Introduction

Unsaturated fatty acids (UFAs) play a key role in maintenance of optimal physical and biological properties of cell membranes [1]. UFAs are biosynthesized from saturated fatty acids by two evolutionary unrelated classes of fatty acid desaturases: soluble fatty acid desaturases, which are expressed exclusively in plant plastids, and membrane-bound fatty acid desaturases (subsequent referred to as FADs), which are widespread in eukaryotes and some prokaryotes [2].

FADs are highly specific towards their fatty acyl substrates (either acyl-CoA or acyl-lipid) and towards the position and geometric configuration of the newly introduced double bond. A range of desaturase regioselectivities dependent on the reference point used by FADs to position the introduced double bond has been described. The main regioselective modes are: (1) the double bond is introduced between specific carbon atoms counted from the carboxy terminus (AX) or (2) methyl terminus (αX) of the fatty acyl substrate, and (3) a subsequent double bond is introduced a specific number of carbon atoms (usually three) counted from the pre-existing double bond (v+3) [3]. However, these FAD regioselectivities are not mutually exclusive, and Meesapyodsuk et al. [4] suggested assigning them primary and secondary modes to more precisely describe FAD regioselectivity. For example, multifunctional FADs preferentially producing Δ9,Δ12-18:2 and Δ9,Δ12-16:2 from Δ9-UFAs but also capable of producing minor amounts of Δ9,Δ12,Δ15-18:3 and Δ9,Δ12,Δ15-16:3 might be termed as v+3 (Δ12), indicating that the primary desaturase regioselectivity is v+3 and the preferred regioselectivity is Δ12. However, this nomenclature may require detailed information on FAD diagnostic substrates and products, which are often not available in the literature. Therefore, we will adhere to nomenclature designating FADs as Δ12- and/or Δ15-FADs. In fungal species such as Saccharomyces cerevisiae or Candida glabrata, the main UFAs are Δ9-UFAs. By contrast, in numerous Basidiomycota, Zygomycota and Ascomycota fungal species, such as Coprinus cinereus [5], Mortierella alpina [6–8], Fusarium moniliforme [9], Aspergillus nidulans [10], Pichia pastoris [11,12], Klyuyveromyces lactis [13], Saccharomyces kluveri [14,15] and the majority of Candida species [16], Δ12- and Δ15-FADs can desaturate Δ9-UFAs to polyunsaturated fatty acids (PUFAs).

Bifunctional Δ12/Δ15-FADs have been characterized in Ascomycota [4,9,10,17], Basidiomycota [5] and the protozoon Acanthamoeba castellan [18]. Additionally, bi- or multifunctional FADs that had previously been identified as FADs with a single desaturase regioselectivity [8,19] were recently reported [20,21]. As a result of the similarity between desaturation and hydroxylation reaction pathways, FADs from different kingdoms additionally produce minor amounts of hydroxy fatty acids [22–24].

The role of Δ9-FADs in pathogenic Candida sp. has been demonstrated in C. albicans, in which partial repression of Ole1p...
Δ9-FAD blocks hyphae and chlamydospore formation [25]. In *C. parapsilosis*, gene deletion of OLE1 impaired invasive growth, pseudohyphae formation and virulence in mice and increased susceptibility to macrophages and various stress factors such as SDS, salts and H₂O₂ [26].

The role of PUFA is more elusive than that of Δ9-UFAs, and PFAs are probably not essential under all growth conditions. Murayama et al. [27] demonstrated that the loss of PUFA production in *C. albicans* via targeted disruptions of the Δ12- and Δ15-FAD genes GAD2 and GAD3, respectively, did not lead to any changes in growth rate under low temperature, chlamydospore formation, hyphal formation or virulence under the conditions tested. In contrast, the hyphal form of *C. albicans* is enriched in PFAs compared to the yeast form which suggests a specific role for PFAs in *C. albicans* morphogenesis [28].

In this study, we isolated the coding regions of GAD2 and GAD3, homologs of fungal Δ12- and Δ15-FADs, respectively, from *C. parapsilosis*, an emerging fungal pathogen [29]. We expressed these FADs in a *Saccharomyces cerevisiae* expression system and identified a broad range of novel polyunsaturated and hydroxylated FA products, including an unusual Δ9,Δ12,Δ15-16:3 with a terminal double bond.

**Methods**

**Strains, media and growth conditions**

*S. cerevisiae* strain BY4741 (MATa his3Δ1 leu2Δ met15Δ ura3Δ; EUROSCARF, Germany) was cultivated in liquid medium lacking uracil (YNB-U: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented with Brent supplement mix without uracil according to the manufacturer’s instructions). In cultivation media, 2% glucose was used as the carbon source. Heterologous protein expression was induced in YNB-U media containing 2% galactose as the sole carbon source (YNBglc-U). When indicated, media were supplemented with 0.5 mM linoleic acid (Sigma-Aldrich), 0.25 mM hexadecadienoic acid (Saccharomyces cerevisiae) (22:2,12-16:2; Larodan), 1% tergitol and 0.65 M NaCl. *C. parapsilosis* (clinical isolate P-69 obtained from the mycological collection of the Faculty of Medicine, Palacky University, Olomouc, Czech Republic) was cultivated prior to lipid extraction and fatty acid methyl ester preparation.

**Lipid extraction and fatty acid methyl ester preparation**

Yeast cells were harvested and total cellular lipids were extracted as described by Buček et al. [34], and fatty acid methyl esters (FAMEs) were prepared according to Matoušková et al. [35]. The resulting FAMEs were extracted with hexane (600 μL), and the extracts were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) using the conditions described below. The relative abundances of individual fatty acids were calculated from the peak areas in GC/MS total ion current chromatograms.

**DMOX derivatization**

Fatty acid 4,4-dimethyloxazoline (DMOX) derivatives were prepared from FAME extracts according to Fay and Richli [36]. Briefly, hexane solvent was evaporated under a stream of nitrogen, and FAMEs were heated at 180°C overnight with 0.5 g 2-amino-2-methylpropanol. After cooling, the DMOX derivatives were dissolved in 5 ml dichloromethane and washed three times with 2 ml distilled water. The dichloromethane phase was dried over anhydrous MgSO₄, evaporated under a stream of nitrogen and redissolved in hexane. DMOX derivatives were analyzed using GC/MS under the conditions described below and identified by comparing their mass spectra to previously described spectra and using inferred empirical rules [37].

**TMS derivatization**

Hydroxy FAs were analyzed in the form of their trimethyloxyl (TMS) derivatives by treating the FAME extracts with excess N,O-bis(trimethylsilyl)acetamide (Sigma-Aldrich) in acetonitrile (10 min, 40°C), evaporated under a gentle stream of N₂ and redissolved in chloroform [24]. Freshly prepared TMS derivatives were analyzed using GC/MS under the conditions described below, and their mass spectra and retention behavior were compared to the previously published mass spectra of FAME TMS derivatives [24] and to that of a TMS derivative prepared from 1 mg methyl ricinoleate (methyl 12-hydroxyoleate, Sigma-Aldrich).

**Sequence and phylogenetic analysis**

The assembly of shotgun reads of *C. parapsilosis* genome publicly available in Candida Genome database was searched using the BLAST tool (http://www.candidagenome.org/cgi-bin/compute/blast_clade.pl) with previously characterized Δ12- and Δ15-FADs as a query.

Amino acid sequences of FADs were aligned using the Muscle algorithm [30], and the phylogeny of FADs was reconstructed with MEGA5 software [31] by neighbor-joining method with calculating the bootstrap support from 1,000 bootstrap replicates as a measure of statistical reliability. Desaturase topology was predicted using the programs TMHMM 2.0 [32] and HMMTOP [33]. Pairwise sequence alignment was performed using the EMBOSs Needle web-based tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Known FAD sequences were retrieved from GenBank. The FAD sequences reported here were deposited into GenBank under accession numbers FN380265 and FN380266.
GC/MS analysis

The FAME extracts and respective DMOX and TMS derivatives were analyzed with a 7890A gas chromatograph coupled to a 5975C mass spectrometer, equipped with electron ionisation and quadruple analyser (Agilent Technologies, Santa Clara, CA, USA) using DB-5MS or DB-WAX capillary columns (both J&W Scientific, Folsom, CA, USA; 30 m × 0.25 mm, film thickness 0.25 mm); the electron ionisation was set at 70 eV.

Conditions for the analysis of FAMEs and DMOX derivatives were as follows: carrier gas, He: 1 ml/min; 10:1 split ratio, injection volume 2 µl; injector temperature 220°C; thermal gradient 140°C to 245°C at 3°C/min, then at 8°C/min to 280°C and temperature held for 5 min. The temperature program was terminated at 245°C and held at this temperature for 10 min when the DB-WAX column was used. The TMS derivatives were analyzed on a DB-5MS column using the following thermal gradient: 50°C to 140°C at 10°C/min, then 1°C/min to 190°C, 3°C/min to 320°C and temperature held for 3 min.

Results

Sequence and phylogenetic analysis of CpFAD2 and CpFAD3 open reading frames

In the genome database of C. parapsilosis [38], we found two open reading frames (ORFs) homologous to fungal Δ12- and Δ15-FADs, and termed them CpFAD2 and CpFAD3, respectively. CpFad2 shares high amino acid (aa) sequence identity with C. albicans Δ12-FAD (80%) [27] and other Δ12-FADs from budding yeasts (Saccharomycotina) (>59%). CpFad2 shares lower sequence identity with bifunctional Δ15-FADs from filamentous Ascomycota (Pezizomycotina) (>36%) and Zygomy- cota (Mortierella alpina) (>34%). CpFad3 shares high sequence identity (79%) with Δ15-FAD from C. albicans and with Δ15-FADs from other budding yeasts (61%). CpFad3 also shares lower sequence identity with bifunctional Δ15-Δ12-FADs from filamentous Ascomycota (>36%) and Zygomy-cota (Mortierella alpina) (34%).

The CpFad2 sequence encodes a 426-aa putative polypeptide; CpFad3 encodes a 432-aa putative polypeptide. CpFad2 and CpFad3 share 57% identity and 74% similarity and contain up to six predicted transmembrane helices and a tripartite conserved...
hystidine-rich motif HX₃JH, HX₉JHH, (H/Q)X₉JHH, which indicates that these proteins belong to a non-heme iron enzyme family [3] (Fig. S1).

In the reconstructed phylogenetic tree of functionally characterized fungal Δ12- and Δ15-FADs, all FADs cluster into clades according to the class or subphylum of the source organism, with the exception of FADs from the filamentous yeasts Aspergillus nidulans, Fusarium moniliforme and Magnaporthe grisea, which create a group of multifunctional Δ15/Δ12 FADs [9]. Within the clades, FADs cluster generally into “Δ12-FAD” subclades and “Δ15-FAD” subclades. CpFad2 clusters within a subclade of strictly monofunctional Δ12-FADs, whereas CpFad3 belongs to a subclade of Δ15-FADs (Fig. 1).

Functional characterization of CpFad2 and CpFad3 in S. cerevisiae

We designed two pairs of specific primers to amplify the CpFad2 and CpFad3 ORFs, and we obtained a single amplicon for each primer pair from genomic DNA isolated from C. parapsilosis. Sequencing of CpFad2 and CpFad3 confirmed that the nucleotide sequences of the amplified coding regions are identical to those obtained from the C. parapsilosis genome database.

The sequence comparison of CpFad2 and CpFad3 with previously characterized FADs strongly suggests that CpFad2 is a Δ12-FAD, whereas CpFad3 is a Δ15-FAD. However, the homology-based functional annotation of FADs may fail to predict FAD substrate specificity [39] or regioselectivity [14]. To experimentally determine the specificities and regioselectivities of CpFad2 and CpFad3, we cloned their coding regions into the pYES2 vector under control of a galactose-inducible promoter. We transformed the resulting plasmids into S. cerevisiae, generating the CpFad2 and CpFad3 strains.

The total lipidic extracts of galactose-induced CpFad2 and CpFad3 strains were transesterified, and the resulting fatty acid methyl esters (FAMEs) were compared to the FAME extract from a control strain transformed with an empty plasmid (Empty strain).

In the FAME extract from the induced CpFad2 strain, we detected multiple PUFAs that were not present in the FAME extract from the control strain (Fig. 2 and Table 1). The double bond position of all detected PUFAs was confirmed by the presence of characteristic MS fragment ions of DMOX derivatives (Fig. 3). In the CpFad2 strain, the most abundant novel PUFAs were linoleic acid (Δ9,Δ12-18:2; 11.88% ± 0.99%) and hexadecadienoic acid (Δ9,Δ12-16:2; 5.25% ± 0.65%). Traces of an octadecadienoic acid isomer with a characteristic 12 atomic mass unit gap between m/z 224 to 236 and 264 to 276 were detected, which is indicative of a Δ11,Δ14 double bond position (Fig. S2). We presume that Δ11,Δ14-18:2 is an elongation product of Δ9,Δ12-16:2. Unexpectedly, we also detected α-linolenic acid (Δ9,Δ12,Δ15-18:3; 0.12% ± 0.11%) in the CpFad2 strain, which indicates that the α-linolenic acid is synthesized from linoleic acid via minor Δ15-desaturase activity of CpFad2 desaturase (Fig. 2).

Additionally, a hexadecatrienoate (0.77% ± 0.09%) with double bonds in position Δ9 and Δ12 and a third double bond in either the Δ14- or Δ15-position was identified in the CpFad2 strain (Fig. 2). The DMOX derivatives of FAMEs with terminal double bonds do not produce the characteristic fragmentation pattern exhibiting a 12 atomic mass unit gap [40]. However, we could unambiguously identify the triunsaturated product of CpFad2 as Δ9,Δ12,Δ15-16:3 with terminal (n-1) double bond by comparing its spectrum to that of α,Δ9,Δ12,Δ15-16:3 methylester [41] (Fig. S3) and to the mass spectrum of a previously described DMOX derivative of α,Δ9,Δ12,Δ15-16:3 [18,21] (Fig. 3B). To confirm the production of Δ9,Δ12,Δ15-16:3 via desaturation of Δ9,Δ12-16:2, the CpFad2 strain was supplemented with Δ9,Δ12-16:2. The increase in relative abundance of Δ9,Δ12,Δ15-16:3 in the supplemented CpFad2 strain indicated that Δ9,Δ12,Δ15-16:3 is produced by Δ15-desaturation (Fig. 3B, C).

In the CpFad3 strain, supplementing the cultivation medium with linoleic acid led to production of α-linolenic acid (3.62% ± 0.15%). No polyunsaturated products were detected in the
CpFAD3 strain cultivated without PUFA supplementation (Fig. 2 and Table 1). Surprisingly, supplementing the CpFAD3 cultivation medium with \( \Delta 9,\Delta 12-16:2 \) led to production of \( \Delta 9,\Delta 12,\Delta 15-16:3 \) (Fig. 4D). To rule out the possible interference of yeast \( \Delta 9 \)-FAD in the metabolism of \( \Delta 9,\Delta 12-16:2 \), the Empty strain was also supplemented with \( \Delta 9,\Delta 12-16:2 \). GC/MS analysis confirmed that \( \Delta 9,\Delta 12-16:2 \) is not desaturated to hexadecatrienoic acid in the Empty strain (Fig. 4A).

To determine the distribution of novel PUFAs in individual lipid classes, the total lipidic extract was analyzed using liquid chromatography with mass spectrometric analysis. The preliminary data indicates that PUFAs are distributed in phospholipids, i.e., phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, and triacylglycerols (data not shown). We have also investigated the effect of accumulation of PUFAs in the CpFAD2 and CpFAD3 strains on their growth rate and tolerance to alkali.

### Table 1. Relative abundances of fatty acids in FAME extracts of total cellular lipids from yeast strains.

| Fatty acid composition (%) | Fatty acid | Empty | Empty+ | CpFAD2 | CpFAD3 | CpFAD3+ |
|---------------------------|-----------|-------|--------|--------|--------|---------|
| 16:0                      | 27.22 ± 0.98 | 24.64 ± 1.01 | 27.04 ± 0.12 | 29.32 ± 0.58 | 25.84 ± 0.56 |
| \( \Delta 9,\Delta 12-16:1 \) | 41.99 ± 1.15 | 5.07 ± 0.06 | 29.71 ± 0.10 | 36.56 ± 0.48 | 3.45 ± 0.74 |
| \( \Delta 9,\Delta 12-16:2 \) | n.d. | n.d. | 5.25 ± 0.65 | n.d. | n.d. |
| \( \Delta 9,\Delta 12-16:3 \) | n.d. | n.d. | 0.77 ± 0.09 | n.d. | n.d. |
| 18:0                      | 6.41 ± 0.51 | 6.21 ± 1.06 | 8.97 ± 0.30 | 8.72 ± 0.36 | 8.23 ± 0.61 |
| \( \Delta 9,\Delta 12-18:2 \) | 24.38 ± 1.07 | 2.72 ± 0.26 | 16.26 ± 1.41 | 25.41 ± 0.43 | 2.74 ± 0.41 |
| \( \Delta 9,\Delta 12-18:3 \) | n.d. | n.d. | 61.36 ± 1.99 | 11.88 ± 0.99 | n.d. | 52.79 ± 1.15 |
| \( \Delta 11,\Delta 14-18:2 \) | 0.35 ± 0.01 | n.d. | n.d. | n.d. |
| \( \Delta 9,\Delta 12-15-18:3 \) | n.d. | n.d. | 0.12 ± 0.11 | n.d. | 6.95 ± 0.99 |

The relative amount of fatty acids is expressed as a percentage of total fatty acid methyl esters. Strains supplemented with 0.5 mM linoleic acid and 1% tertigol are marked with “+”. Values represent means of three cultivations ± standard deviation. n.d.: FAME not detected.

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Figure 3. Mass spectra of DMOX derivatives of PUFAs detected in FAME extracts from CpFAD2 and CpFAD3 yeast strains. (A) \( \Delta 9,\Delta 12-16:2 \); (B) \( \Delta 9,\Delta 12,\Delta 15-16:3 \); (C) \( \Delta 9,\Delta 12-18:2 \); (D) \( \Delta 9,\Delta 12,\Delta 15-18:3 \). Characteristic fragments are highlighted and fragmentation patterns of DMOX derivatives are shown.

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metal cations. When compared to the Empty strain, we observed a decreased growth rate for yeasts expressing \( \text{CpFAD2} \) on YNBgal-U agar plates (Fig. S4B). The decreased growth rate of the \( \text{CpFAD2} \) strain became even more prominent on YNBgal-U containing 0.65 M NaCl (Fig. S4C). The growth rate of the \( \text{CpFAD3} \) strain on YNBgal-U or YNBgal-U supplemented with linoleic acid was unaltered compared to that of the Empty strain (Fig. S4B, D).

Hydroxylation activity of \( \text{CpFad2} \) and \( \text{CpFad3} \)
To determine whether \( \text{CpFad2} \) and \( \text{CpFad3} \) are capable of FA hydroxylation, we treated the FAME extracts with \( \text{N,O-bis(trimethylsilyl)acetamide} \) to convert hydroxy FAMEs to their corresponding trimethylsilyl-FAMEs, which exhibit better chromatographic properties and provide MS fragment ions characteristic of particular TMS group locations [42]. Trace amounts of TMS derivatives of methyl 9-hydroxypalmitic acid (MS fragment ions at \( m/z \) 201 and 259) and methyl 9-hydroxystearic acid (MS fragment ions at \( m/z \) 229 and 259) were detected in all transformed yeast strains. Characteristic fragments are highlighted in bold, and fragmentation patterns of TMS derivatives are shown above the spectra. doi:10.1371/journal.pone.0093322.g005

Figure 5. Mass spectra of TMS derivatives of hydroxy fatty acid methyl esters. (A) TMS-methyl ricinoleate detected in FAME extracts from \( \text{CpFAD2} \) yeast strain. (B) TMS- methyl hydroxypalmitate detected in all yeast strains. (C) TMS-methyl hydroxyoleate detected in all yeast strains. The growth rate of the \( \text{CpFAD3} \) strain on YNBgal-U or YNBgal-U supplemented with linoleic acid was unaltered compared to that of the Empty strain (Fig. S4B, D).

Figure 4. GC chromatograms of FAME extracts from yeasts supplemented with hexadecadienoic acid (\( \Delta_9,\Delta_{12}-16:2 \)). GC/MS analysis of FAME extracts from (A) Empty strain supplemented with hexadecadienoic acid (\( \Delta_9,\Delta_{12}-16:2 \)), (B) \( \text{CpFAD2} \) strain, (C) \( \text{CpFAD2} \) strain supplemented with \( \Delta_9,\Delta_{12}-16:2 \) and (D) \( \text{CpFAD3} \) strain supplemented with \( \Delta_9,\Delta_{12}-16:2 \). Hexane containing internal standard (hexadecane at concentration of 20 \( \mu \)g/ml) was used in preparation of all extracts. doi:10.1371/journal.pone.0093322.g004
Table 2. Relative abundances of TMS derivatives of hydroxy FAs in FAME extracts from CpFAD2, CpFAD3 and Empty strains.

| TMS-derivative                      | Empty          | Empty+         | CpFAD2         | CpFAD3         | CpFAD3+        |
|-------------------------------------|----------------|----------------|----------------|----------------|----------------|
| TMS-methyl hydroxypalmitate         | 0.09 ± 0.01    | 0.05 ± 0.01    | 0.10 ± 0.03    | 0.09 ± 0.01    | 0.04 ± 0.01    |
| TMS-methyl ricinoleate              | n.d.           | n.d.           | 0.10 ± 0.02    | n.d.           | n.d.           |
| TMS-methyl hydroxysterate           | 0.06 ± 0.00    | 0.02 ± 0.00    | 0.09 ± 0.00    | 0.07 ± 0.01    | 0.02 ± 0.00    |

The relative amount of FAME-TMS derivatives is expressed as a percentage of total fatty acid methyl esters. Values represent means of three cultivations ± standard deviation. n.d.: TMS-derivative not detected.

Table 3. Relative abundance of fatty acids in FAME extracts from C. parapsilosis.

| Fatty acid       | C. parapsilosis (%) |
|------------------|---------------------|
| 16:0             | 12.54 ± 0.07        |
| Δ9-16:1          | 3.38 ± 0.04         |
| Δ9,12-16:2       | 0.05 ± 0.01         |
| Δ9,12,15-16:3    | n.d.                |
| 18:0             | 4.31 ± 0.09         |
| Δ9,18-1          | 54.96 ± 0.40        |
| Δ9,12-18:2       | 22.89 ± 0.15        |
| Δ9,12,15-18:3    | 1.90 ± 0.24         |

The relative amount of fatty acids is expressed as a percentage of total fatty acid methyl esters. Values represent means of three cultivations ± standard deviation. n.d.: FAME not detected.
into a bifunctional Δ12-FAD/hydroxylase [22,50]. Based on these observations and our current results, we hypothesize that minor Δ15-desaturase regioselectivity and hydroxylase activity might be present in numerous functionally characterized Δ12-FADs from budding yeasts, including Cpfad2 from C. albicans [27]. The fact that minor PUFA and hydroxylated products were rarely detected in previously described Δ12-FADs from budding yeast might be caused by a low level of desaturase expression under the experimental conditions, by a low level of accumulation of PUFAs and hydroxylated fatty acids or by the low sensitivity of the FAME analysis procedure. The reason underlying the absence of specific Δ15-hydroxylated FAs in Cpfad3 strain supplemented with linoleic acid might be the absence of Cpfad3 Δ15-hydroxylase activity and/or the overall low enzymatic activity of Cpfad3 compared to Cpfad2 in our expression system.

Based on the range of observed desaturase specificities of Cpfad2 [its capability of introducing a double bond at the Δ15 (ω1) position in Δ9,Δ12,Δ15-16:3 and Δ15(ω3) position in Δ9,Δ12,Δ15-18:3 and the lack of Δ9,Δ15-18:2 products], we propose that the primary regioselective mode of Cpfad2 is ω4-3 and the secondary mode is Δ12. This would imply, in accordance with the FAD regioselectivity classification described by Meesapongsuk et al. [4], that the preexisting double bond serves as a reference point for positioning of the introduced double bond and that the Δ12-position is preferred over the Δ15-position.

The Cpfad3 strain produced Δ9,Δ12,Δ15-18:3 and Δ9,Δ12,Δ15-16:3 when supplemented with Δ9,Δ12-18:2 and Δ9,Δ12-16:2 PUFAs, respectively. The complete absence of novel PUFAs in the Cpfad3 strain cultivated without addition of linoleic acid or hexadecadienoic acid (ω6 substrates) indicates that Cpfad3 cannot desaturate the naturally present Δ9-FAs, in contrast to, for example, Ω3-FADs from Caenorhabditis elegans [51] or Saccharomyces kluveri [52]. Together, this data indicates that Cpfad3 exhibits Δ15-regioselectivity requiring a preexisting Δ12-double bond and is capable of introducing a terminal double bond.

Overexpression of Δ12- and Δ15(ω3)-FADs in S. cerevisiae provides a tool to study the influence of PUFAs on yeast physiology [19,53–55]. The PUFAs were present in phospholipid fraction of the Cpfad2 and Cpfad3 strain supplemented with linoleic acid, suggesting that they might influence the membrane properties and the yeast phenotype. However, we did not observe any increase in tolerance to NaCl, which has been observed in yeast strains producing PUFAs [55]. A decreased growth rate of yeasts heterologously expressing Δ12-FADs was previously attributed to impairment of tryptophan uptake [54]. Although we employed the BY4741 yeast strain which is not auxotrophic for tryptophan, the modified structure and physical properties of yeast cell membranes containing PUFAs might affect the properties of numerous cell membrane proteins and result in a decreased growth rate.

In C. parapsilosis, the low amount or complete absence of C16 PUFAs and low amount of palmimetic acid is in general agreement with the results of a previous study by Moss et al. [16] that describes the fatty acid composition of various Candida species. Together, these data suggests that in Candida species, C16 PUFAs do not accumulate. The low content of C16 PUFAs might be a consequence of a low amount of their precursor, palmimetic acid. Alternatively, overexpression of Cpfad2 under control of the GAL1 promoter might result in higher expression and therefore higher desaturase activity of Cpfad2 in S. cerevisiae, as compared to the activity of Cpfad2 in C. parapsilosis.

Taken together, this study provides further evidence that, despite the growing database of functionally characterized FADs, detailed characterization of FADs by mass spectrometry analysis of fatty acid derivatives can reveal surprising desaturase specificities that cannot be inferred solely from sequence comparisons.

Supporting Information

Figure S1 Amino acid sequence alignment of Cpfad2 and Cpfad3. Three conserved histidine-rich regions (H1–H3) are marked by boxes. Predicted transmembrane domains for Cpfad2 and Cpfad3 are indicated by bars above or below the sequence, respectively. The consensus transmembrane region predicted by both HMMTOP and TMHMM 2.0 algorithms are highlighted, and the fragmentation pattern of the DMOX derivative is shown above the spectra. (TIF)

Figure S2 Mass spectra of DMOX derivative of Δ11,Δ14-18:2-methylester detected in FAME extract from Cpfad2 yeast strain. Characteristic fragments are highlighted, and the fragmentation pattern of the DMOX derivative is shown above the spectra. (TIF)

Figure S3 Mass spectra of Δ9,Δ12,Δ15-16:3-methylester identified in FAME extract from Cpfad2 yeast strain. (TIF)

Figure S4 Comparison of growth rates of Cpfad2, Cpfad3 and Empty yeast strains. Yeast suspensions were spotted on (A) YNBglc-U agar plates, which repress heterologous protein expression, (B) YNBagl-U agar plates, (C) YNBgal-U agar plates containing 0.65 M NaCl and (D) YNBgal-U agar plates containing 1% tergitol and 0.5 mM linoleic acid. Prior to plating on solid media, yeast strains were grown on YNBglc-U agar plates and incubated overnight at 4°C. The cells then were resuspended in sterile water to an OD600 of 1.0. Serial 10-fold dilutions were spotted on the YNB agar plate using a replica plater. The agar plates were incubated at 30°C for 3 days and photographed. Representative images are shown. (TIF)

Figure S5 Extracted ion chromatograms of TMS derivatives of hydroxy FAMES. TMS derivatives of FAME extracts from the Cpfad2 strain, Cpfad3 strain supplemented with linoleic acid and Empty strain are displayed in ion chromatograms extracted at m/z values characteristic for individual TMS-hydroxy FAMEs. (A) Ion chromatograms extracted at m/z 201 and 259 characteristic for TMS-methyl hydroxypalmitate, (B) ion chromatograms extracted at m/z 229 and 259 characteristic for TMS-methyl hydroxyoctadecenate and (C) ion chromatograms extracted at m/z 187 and 299 characteristic for TMS-methyl ricinoleate. (TIF)

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Author Contributions

Conceived and designed the experiments: AB IP PM OHH HS. Performed the experiments: AB. Analyzed the data: AB. Contributed reagents/materials/analysis tools: IP HS. Wrote the paper: AB IP OHH.

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