex11 domain in gray. The putative dilysine motif (KKXX) is shown in red. (B) TMHMM profile.](ec.asm.org)
importance of the dilyasine domain in Pex11p is still unclear. However, it is known that this domain is dispensable in the functioning of AtPex11e, trypanosome Pex11p, and rat Pex11e (7, 49). All these results suggest that Y. lipolytica is more similar to higher eukaryotes than to S. cerevisiae when it comes to peroxisome division.

**YIPEX11 is induced by oleic acid.** In S. cerevisiae, Pex11p plays a very important role for growth on oleic acid and is highly induced by this fatty acid (3, 4, 9). Because lipid metabolism is a key process in *Y. lipolytica*, we wanted to understand how the expression of the three putative PEX11 genes, *YIPEX11*, *YALI0C04565*, and *YALI0D25498*, changed in response to oleic acid exposure in the reference strain JMY2900. Cells were grown on YNB (1% glucose) for 15 h and were then transferred into fresh liquid YNB media supplemented with 1% glucose (YNBD$_1$), 3% oleic acid (YNBD$_3$), or both 1% glucose and 3% oleic acid (YNBD$_{1,3}$). Cells were harvested after 2 and 6 h of growth. RT-PCR analysis indicated that (i) *YIPEX11*, *YALI0C04565*, and *YALI0D25498* were expressed under all the conditions tested; (ii) as expected, *YIPEX11* was strongly upregulated in media containing oleic acid; and (iii) *YALI0C04565* and *YALI0D25498* were also strongly induced in oleic acid media, but their expression was lower at 6 h than at 2 h, especially in YNBD$_{0.5}$O$_{0.1}$ and YNBD$_{0.5}$O$_{0.3}$ (Fig. 3). This result also showed that the presence of glucose did not affect the upregulation of *YIPEX11*, *YALI0C04565*, and *YALI0D25498* by oleic acid, showing that glucose catabolite repression does not exist in this yeast, contrary to *S. cerevisiae* (50). This suggests also that *YIPEX11* in *Y. lipolytica* is induced by oleic acid (YNBD$_{0.5}$O$_{0.1}$ and YNBD$_{0.5}$O$_{0.3}$), or both 1% glucose and 3% oleic acid (YNBD$_{1,3}$). *YIPEX11* deletion prevents growth on fatty acids. In *S. cerevisiae*, studies of ScPex11p’s effects on growth on FA media have yielded mixed results. Erdmann and Blobel (3) and Huber et al. (11) found that ΔScpex11 was unable to grow on oleic acid, whereas Marshall et al. (4) and Rottensteiner et al. (12) showed that growth was possible. To better understand the general role of *YlPEX11* and to investigate the specific part it plays in FA oxidation, we created a knockout strain, JMY3227, and examined its growth on the FA substrates because its peroxisomes lacked the closest homologue of ScPex11p. In *S. cerevisiae*, peroxisomal RedStar2SKL shows dose-dependent sensitivity to oleic acid. The fact that ΔYlpe11 failed to grow on oleic acid media could be due to a sensitivity to oleic acid. To determine the minimum concentration of oleic acid that inhibits ΔYlpex11 growth, JMY2900 and ΔYlpex11 were grown for 24 h in YNBD$_1$, media that contained different concentrations of oleic acid (from 0.1% to 3%). Cell survival was determined by running a drop test on YPD plates, and cell morphology was characterized by microscopy (Fig. 5). In the reference strain (JMY2900), the number and size of the lipid bodies increased as oleic acid concentrations increased; in ΔYlpex11, cell morphology was increasingly affected by higher levels of oleic acid (Fig. 5A) and became noticeably different from that of the reference strain when oleic acid concentrations were greater than 0.5%. At oleic acid concentrations of 1% and 3%, several ΔYlpex11 cells appeared to lack vacuoles and lipid bodies, which are dead cells (bleary stained with Bodipy without a defined structure) (Fig. 5A). In accordance with the morphological observations, the drop test showed that ΔYlpex11 survival decreased with increasing levels of oleic acid (Fig. 5B; see also Fig. 8, below). Whereas JMY2900 and ΔYlpex11 had the same levels of survival in media containing 0.1% or 0.3% oleic acid (YNBD$_{0.5}$O$_{0.1}$ and YNBD$_{0.5}$O$_{0.3}$), ΔYlpex11 survival fell dramatically (500 to 1,000 times fewer normal-sized colonies than JMY2900) in media containing 0.5% to 3% oleic acid (YNBD$_{0.5}$O$_{0.5}$ and YNBD$_{0.5}$O$_{3}$) (Fig. 5B). These results showed that ΔYlpex11 is highly sensitive to oleic acid and that a concentration of 0.5% oleic acid is enough to strongly impact cell viability.

**YIPEX11 is involved in peroxisome proliferation.** In *S. cerevisiae*, plants, and mammals, Pex11 is involved in peroxisome fission, and giant peroxisomes are observed in Δpex11 mutants (3–8). In order to determine if this protein has the same function in *Y. lipolytica*, we generated mutants of the wild type and ΔYlpex11 that constitutively expressed a peroxisome-targeted RedStar2 fluorescent protein: JMY3175 (the wild-type JMY2900 with RedStar2SKL) and JMY3170 (the ΔYlpex11 strain expressing RedStar2SKL; RedStar2SKLp) (25). Experiments were performed in minimum media. In yeast grown on YNBD$_1$ (2% glucose), no peroxisomes were observed, probably because they were too small and/or too few in number to be visible (data not shown). However, in yeast grown on YNBD$_{0.5}$O$_{3}$, individual peroxisomes were
easily distinguishable in JMY3165 (JMY330 pTEF-RedStar2SKL) but not in JMY3170 (ΔYlpex11 pTEF-RedStar2SKL). Instead, in JMY3170, red fluorescence increased rapidly overall and prevented accurate observation of peroxisomes, probably because the cells died too fast (data not shown). We therefore attempted a different strategy to observe the peroxisomes in JMY3170: 3% oleic acid (YNBO₃) was added to cells that had been previously grown for 16 h in YNBD₂. Because the oleic acid concentration...
FIG 6 Microscopic images and lipid content of the wild type and ΔYlpex11. (A) Morphology of JMY3175 (the wild-type JMY2900 with RedStar2SKLp) and JMY3170 (the ΔYlpex11 strain expressing RedStar2SKLp). Microscopic images are shown for cells cultivated for 16 h in YNBD to which 3% oleic acid was added (YNB03) at the indicated times after addition of oleic acid. Cell morphology was followed for a period of 48 h (30 min and 1.3, 3, 4.3, 21, and 48 h). Peroxisomes were stained red (RedStar2SKLp, rows 3 and 7), and lipid bodies were stained green (Bodipy, rows 2 and 6). White arrows indicate the peroxisomes. (B) Morphology of JMY4729 (the wild-type JMY2900 with Pot1-GFPp) and JMY4730 (the ΔYlpex11 strain with Pot1-GFPp). Microscopic images are of cells after 6 h of cultivation in YNBD to which 3% oleic acid was added. Peroxisomes were stained green (Pot1-GFPp). White arrows indicate the peroxisomes. (C) Total lipid content (FFAs and TAGs) as a percentage of yeast CDW. Analyses of total lipids were performed after 24 h for strains grown in YNBD2 and YNBD0.5O3 and after 48 h for strains that experienced the addition of 3% oleic acid after having been grown in YNBD2 (YNB03). Gray bars, wild type; black bars, ΔYlpex11.
TABLE 4 Lipid profiles

| FA        | % of FA in oleic acids | JMY2900 | ΔYlpex11 |
|-----------|------------------------|---------|----------|
| C16:0     | 3.9                    | 3.6 ± 0.2 | 4.2 ± 0.6 |
| C16:1(n-9) | 0.9                   | 2.7 ± 0.9 | 1.2 ± 0.3 |
| C16:1(n-7) | 4.7                   | 7 ± 1.9   | 3.4 ± 0.4 |
| C18:0     | 0.7                    | 0.99 ± 0.1 | 0.8 ± 0.2 |
| C18:1(n-9) | 73                    | 68.4 ± 4.1 | 58 ± 8.7 |
| C18:2(n-6) | 7                     | 11 ± 2.2  | 17.4 ± 3.3 |

was low and we eliminated the glucose, it was thought that this process would slow down the growth, thus reducing ΔYlpex11 cell death due to oleic acid toxicity. One hour and 30 min after the addition of the oleic acid, the peroxisomes began to become visible in JMY3165 and JMY3170 cells (Fig. 6A, see particularly the panel for 4 h 30 min with RedStrad2SKLP). Whereas a dozen peroxisomes were visible in each JMY3165 cell, only a few giant peroxisomes (up to four) were visible in each JMY3170 cell (Fig. 6A, see particularly the results at 4 h 30 min, RedStrad2SKLP). The same results were obtained using Pot1-GFPp, a green fluorescent protein (GFP)-tagged 3-ketocyl coenzyme A (CoA) thiolase used previously by Chang et al. (26) to stain peroxisomes. Small peroxisomes were stained in the wild type (JMY4729), while large peroxisomes were stained in the ΔYlpex11 strain (JMY4730) (Fig. 6B). These results strongly suggest that, as in other organisms, YlPex11p is involved in peroxisome fission and inheritance in Y. lipolytica. However, after 21 and 48 h of culture in YNBO3, peroxisomes were physically closer to lipid bodies in both strains, probably to break down the FAs, but we cannot exclude that these results were caused by the size of lipid bodies that filled up most of the cells, changing the geometries of peroxisomes in cells.

The FA contents of the reference strain and ΔYlpex11 were analyzed using GC. The results revealed that FAs accounted for 6%, 25%, and 28% of JMY2900 cell dry weight (CDW) after 24 h or 48 h of growth on YNBD0, YNBD0.5O3, and YNBO3, respectively. In contrast, FAs accounted for 8%, 10%, and 33%, respectively, of ΔYlpex11 CDW under the same conditions (Fig. 6C). Notably, when exposed to nonlethal conditions (YNBD0 and YNBO3), ΔYlpex11 was able to accumulate more FAs than the reference strain. This higher total FA accumulation likely reflected the reduced β-oxidation activity in ΔYlpex11. Indeed, the FA profiles of JMY2900 and ΔYlpex11, obtained from strains grown on YNBD0.5O3, revealed some interesting differences. The main constituents of the oleic acids used in this study were the following: 73% C18:1(n-9), 7% C18:2(n-6), 4.7% C16:1(n-7), 3.9% C16:0, and 0.9% C16:1(n-9) (Table 4). In JMY2900 cells, C16:1(n-9) accounted for 2.7% of the FAs, compared with the 0.9% present in the medium or the 1.2% present in ΔYlpex11 cells (Table 4). C16:1(n-9) cannot be synthesized by cells and is generated by the breakdown of C18:1(n-9). As the FAs that accumulate in Y. lipolytica cells generally reflect the FA composition of the extracellular medium (51), the overrepresentation of C16:1(n-9) in JMY2900 cells indicated that their β-oxidation was fully active. Similarly, the low level of C16:1(n-9) in ΔYlpex11 cells may indicate that β-oxidation, which produces this fatty acid by breaking down the C18:1(n-9) present in the medium, was not functional in this mutant. Moreover, C18:2(n-6) levels were proportionally higher in ΔYlpex11 than in JMY2900: that particular fatty acid represented 17% versus 11% of the total FAs, respectively (Table 4). In Y. lipolytica, C18:2(n-6) can be synthesized by the Δ12 fatty acid desaturase Fad2p, which converts C18:1(n-9) into C18:2(n-6). It is therefore possible that the very high level of C18:2(n-6) in ΔYlpex11 might have been due to the greater availability of C18:1(n-9) resulting from nonfunctional β-oxidation in this mutant.

To determine if YlPex11p is localized to peroxisomes as ScPex11p, a fusion protein was constructed at the C terminus by using YFP. This fusion was introduced into the JMY3170 (ΔYlpex11 pTEF-RedStar2SKL derivative strain to create JMY2616 (ΔYlpex11 pTEF-RedStar2SKL pTEF-YlPEX11-YFP). We first analyzed the capacity of YlPex11-YFPp to complement the deletion of YlPEX11 in a drop test on oleate medium. This protein is only partially functional, since JMY2616 grew slowly on oleate and colonies were smaller (Fig. 7A). Analysis of its localization showed that YlPex11-YFPp is localized at the periphery of peroxisomes (Fig. 7B), demonstrating that its localization is similar to that of ScPex11p at this time of induction.

It still remains unclear why ΔYlpex11 is unable to oxidize FAs. We previously showed that Δpox4 or Δpox5 (acyl-CoA oxidases) mutants, which also form giant peroxisomes, are still able to break down FAs (52). The failure of ΔYlpex11 to grow on oleic acid is thus not a simple consequence of having large peroxisomes. Furthermore, the observation that RedStar2SKLP (PTS1) and Pot1-GFPp (PTS2) correctly targeted the peroxisomes in the reference strain and the ΔYlpex11 mutant indicates that, even in the knockout yeast cells, proteins with PTS1 and PTS2 sequences remained correctly located in peroxisomes at this time of induction. However, we cannot exclude the possibility that other important proteins involved in β-oxidation were not imported into the peroxisomes.

![Fig 7 Complementation of ΔYlpex11 by overexpression of pTEF-YlPEX11-YFP and localization of YlPex11p-YFPp.](image-url)
YIP11 deletion affects the lipid body morphology of yeast grown in oleic acid media. To better understand why ΔYlpex11 did not survive when grown on FA media, we studied the morphology of cells grown on YNBD0.5O3 (Fig. 8). After 4 h 30 min of culture, ΔYlpex11 cells stopped growing (Fig. 8A), which suggests that oleic acid may inhibit the growth of this strain. Similar results have been obtained for S. cerevisiae: when ΔScpex11 was cultivated in the presence of oleic acid and galactose, it stopped growing after 8 h of culture (8). Under the microscope, ΔYlpex11, after 3 h of growth, had a higher number of lipid bodies. However, at this time, we observed a typical shape (round) as with the reference strain, JMY2900 (Fig. 8B). However, after 4 h 30 min, ΔYlpex11 cells contained lipid bodies with seemingly modified shapes, and the lipid bodies appeared increasingly diffuse over time (Fig. 8B). At 24 h of growth, ΔYlpex11 cells had a round morphology; they were devoid of lipid bodies and vacuoles due to cell death (Fig. 8B). This phenotype has not been previously observed in mutants in which β-oxidation has been deleted, such as the Δpex1-6 mutant (51, 53) or the Δmfe mutant (53, 54), which suggests that the lack of functional β-oxidation could not by itself be responsible for the phenotype we observed. The survival rate analysis indicated that, when ΔYlpex11 and the reference strain were grown on YNBD0.5O3, strain survival rates were similar after the first 3 h of culture (84% and 97% live cells, respectively). Survival stayed constant for the reference strain (85 to 96%) (Fig. 8C and D) but decreased dramatically for ΔYlpex11: from 68% at 4 h 30 min of culture to 5% at 6 h 30 min and 7 h 30 min of culture, and finally to 1% at 24 h of culture (96-fold lower than the reference strain result) (Fig. 8C to E). These data were confirmed in a drop test (Fig. 8D). Taken together, these findings suggest that the ΔYlpex11 deletion mutant was not viable in the presence of oleic acid, probably due to improper lipid homeostasis (lipid body lysis).

Oleic acid toxicity may be due to profoundly altered triglyceride metabolism in yeast. One potential explanation for the appearance of diffuse lipid bodies in ΔYlpex11 (Fig. 8B) could be an increased remobilization of the triacylglycerols (TAGs) that make up most lipid bodies. Under this scenario, cell death would be caused by the liberation of FFAs into the cytoplasm. In order to better understand what happens when ΔYlpex11 is grown in YNBD0.5O3, the expression levels of genes involved in TAG storage (such as DGA1, LRO1, and particularly DGA2 [23]) and TAG remobilization (such as Tgl4p [24, 53]) were analyzed using RT-
PCR (Fig. 9). Surprisingly, the global expression levels of TGL4, DGA1, DGA2, and LRO1 were lower in ΔYlpex11 than in the reference strain, particularly at 24 h of growth (Fig. 9A). At 3 h of growth, expression levels of TGL4, DGA1, and LRO1 were quite similar in JMY2900 and ΔYlpex11 cells (Fig. 9A). However, also at 3 h of growth, DGA2 showed a higher level of expression in ΔYlpex11 cells, suggesting that TAG synthesis at this time was more active in ΔYlpex11 than it was in the reference strain (Fig. 9A). One possible explanation for this is that the decreased FA consumption in the mutant allowed for increased TAG storage. Interestingly, in JMY2900 after 4 h 30 min of growth, expression of TGL4, DGA1, and LRO1 stayed stable and expression of DGA2 increased, which resulted in TAG accumulation into the lipid bodies. In contrast, in ΔYlpex11, expression of DGA2 and LRO1 decreased, suggesting that TAG synthesis decreased also, and FFA levels increased (Fig. 9A). After 6 h 30 min and 7 h 30 min of culture, the reference strain demonstrated increased expression of all four genes; in contrast, only TGL4 expression increased in ΔYlpex11 (Fig. 9A). This pattern suggests that there was a change in lipid homeostasis in ΔYlpex11 that resulted in the presence of more and more FFAs in the cells, whereas in the reference strain, increased TGL4 expression was probably compensated by increased DGA1, DGA2, and LRO1 expression (Fig. 9A), leading to a greater level of TAGs. Analyses of the ratio of FFA versus TAG by HPLC between ΔYlpex11 and the reference strain confirmed this hypothesis. Indeed, the FFA/TAG ratio was increasing after 4 h 30 min of culture for ΔYlpex11 and reached 100-fold more than the wild type at 24 h (Fig. 9B). It may be that part of the cell death observed when ΔYlpex11 was grown on YNBD0.5O3 can be explained by the liberation of FFAs into the cytoplasm following lysis of the lipid bodies.

**TGL4 and DGA2 are partially involved in lipid body lysis.** In *Y. lipolytica*, DGA2 and TGL4 are the most important genes regulating TAG synthesis and remobilization, respectively (19, 24). It is therefore likely that they play a role in the lysis of lipid bodies. To test this hypothesis, we created ΔYlpex11 strains in which TGL4 was deleted and/or DGA2 was overexpressed. The strains were grown in YNBD0.5O3 and their cell morphology was characterized using microscopy. While ΔYlpex11 Δtg14 and ΔYlpex11 pTEF-DGA2 had similar growth patterns as ΔYlpex11, ΔYlpex11 Δtg14 pTEF-DGA2 demonstrated an intermediate growth pattern (Fig. 10A), suggesting that combining a TGL4 deletion with DGA2 overexpression partially rescued the ΔYlpex11 growth defect by reducing the strain’s sensitivity to oleic acid. Microscopic observations of ΔYlpex11 Δtg14 pTEF-DGA2 grown in YNBD0.5O3 supported this hypothesis; indeed, lysis of the lipid bodies occurred 2 h later than in ΔYlpex11 (Fig. 10B). In addition, we observed a multiplicity of small lipid bodies surrounding a large lipid body. Moreover, the drop test indicated that ΔYlpex11 Δtg14 pTEF-DGA2 cultivated in YNBD0.5O3 grew faster than ΔYlpex11 on YPD plates (data not shown). However, in ΔYlpex11 at 24 h of growth, neither TGL4 deletion nor DGA2 overexpression prevented cell
death (Fig. 10B and data not shown). Interestingly, the lipid bodies conserved a shape (albeit not corresponding to that of the wild type, JMY2900) which suggested that TGL4 deletion and DGA2 overexpression counteracted lipid body lysis but were not able to fully prevent it from occurring. Therefore, other genes are probably involved in the cell death of ΔYlpex11 grown in oleic acid media.

**Conclusions.** The aim of this study was to explore the role played by Pex11p in the oleaginous yeast *Y. lipolytica*. Studies with *S. cerevisiae* have yielded mixed results regarding ΔScpex11 growth on oleic acid; indeed, two previous studies that used the same strain, UTL-7A, had contradictory results (3, 12). We demonstrated here that ΔYlpex11 was unable to grow on FA media, suggesting that Pex11p is essential in allowing *Y. lipolytica* to grow on oleic acid. As in other organisms (3–8, 10, 33, 55), Ylpex11p is necessary for peroxisome fission, and therefore the deletion of Ylpex11 generates giant peroxisomes when yeast are grown in minimum oleic acid medium. In our study, these giant peroxisomes were able to transport RedStar2SKLp or Pot1-GFPp, an observation which suggests that, despite other changes to peroxisome function, all the proteins containing a PTS1 (SKL) or PTS2 [(R,K)-(L,V,I)-X5-(H,Q)-(L,A,F)] close to the N terminal, such as Pot1p] sequences were still correctly addressed to the peroxisome in minimum media. In addition to the difference in growth on oleic acid media, three other factors suggest that *S. cerevisiae* and *Y. lipolytica* have distinct peroxisome fission mechanisms: (i) putative phosphorylation sites differ between Ylpex11p and ScPex11p; (ii) *Y. lipolytica* has two distinct Pex11-like proteins (YALI0C04565p and YALI0D25498p); (iii) *Y. lipolytica* lacks homologues of the Pex25p, Pex27p, and Pex34p proteins found in *S. cerevisiae*. It seems likely that increased understanding of peroxisome fission in *Y. lipolytica* could be a promising line of research in the near future, especially because the peroxisome fission mechanism of *Y. lipolytica* is more similar to that in higher eukaryotes than in *S. cerevisiae*. Cell death of ΔYlpex11 mutants grown on minimum and rich oleic acid media may be mediated by an alteration of lipid homeostasis resulting in an increase of the FFA/TAG ratio and a modification of lipid body morphology that probably results in the release of FFAs into the cytoplasm. The underlying cause of the lipid body lysis is still not well understood, but it may involve TGL4 and DGA2. An imbalance in their expression (*TGL4* being overexpressed relative to *DGA2*) may contribute to the breakdown of lipid bodies, ultimately leading to their lysis. Indeed, *TGL4* deletion coupled with *DGA2* overexpression partially protected ΔYlpex11 against oleic acid toxicity, delaying cell death. Lipid homeostasis could also perturb sphingolipid metabolism. Sphingolipids are involved in a variety of biological processes, and the metabolites of these sphingolipids, such as ceramide, sphingosine, and sphingosine-1-phosphate, can regulate apoptosis (56). This could explain why the overexpression of *DGA2* and deletion of *TGL4* are not sufficient to save the phenotype of ΔYlpex11. All these results demonstrate that Ylpex11p is involved in lipid homeostasis in *Y. lipolytica* and that further research should be carried out in the near future to better understand the role of Ylpex11p in this process.

During the revision of the manuscript, complementary work on PEX11 function was performed in rich media and showed a defect in peroxisome biogenesis and a defect in the targeting of peroxisomal proteins (57). However, those authors did not report the effect on lipid body structure or number, in either the ΔYlpex11 cells showing abnormal small lipid bodies or in the ΔYlpex11 strain complemented by overexpression of YPEX11, which showed large egg-shaped lipid bodies. Taken together, these results show that Pex11p is involved in lipid homeostasis, maybe by connecting peroxisome biogenesis, lipid storage, and lipid remobilization. Further experiments have to be performed in minimum media containing lower oleic acid concentrations to reduce the effects of peroxisomal protein targeting and the effect of oleic acid toxicity.

**ACKNOWLEDGMENTS**

This work was supported by FIDOP/FASO funds (Fonds d’action stratégique des oléagineux) from the French vegetable oil and protein production industry.

We thank R. Rachubinski for the pUB4-pTEF-POT1-GFP vector. We also thank T. Rossignol for plasmid JMP1822 and C. Gaillardin for his helpful comments. We also thank J. Pearce and L. Higgins for their language editing services.

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