



# $\Delta$ 12-Fatty Acid Desaturase from *Candida parapsilosis* Is a Multifunctional Desaturase Producing a Range of Polyunsaturated and Hydroxylated Fatty Acids

Aleš Buček<sup>1</sup>, Petra Matoušková<sup>1</sup>, Hana Sychrová<sup>2</sup>, Iva Pichová<sup>1\*</sup>, Olga Hrušková-Heidingsfeldová<sup>1\*</sup>

<sup>1</sup> Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic, <sup>2</sup> Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

## Abstract

Numerous  $\Delta$ 12-,  $\Delta$ 15- and multifunctional membrane fatty acid desaturases (FADs) have been identified in fungi, revealing great variability in the enzymatic specificities of FADs involved in biosynthesis of polyunsaturated fatty acids (PUFAs). Here, we report gene isolation and characterization of novel  $\Delta$ 12/ $\Delta$ 15- and  $\Delta$ 15-FADs named CpFad2 and CpFad3, respectively, from the opportunistic pathogenic yeast *Candida parapsilosis*. Overexpression of CpFad3 in *Saccharomyces cerevisiae* strains supplemented with linoleic acid ( $\Delta$ 9, $\Delta$ 12-18:2) and hexadecadienoic acid ( $\Delta$ 9, $\Delta$ 12-16:2) leads to accumulation of  $\Delta$ 15-PUFAs, i.e.,  $\alpha$ -linolenic acid ( $\Delta$ 9, $\Delta$ 12, $\Delta$ 15-18:3) and hexadecatrienoic acid with an unusual terminal double bond ( $\Delta$ 9, $\Delta$ 12, $\Delta$ 15-16:3). CpFad2 produces a range of  $\Delta$ 12- and  $\Delta$ 15-PUFAs. The major products of CpFad2 are linoleic and hexadecadienoic acid ( $\Delta$ 9, $\Delta$ 12-16:2), accompanied by  $\alpha$ -linolenic acid and hexadecatrienoic acid ( $\Delta$ 9, $\Delta$ 12, $\Delta$ 15-16:3). Using GC/MS analysis of trimethylsilyl derivatives, we identified ricinoleic acid (12-hydroxy-9-octadecenoic acid) as an additional product of CpFad2. These results demonstrate that CpFAD2 is a multifunctional FAD and indicate that detailed analysis of fatty acid derivatives might uncover a range of enzymatic selectivities in other  $\Delta$ 12-FADs from budding yeasts (Ascomycota: Saccharomycotina).

**Citation:** Buček A, Matoušková P, Sychrová H, Pichová I, Hrušková-Heidingsfeldová O (2014)  $\Delta$ 12-Fatty Acid Desaturase from *Candida parapsilosis* Is a Multifunctional Desaturase Producing a Range of Polyunsaturated and Hydroxylated Fatty Acids. PLoS ONE 9(3): e93322. doi:10.1371/journal.pone.0093322

**Editor:** Vishnu Chaturvedi, California Department of Public Health, United States of America

**Received:** November 19, 2013; **Accepted:** March 3, 2014; **Published:** March 28, 2014

**Copyright:** © 2014 Buček et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This study was funded by project TA01011461 from Technology Agency of the Czech Republic and by the research project RVO 613 88 963. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: iva.pichova@uochb.cas.cz (IP); olga-hh@uochb.cas.cz (OHH)

## 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 (subsequently 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 ( $\Delta$ X) or (2) methyl terminus ( $\omega$ X) 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  $\Delta$ 9, $\Delta$ 12-18:2 and  $\Delta$ 9, $\Delta$ 12-16:2 from  $\Delta$ 9-UFAs but also capable of producing minor

amounts of  $\Delta$ 9, $\Delta$ 12, $\Delta$ 15-18:3 and  $\Delta$ 9, $\Delta$ 12, $\Delta$ 15-16:3 might be termed as  $v+3$  ( $\Delta$ 12), indicating that the primary desaturase regioselectivity is  $v+3$  and the preferred regioselectivity is  $\Delta$ 12. 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  $\Delta$ 12- and/or  $\Delta$ 15-FADs.

In fungal species such as *Saccharomyces cerevisiae* or *Candida glabrata*, the main UFAs are  $\Delta$ 9-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], *Kluyveromyces lactis* [13], *Saccharomyces kluyveri* [14,15] and the majority of *Candida* species [16],  $\Delta$ 12- and  $\Delta$ 15-FADs can desaturate  $\Delta$ 9-UFAs to polyunsaturated fatty acids (PUFAs).

Bifunctional  $\Delta$ 12/ $\Delta$ 15-FADs have been characterized in Ascomycota [4,9,10,17], Basidiomycota [5] and the protozoan *Acanthamoeba castellanii* [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  $\Delta$ 9-FADs in pathogenic *Candida* sp. has been demonstrated in *C. albicans*, in which partial repression of Ole1p

$\Delta 9$ -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 H<sub>2</sub>O<sub>2</sub> [26].

The role of PUFAs is more elusive than that of  $\Delta 9$ -UFAs, and PUFAs 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  $\Delta 12$ - and  $\Delta 15$ -FAD genes *CaFAD2* and *CaFAD3*, 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 PUFAs compared to the yeast form which suggests a specific role for PUFAs in *C. albicans* morphogenesis [28].

In this study, we isolated the coding regions *CpFAD2* and *CpFAD3*, homologs of fungal  $\Delta 12$ - and  $\Delta 15$ -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  $\Delta 9, \Delta 12, \Delta 15$ -16:3 with a terminal double bond.

## Methods

### Strains, media and growth conditions

*Saccharomyces cerevisiae* strain BY4741 (MATa his3 $\Delta$ 1 leu2 $\Delta$  met15 $\Delta$  ura3 $\Delta$ ; EUROSCARF, Germany) was cultivated in liquid medium lacking uracil (YNBglc-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 (YNBgal-U). When indicated, media were supplemented with 0.5 mM linoleic acid (Sigma-Aldrich), 0.25 mM hexadecadienoic acid ( $\zeta 9, \zeta 12$ -16:2; Larodan), 1% tertgitol 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 in YNBglc medium at 37°C until the culture reached a stationary growth phase.

### 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](http://www.candidagenome.org/cgi-bin/compute/blast_clade.pl)) with previously characterized  $\Delta 12$ - and  $\Delta 15$ -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 calculated 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/](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 FN386265 and FN386266.

### Heterologous expression in *Saccharomyces cerevisiae*

Total cellular DNA was extracted from *C. parapsilosis* strain CP-69 and served as a template for PCR amplification of the *CpFAD2* coding region using the following primers: *CpFAD2*-5' (CTG GAG CTC ATG TCT TCA GCC ACA ACT TC) and *CpFAD2*-3' (CTG CTC GAG TTA TTT CTC TTT CTT TGG GT) (*SacI* and *XhoI* restriction sites are underlined; start and stop codons are italicized). The *CpFAD3* coding region was amplified using the primers *CpFAD3*-5' (CAG GAG CTC ATG AGT ACG GTC CAT GCA TC) and *CpFAD3*-3' (CTG CTC GAG CTA GTT TCT TGG TTT GAC AC). The resulting fragments were cloned into the vector pYES2 (Invitrogen) under control of the galactose-inducible *GALI* promoter, generating plasmids *CpFAD2*-pYES and *CpFAD3*-pYES. The constructs were verified by sequencing.

*S. cerevisiae* strain BY4741 was transformed with *CpFAD2*-pYES, *CpFAD3*-pYES and empty pYES2 plasmid by electroporation. The resulting *CpFAD2*, *CpFAD3* and Empty yeast strains were selected on YNBglc-U agar plates. Single colonies were inoculated into 20 ml of YNBglc-U and cultivated in a rotary shaker (30°C, 220 RPM). At the early stationary phase, yeast cells were harvested by centrifugation (3 min, 1000 g, 20°C), washed with 20 ml of YNBgal-U and transferred into 20 ml of fresh YNBgal-U (optionally supplemented with linoleic acid, hexadecadienoic acid and tertgitol). Yeast strains were cultivated for 72 h.

### Lipid extraction and fatty acid methyl ester preparation

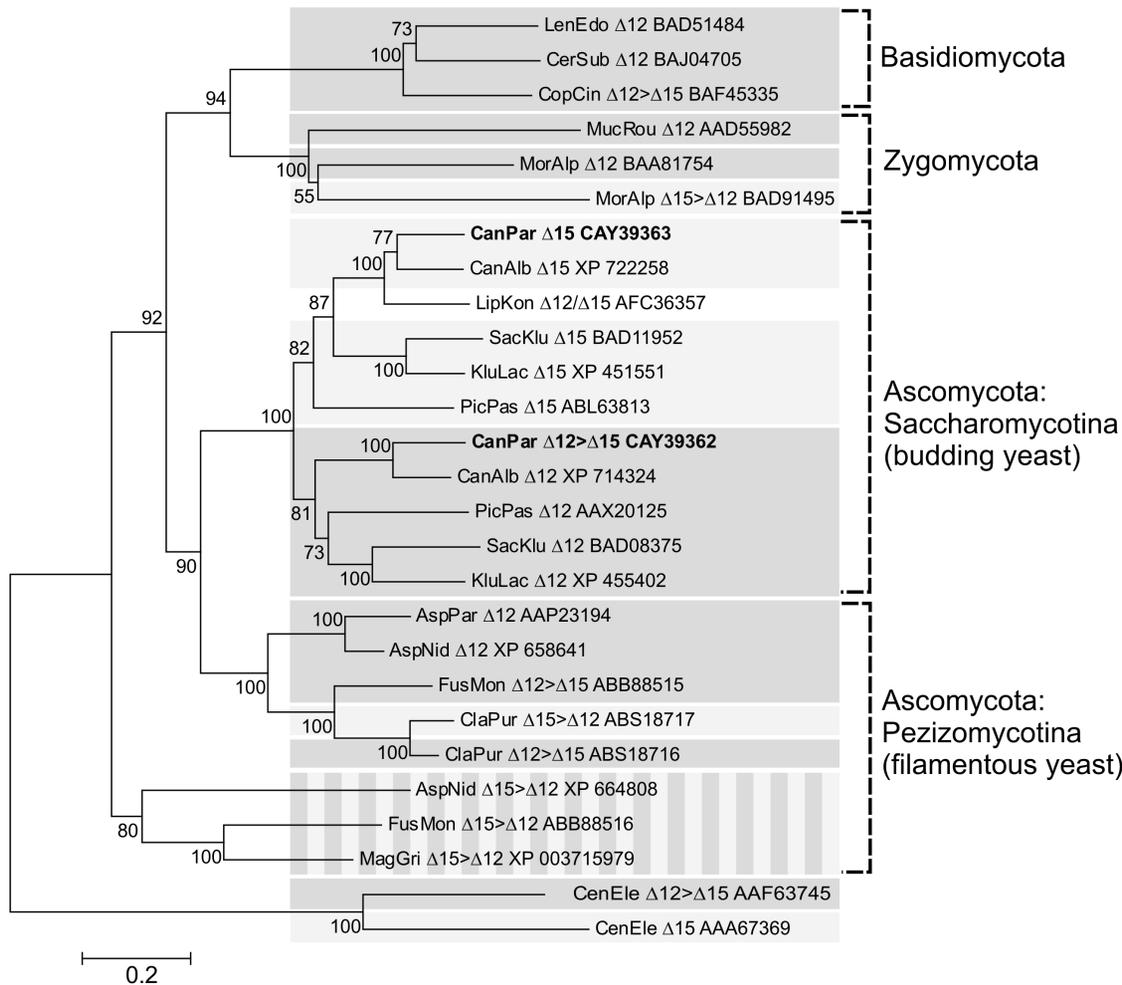
Yeast cells were harvested and total cellular lipids were extracted as described by Buček *et al.* [34], and fatty acid methyl esters (FAMES) were prepared according to Matoušková *et al.* [35]. The resulting FAMES were extracted with hexane (600  $\mu$ l), and the extracts were analyzed by gas chromatography 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-dimethylloxazoline (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 MgSO<sub>4</sub>, 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 trimethylsilyl (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 N<sub>2</sub> 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).



**Figure 1. Phylogenetic tree of fungal  $\Delta 12$ - and  $\Delta 15$ -FADs.** The species name is abbreviated and followed by experimentally determined desaturase regioselectivity and GenBank protein accession number. For multifunctional FADs, regioselectivity is indicated by ">" (e.g.,  $\Delta 12 > \Delta 15$  indicates a preference for  $\Delta 12$ -desaturation) if available in the literature. Dark grey background denotes  $\Delta 12$ -FADs and  $\Delta 12 > \Delta 15$ -FADs, light grey background denotes  $\Delta 15$ -FADs and  $\Delta 15 > \Delta 12$ -FADs and striped background highlights a clade of bifunctional  $\Delta 15 > \Delta 12$ -FADs. *CpFAD2* and *CpFAD3* are highlighted in bold.  $\Delta 12$ -FAD and  $\Delta 15$ -FAD from the nematode *Caenorhabditis elegans* were added as an outgroup. doi:10.1371/journal.pone.0093322.g001

## 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 quadrupole analyser (Agilent Technologies, Santa Clara, CA, USA) using DB-5MS or DB-WAX capillary columns (both J&W Scientific, Folsom, CA, USA; 30 m  $\times$  0.25 mm, film thickness 0.25 mm); the electron ionisation was set at 70 eV.

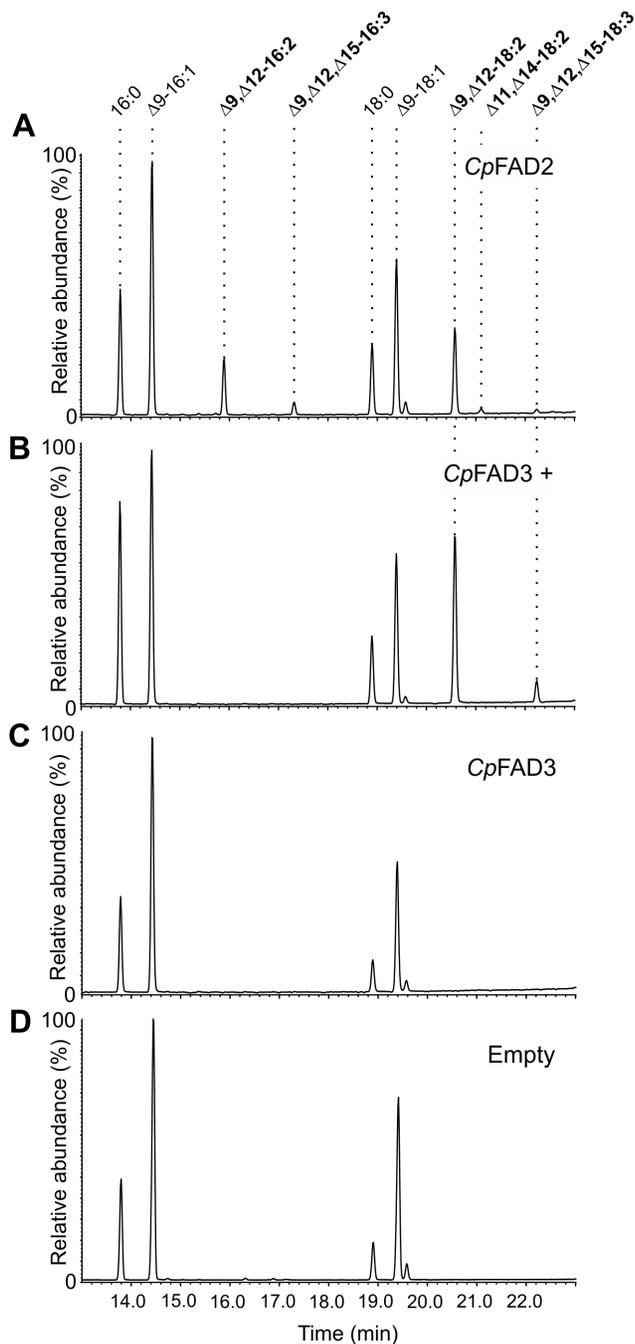
Conditions for the analysis of FAMES and DMOX derivatives were as follows: carrier gas, He: 1 ml/min; 10:1 split ratio, injection volume 2  $\mu$ l; injector temperature 220°C; thermal gradient 140°C to 245°C at 3°C/min, then at 8°C/min to 280°C and temperature held for 5 min. The temperature program 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 198°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 FAD2 and FAD3 desaturases and termed them *CpFAD2* and *CpFAD3*, respectively. *CpFAD2* shares high amino acid (aa) sequence identity with *C. albicans*  $\Delta 12$ -FAD (80%) [27] and other  $\Delta 12$ -FADs from budding yeasts (Saccharomycotina) (>59%). *CpFAD2* shares lower sequence identity with  $\Delta 12$ -FADs from filamentous Ascomycota (Pezizomycotina) (>44%), Basidiomycota (>33%) and Zygomycota (>32%). *CpFAD3* shares high sequence identity (79%) with  $\Delta 15$ -FAD from *C. albicans* and with  $\Delta 15$ -FADs from other budding yeasts (61%). *CpFAD2* also shares lower sequence identity with bifunctional  $\Delta 15 > \Delta 12$ -FADs from filamentous Ascomycota (>36%) and Zygomycota *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



**Figure 2. GC chromatograms of FAME extracts from *S. cerevisiae* strains.** Analysis of extracts from (A) strain expressing *CpFad2*, (B) strain expressing *CpFad3* grown in media supplemented with 0.5 mM linoleic acid and 1% tergitol, (C) strain expressing *CpFad3* and (D) strain bearing empty pYES2 plasmid. doi:10.1371/journal.pone.0093322.g002

histidine-rich motif HX<sub>3-4</sub>H, HX<sub>2-3</sub>HH, (H/Q)X<sub>2-3</sub>HH, 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  $\Delta 12$ - and  $\Delta 15$ -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  $\Delta 15/\Delta 12$  FADs [9]. Within the clades, FADs cluster generally into “ $\Delta 12$ -FAD” subclades and “ $\Delta 15$ -FAD” subclades. *CpFad2* clusters within a subclade of strictly monofunctional  $\Delta 12$ -FADs, whereas *CpFad3* belongs to a subclade of  $\Delta 15$ -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.

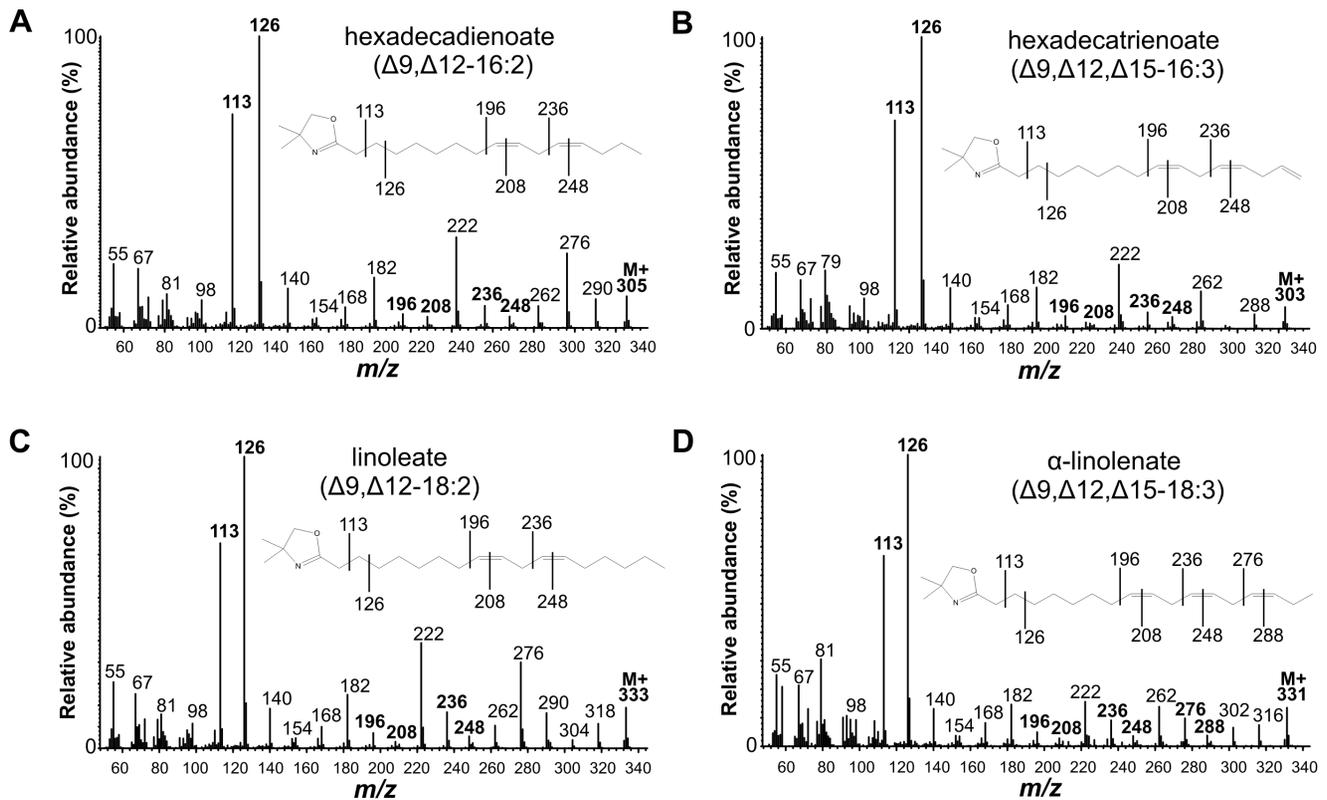
The sequence comparison of *CpFad2* and *CpFad3* with previously characterized FADs strongly suggests that *CpFad2* is a  $\Delta 12$ -FAD, whereas *CpFad3* is a  $\Delta 15$ -FAD. However, the homology-based functional annotation of FADs may fail to predict FAD substrate specificity [39] or regioselectivity [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 ( $\Delta 9,\Delta 12$ -18:2; 11.88%  $\pm$  0.99%) and hexadecadienoic acid ( $\Delta 9,\Delta 12$ -16:2; 5.25%  $\pm$  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  $\Delta 11,\Delta 14$  double bond position (Fig. S2). We presume that  $\Delta 11,\Delta 14$ -18:2 is an elongation product of  $\Delta 9,\Delta 12$ -16:2. Unexpectedly, we also detected  $\alpha$ -linolenic acid ( $\Delta 9,\Delta 12,\Delta 15$ -18:3; 0.12%  $\pm$  0.11%) in the *CpFAD2* strain, which indicates that the  $\alpha$ -linolenic acid is synthesized from linoleic acid *via* minor  $\Delta 15$ -desaturase activity of *CpFAD2* desaturase (Fig. 2).

Additionally, a hexadecatrienoate (0.77%  $\pm$  0.09%) with double bonds in position  $\Delta 9$  and  $\Delta 12$  and a third double bond in either the  $\Delta 14$ - or  $\Delta 15$ -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  $\Delta 9,\Delta 12,\Delta 15$ -16:3 with terminal (n-1) double bond by comparing its spectrum to that of  $\Delta 9,\Delta 12,\Delta 15$ -16:3 methyl ester [41] (Fig. S3) and to the mass spectrum of a previously described DMOX derivative of  $\Delta 9,\Delta 12,\Delta 15$ -16:3 [18,21] (Fig. 3B). To confirm the production of  $\Delta 9,\Delta 12,\Delta 15$ -16:3 *via* desaturation of  $\Delta 9,\Delta 12$ -16:2, the *CpFAD2* strain was supplemented with  $\Delta 9,\Delta 12$ -16:2. The increase in relative abundance of  $\Delta 9,\Delta 12,\Delta 15$ -16:3 in the supplemented *CpFAD2* strain indicated that  $\Delta 9,\Delta 12,\Delta 15$ -16:3 is produced by  $\Delta 15$ -desaturation (Fig. 4B, C).

In the *CpFAD3* strain, supplementing the cultivation medium with linoleic acid led to production of  $\alpha$ -linolenic acid (3.62%  $\pm$  0.15%). No polyunsaturated products were detected in the



**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.**

doi:10.1371/journal.pone.0093322.g003

*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).

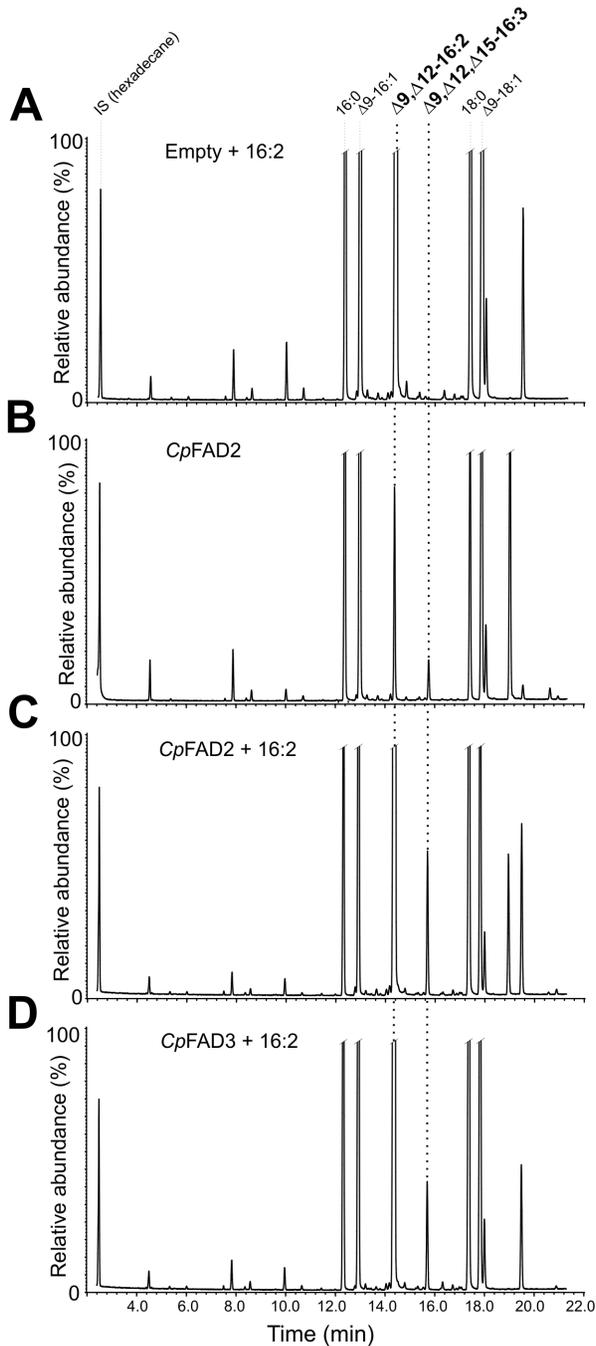
To determine the distribution of novel PUFAs in individual lipid classes, the total lipidic extract was analyzed using liquid chromatography with mass spectrometric analysis. The preliminary data indicates that PUFAs are distributed in phospholipids, *i.e.*, phosphatidylserine, phosphatidylethanolamine, 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	Fatty acid composition (%)				
	Empty	Empty+	<i>CpFAD2</i>	<i>CpFAD3</i>	<i>CpFAD3+</i>
16:0	27.22 ± 0.98	24.64 ± 1.01	27.04 ± 0.12	29.32 ± 0.58	25.84 ± 0.56
$\Delta 9-16:1$	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.
$\Delta 9, \Delta 12, \Delta 15-16:3$	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
$\Delta 9-18:1$	24.38 ± 1.07	2.72 ± 0.26	16.26 ± 1.41	25.41 ± 0.43	2.74 ± 0.41
$\Delta 9, \Delta 12-18:2$	n.d.	61.36 ± 1.99	11.88 ± 0.99	n.d.	52.79 ± 1.15
$\Delta 11, \Delta 14-18:2$	n.d.	n.d.	0.35 ± 0.01	n.d.	n.d.
$\Delta 9, \Delta 12, \Delta 15-18:3$	n.d.	n.d.	0.12 ± 0.11	n.d.	6.95 ± 0.99

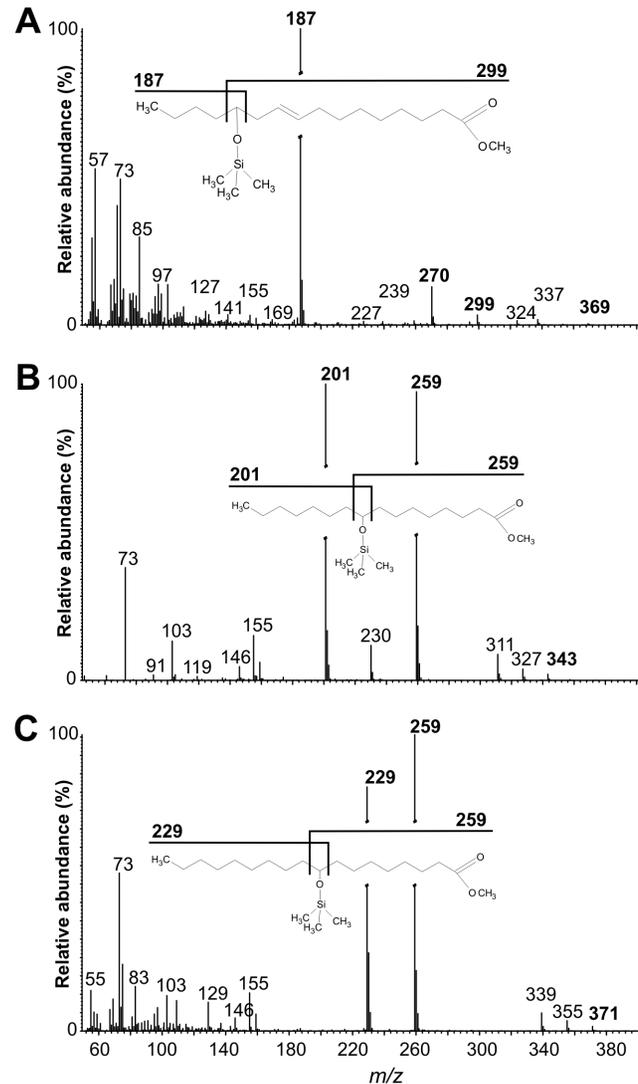
The relative amount of fatty acids is expressed as a percentage of total fatty acid methyl esters. Strains supplemented with 0.5 mM linoleic acid and 1% tertgitol are marked with "+". Values represent means of three cultivations ± standard deviation. n.d.: FAME not detected.

doi:10.1371/journal.pone.0093322.t001



**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) *CpFAD2* strain, (C) *CpFAD2* strain supplemented with  $\Delta 9, \Delta 12-16:2$  and (D) *CpFAD3* strain supplemented with  $\Delta 9, \Delta 12-16:2$ . Hexane containing internal standard (hexadecane at concentration of 20  $\mu\text{g}/\text{ml}$ ) was used in preparation of all extracts.  
doi:10.1371/journal.pone.0093322.g004

metal cations. When compared to the Empty strain, we observed a decreased growth rate for yeasts expressing *CpFAD2* on YNBgal-U agar plates (Fig. S4B). The decreased growth rate of the *CpFAD2* strain became even more prominent on YNBgal-U containing 0.65 M NaCl (Fig. S4C). The growth rate of the *CpFAD3* strain on



**Figure 5. Mass spectra of TMS derivatives of hydroxy fatty acid methyl esters.** (A) TMS-methyl ricinoleate detected in FAME extracts from *CpFAD2* yeast strain. (B) TMS-methyl hydroxypalmitate detected in all yeast strains. (C) TMS-methyl hydroxystearate detected in all 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

YNBgal-U or YNBgal-U supplemented with linoleic acid was unaltered compared to that of the Empty strain (Fig. S4B, D).

#### Hydroxylation activity of *CpFad2* and *CpFad3*

To determine whether *CpFad2* and *CpFad3* are capable of FA hydroxylation, we treated the FAME extracts with *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 (Fig. 5, S5 and Table 2), providing evidence of the intrinsic  $\Delta 9$ -fatty acid hydroxylase activity of yeast  $\Delta 9$ -FAD Ole1p [22,24]. Notably, in the *CpFAD2* strain, an additional TMS derivative with low abundance ( $0.10\% \pm 0.02\%$ ) was detected (Table 2). This was

**Table 2.** Relative abundances of TMS derivatives of hydroxy FAs in FAME extracts from *CpFAD2*, *CpFAD3* and Empty strains.

TMS-derivative	TMS-derivative composition (%)				
	Empty	Empty+	<i>CpFAD2</i>	<i>CpFAD3</i>	<i>CpFAD3+</i>
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 hydroxystearate	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. Strains supplemented with 0.5 mM linoleic acid and 1% tertgitol are marked with "+". Values represent means of three cultivations ± standard deviation. n.d.: TMS-derivative not detected.  
doi:10.1371/journal.pone.0093322.t002

determined to be a TMS derivative of methyl ricinoleate (methyl 12-hydroxyoleate) based on its retention time, which was identical to that of TMS derivative of methyl ricinoleate standard, and on the presence of characteristic fragment ions, namely (M-15) ion at  $m/z$  369 and ions at  $m/z$  187, 270 and 299 (Fig. 5A). This observation indicates that *CpFAD2* exhibits 12-hydroxylation activity.

### PUFA content in *C. parapsilosis*

Although the relative abundance of linoleic acid in *C. parapsilosis* (22.18%) was higher than that in the *CpFAD2 S. cerevisiae* strain (11.88%), we detected only trace amounts of  $\Delta 9, \Delta 12$ -16:2 (0.04%) in *C. parapsilosis* in contrast to moderately abundant  $\Delta 9, \Delta 12$ -16:2 (5.25%) in the *CpFAD2* strain. Notably,  $\Delta 9, \Delta 12, \Delta 15$ -16:3 was completely absent in *C. parapsilosis* under the conditions tested (Table 3). No additional PUFAs were detected under low cultivation temperatures (25°C or 30°C) (data not shown).

### Discussion

Fungal FAD research is largely motivated by the search for novel FADs that could be used in metabolic engineering of microorganisms to produce PUFAs and other valuable UFAs on an industrial scale [43,44]. Additionally, desaturation, as a part of fungal fatty acid metabolism, has been shown to play a crucial role in the growth and morphogenesis of pathogenic yeast species in plants and humans. Therefore, FADs have been suggested as potential targets for antifungal drugs [25–27,45,46].

Nguyen *et al.* [26] described the role of  $\Delta 9$ -FAD in the pathobiology of *C. parapsilosis*, a fungal pathogen that forms persistent biofilms on implanted medical devices such as catheters [29]. In addition to saturated and monounsaturated fatty acids, *C. parapsilosis* cellular lipids contain a major fraction of PUFAs, suggesting activity of  $\Delta 12$ - and  $\Delta 15$ -FADs [16]. In this study, we identified and characterized *CpFad2* and *CpFad3* desaturases responsible for production of PUFAs in *C. parapsilosis*.

Sequence analysis of *CpFad2* and *CpFad3* using TMHMM 2.0 and HMMTOP topology prediction algorithms indicated the presence of more than four transmembrane helices. The proposed topology, however, does not satisfy the requirement of arrangement of conserved histidine motifs on a common, cytosolic ER membrane face [47,48] (Fig. S1). Therefore, the third and fourth transmembrane helix likely represents a hydrophobic region peripherally associated with an ER membrane. This topology was previously proposed by Hoffmann *et al.* for  $\Delta 12$ -FAD and a bifunctional  $\Delta 12/\Delta 15$ -FAD from *Aspergillus nidulans* [10]. Alternatively, some of the additional TM helices might be correctly predicted and play a role in binding the acyl-lipid substrate, as hypothesized by Diaz *et al.* [49].

Several identified FADs can produce unusual terminally unsaturated  $\Delta 9, \Delta 12, \Delta 15$ -16:3 fatty acids, including FADs from the protozoan *Acanthamoeba castellanii* [18], the basidiomycete fungus *Coprinus cinereus* [5], the ascomycete filamentous fungus *Claviceps purpurea* [4], and the ascomycete budding yeast *Lipomyces kononenkoae* [17].  $\Delta 9, \Delta 12, \Delta 15$ -16:3 also has been identified as an initially undiscerned product of  $\Delta 15$ -FAD from the zygomycete fungus *Mortierella alpina* [21] and  $\Delta 12$ -FAD from the nematode *Caenorhabditis elegans* [20].

Previously,  $\Delta 9, \Delta 12, \Delta 15$ -16:3 was not detected as a product of *CaFad2* heterologously expressed in *S. cerevisiae* under control of the *GALI* promoter. *CaFad2* is a  $\Delta 12$ -FAD from *C. albicans*, which can produce  $\Delta 9, \Delta 12$ -16:2 and  $\Delta 9, \Delta 12$ -18:2 fatty acids [27]. However, the presence of  $\Delta 9, \Delta 12, \Delta 15$ -16:3 might be easily missed due to its overall low abundance or due to the low abundance of the molecular ion at  $m/z$  264 and low abundance of fragments at  $m/z$  74 and 87 characteristic of FAMES (Fig. S3).

Production of  $\alpha$ -linolenic, hexadecatrienoic ( $\Delta 9, \Delta 12, \Delta 15$ -16:3) and ricinoleic acid (12-hydroxyoleate) by *CpFad2* suggests that *CpFad2* is a bifunctional  $\Delta 12/\Delta 15$ -FAD. The  $\Delta 15$ -desaturase activity of  $\Delta 12$ -FAD from budding yeast (*Saccharomyces*) has been reported only by Yan *et al.* [17], and the hydroxylase activity has not been investigated. Damude *et al.* suggested that  $\Delta 15$ -FADs independently evolved from ancestral  $\Delta 12$ -FADs multiple times [9]. The facile evolutionary shift between  $\Delta 12$ - and  $\Delta 15$ -FAD regioselectivities was further supported by the effect of site-directed mutagenesis of a single amino acid residue [4] and by domain-swapping experiments [4,10]. Analogously, one to four amino acid mutations were found to be sufficient to convert plant  $\Delta 12$ -FAD

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

Fatty acid	Fatty acid composition (%)
	<i>C.parapsilosis</i>
16:0	12.54 ± 0.07
$\Delta 9$ -16:1	3.38 ± 0.04
$\Delta 9, \Delta 12$ -16:2	0.05 ± 0.01
$\Delta 9, \Delta 12, \Delta 15$ -16:3	n.d.
18:0	4.31 ± 0.09
$\Delta 9$ -18:1	54.96 ± 0.40
$\Delta 9, \Delta 12$ -18:2	22.89 ± 0.15
$\Delta 9, \Delta 12, \Delta 15$ -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.

doi:10.1371/journal.pone.0093322.t003

into a bifunctional  $\Delta 12$ -FAD/hydroxylase [22,50]. Based on these observations and our current results, we hypothesize that minor  $\Delta 15$ -desaturase regioselectivity and hydroxylase activity might be present in numerous functionally characterized  $\Delta 12$ -FADs from budding yeasts, including *CpFad2* from *C. albicans* [27]. The fact that minor PUFA and hydroxylated products were rarely detected in previously described  $\Delta 12$ -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  $\Delta 15$ -hydroxylated FAs in *CpFAD3* strain supplemented with linoleic acid might be the absence of *CpFad3*  $\Delta 15$ -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  $\Delta 15$  ( $\omega 1$ ) position in  $\Delta 9, \Delta 12, \Delta 15$ -16:3 and  $\Delta 15$  ( $\omega 3$ ) position in  $\Delta 9, \Delta 12, \Delta 15$ -18:3 and the lack of  $\Delta 9, \Delta 15$ -18:2 products], we propose that the primary regioselective mode of *CpFad2* is  $v+3$  and the secondary mode is  $\Delta 12$ . This would imply, in accordance with the FAD regioselectivity classification described by Meesapyodsuk *et al.* [4], that the preexisting double bond serves as a reference point for positioning of the introduced double bond and that the  $\Delta 12$ -position is preferred over the  $\Delta 15$ -position.

The *CpFAD3* strain produced  $\Delta 9, \Delta 12, \Delta 15$ -18:3 and  $\Delta 9, \Delta 12, \Delta 15$ -16:3 when supplemented with  $\Delta 9, \Delta 12$ -18:2 and  $\Delta 9, \Delta 12$ -16:2 PUFAs, respectively. The complete absence of novel PUFAs in the *CpFAD3* strain cultivated without addition of linoleic acid or hexadecadienoic acid ( $\omega 6$  substrates) indicates that *CpFAD3* cannot desaturate the naturally present  $\Delta 9$ -FAs, in contrast to, for example,  $\omega 3$ -FADs from *Caenorhabditis elegans* [51] or *Saccharomyces kluyveri* [52]. Together, this data indicates that *CpFad3* exhibits  $\Delta 15$ -regioselectivity requiring a preexisting  $\Delta 12$ -double bond and is capable of introducing a terminal double bond.

Overexpression of  $\Delta 12$ - and  $\Delta 15$  ( $\omega 3$ )-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  $\Delta 12$ -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 palmitoleic 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, palmitoleic acid. Alternatively, overexpression of *CpFad2* under control of the *GALI* 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 indicated by solid bars; transmembrane regions predicted by only one algorithm are indicated by dashed bars. Identical residues are indicated by a black background, similar residues by a grey background.

(TIF)

**Figure S2 Mass spectra of DMOX derivative of  $\Delta 11, \Delta 14$ -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  $\Delta 9, \Delta 12, \Delta 15$ -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) YNBgal-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 OD<sub>600</sub> of 1.0. Serial 10-fold dilutions were spotted on the YNB agar plates 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 hydroxystearate and (C) ion chromatograms extracted at  $m/z$  187 and 299 characteristic for TMS-methyl ricinoleate.

(TIF)

## Acknowledgments

We thank Dr. Petr Hamal (University Hospital in Olomouc, Czech Republic) for providing the clinical isolate of *C. parapsilosis* and H. Hoffman for proofreading of the manuscript.

## 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.

## References

- Aguilar PS, de Mendoza D (2006) Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. *Mol Microbiol* 62: 1507–1514.
- Sperling P, Ternes P, Zank TK, Heinz E (2003) The evolution of desaturases. *Prostaglandins Leukot Essent Fatty Acids* 68: 73–95.
- Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. *Annu Rev Plant Physiol Plant Mol Biol* 49: 611–641.
- Meesapyodsuk D, Reed DW, Covello PS, Qiu X (2007) Primary structure, regioselectivity, and evolution of the membrane-bound fatty acid desaturases of *Claviceps purpurea*. *J Biol Chem* 282: 20191–20199.
- Meesapyodsuk D, Reed DW, Covello PS, Qiu X (2007) Primary structure, regioselectivity, and evolution of the membrane-bound fatty acid desaturases of *Claviceps purpurea*. *J Biol Chem* 282: 20191–20199.
- Sakuradani E, Kobayashi M, Ashikari T, Shimizu S (1999) Identification of Delta12-fatty acid desaturase from arachidonic acid-producing *Mortierella* fungus by heterologous expression in the yeast *Saccharomyces cerevisiae* and the fungus *Aspergillus oryzae*. *Eur J Biochem* 261: 812–820.
- Huang YS, Chaudhary S, Thurmond JM, Bobik EG, Yuan L, et al. (1999) Cloning of delta12- and delta6-desaturases from *Mortierella alpina* and recombinant production of gamma-linolenic acid in *Saccharomyces cerevisiae*. *Lipids* 34: 649–659.
- Sakuradani E, Abe T, Iguchi K, Shimizu S (2005) A novel fungal omega3-desaturase with wide substrate specificity from arachidonic acid-producing *Mortierella alpina* 1S-4. *Appl Microbiol Biotechnol* 66: 648–654.
- Damude HG, Zhang H, Farrall L, Ripp KG, Tomb J-F, et al. (2006) Identification of bifunctional delta12/omega3 fatty acid desaturases for improving the ratio of omega3 to omega6 fatty acids in microbes and plants. *Proc Natl Acad Sci U S A* 103: 9446–9451.
- Hoffmann M, Hornung E, Busch S, Kassner N, Ternes P, et al. (2007) A small membrane-peripheral region close to the active center determines regioselectivity of membrane-bound fatty acid desaturases from *Aspergillus nidulans*. *J Biol Chem* 282: 26666–26674.
- Wei DS, Li MC, Zhang XX, Zhou H, Xing LJ (2006) A novel Delta12-fatty acid desaturase gene from methylotrophic yeast *Pichia pastoris* GS115. *Acta Biochim Pol* 53: 753–759.
- Zhang X, Li M, Wei D, Xing L (2008) Identification and characterization of a novel yeast omega3-fatty acid desaturase acting on long-chain n-6 fatty acid substrates from *Pichia pastoris*. *Yeast* 25: 21–27.
- Kainou K, Kamisaka Y, Kimura K, Uemura H (2006) Isolation of Delta12 and omega3-fatty acid desaturase genes from the yeast *Kluyveromyces fragilis* and their heterologous expression to produce linoleic and alpha-linolenic acids in *Saccharomyces cerevisiae*. *Yeast* 23: 605–612.
- Oura T, Kajiwara S (2004) *Saccharomyces kluyveri* FAD3 encodes an omega3 fatty acid desaturase. *Microbiology* 150: 1983–1990.
- Watanabe K, Oura T, Sakai H, Kajiwara S (2004) Yeast Delta 12 fatty acid desaturase: gene cloning, expression, and function. *Biosci Biotechnol Biochem* 68: 721–727.
- Moss CW, Shinoda T, Samuels JW (1982) Determination of cellular fatty acid compositions of various yeasts by gas-liquid chromatography. *J Clin Microbiol* 16: 1073–1079.
- Yan Z, Zhuo L, Mula J, Xia W, Yangmin G, et al. (2013) Clone and identification of bifunctional  $\Delta 12/\Delta 15$  fatty acid desaturase LKFAD15 from *Lipomyces kononenkoae*. *Food Sci Biotechnol* 22: 573–576.
- Sayanova O, Haslam R, Guschina I, Lloyd D, Christie WW, et al. (2006) A bifunctional Delta12,Delta15-desaturase from *Acanthamoeba castellanii* directs the synthesis of highly unusual n-1 series unsaturated fatty acids. *J Biol Chem* 281: 36533–36541.
- Peyou-Ndi MM, Watts JL, Browse J (2000) Identification and characterization of an animal delta(12) fatty acid desaturase gene by heterologous expression in *Saccharomyces cerevisiae*. *Arch Biochem Biophys* 376: 399–408.
- Zhou X-R, Green AG, Singh SP (2011) *Caenorhabditis elegans* Delta12-desaturase FAT-2 is a bifunctional desaturase able to desaturate a diverse range of fatty acid substrates at the Delta12 and Delta15 positions. *J Biol Chem* 286: 43644–43650.
- Kikukawa H, Sakuradani E, Kishino S, Park S-B, Ando A, et al. (2013) Characterization of a trifunctional fatty acid desaturase from oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast expression system. *J Biosci Bioeng*.
- Broadwater JA, Whittle E, Shanklin J (2002) Desaturation and hydroxylation. Residues 148 and 324 of Arabidopsis FAD2, in addition to substrate chain length, exert a major influence in partitioning of catalytic specificity. *J Biol Chem* 277: 15613–15620.
- Serra M, Gauthier LT, Fabrias G, Buist PH (2006) Delta11 desaturases of *Trichoplusia ni* and *Spodoptera littoralis* exhibit dual catalytic behaviour. *Insect Biochem Mol Biol* 36: 822–825.
- Carvalho F, Gauthier LT, Hodgson DJ, Dawson B, Buist PH (2005) Quantitation of hydroxylated byproduct formation in a *Saccharomyces cerevisiae* Delta9 desaturating system. *Org Biomol Chem* 3: 3979–3983.
- Krishnamurthy S, Plaine A, Albert J, Prasad T, Prasad R, et al. (2004) Dosage-dependent functions of fatty acid desaturase Ole1p in growth and morphogenesis of *Candida albicans*. *Microbiology* 150: 1991–2003.
- Nguyen LN, Gacser A, Nosanchuk JD (2011) The stearoyl-coenzyme A desaturase 1 is essential for virulence and membrane stress in *Candida parapsilosis* through unsaturated fatty acid production. *Infect Immun* 79: 136–145.
- Murayama SY, Negishi Y, Umeyama T, Kaneko A, Oura T, et al. (2006) Construction and functional analysis of fatty acid desaturase gene disruptants in *Candida albicans*. *Microbiology* 152: 1551–1558.
- Ghannoum MA, Janini G, Khamis L, Radwan SS (1986) Dimorphism-associated variations in the lipid composition of *Candida albicans*. *J Gen Microbiol* 132: 2367–2375.
- Trofa D, Gacser A, Nosanchuk JD (2008) *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* 21: 606–625.
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32: 1792–1797.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739.
- Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305: 567–580.
- Tusnády GE, Simon I (2001) The HMMTOP transmembrane topology prediction server. *Bioinformatics* 17: 849–850.
- Buček A, Vogel H, Matoušková P, Prchalová D, Záček P, et al. (2013) The role of desaturases in the biosynthesis of marking pheromones in bumblebee males. *Insect Biochem Mol Biol* 43: 724–731.
- Matoušková P, Luxová A, Matoušková J, Jiroš P, Svatoš A, et al. (2008) A delta9 desaturase from *Bombus lucorum* males: investigation of the biosynthetic pathway of marking pheromones. *Chembiochem* 9: 2534–2541.
- Fay L, Richli U (1991) Location of double bonds in polyunsaturated fatty acids by gas chromatography-mass spectrometry after 4,4-dimethylloxazoline derivatization. *J Chromatogr A* 541: 89–98.
- Spitzer V (1996) Structure analysis of fatty acids by gas chromatography-low resolution electron impact mass spectrometry of their 4,4-dimethylloxazoline derivatives—a review. *Prog Lipid Res* 35: 387–408.
- Butler G, Rasmussen MD, Lin MF, Santos MAS, Sakthikumar S, et al. (2009) Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* 459: 657–662.
- Pereira SL, Huang Y-S, Bobik EG, Kinney AJ, Stecca KL, et al. (2004) A novel omega3-fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid. *Biochem J* 378: 665–671.
- Christie WW, Robertson GW, McRoberts WC, Hamilton JTG (2000) Mass spectrometry of the 4, 4-dimethylloxazoline derivatives of isomeric octadecenoates (monoenes). *Eur J Lipid Sci Technol* 102: 23–29.
- Pan Z, Rimando AM, Baerson SR, Fishbein M, Duke SO (2007) Functional characterization of desaturases involved in the formation of the terminal double bond of an unusual 16:3Delta(9,12,15) fatty acid isolated from *Sorghum bicolor* root hairs. *J Biol Chem* 282: 4326–4335.
- Nicolaides N, Soukup VG, Ruth EC (1983) Mass spectrometric fragmentation patterns of the acetoxy and trimethylsilyl derivatives of all the positional isomers of the methyl hydroxypalmitates. *Biol Mass Spectrom* 10: 441–449.
- Certik M, Shimizu S (1999) Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *J Biosci Bioeng* 87: 1–14.
- Uemura H (2012) Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives. *Appl Microbiol Biotechnol* 95: 1–12.
- Wilson RA, Calvo AM, Chang P-K, Keller NP (2004) Characterization of the *Aspergillus parasiticus* delta12-desaturase gene: a role for lipid metabolism in the *Aspergillus*-seed interaction. *Microbiology* 150: 2881–2888.
- Calvo AM, Gardner HW, Keller NP (2001) Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans*. *J Biol Chem* 276: 25766–25774.
- Prasad MR, Sreekrishna K, Joshi VC (1980) Topology of the delta 9 terminal desaturase in chicken liver microsomes and artificial micelles. Inhibition of the enzyme activity by the antibody and susceptibility of the enzyme to proteolysis. *J Biol Chem* 255: 2583–2589.
- Stukey JE, McDonough VM, Martin CE (1990) The OLE1 gene of *Saccharomyces cerevisiae* encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. *J Biol Chem* 265: 20144–20149.
- Diaz AR, Mansilla MC, Vila AJ, de Mendoza D (2002) Membrane topology of the acyl-lipid desaturase from *Bacillus subtilis*. *J Biol Chem* 277: 48099–48106.
- Broun P, Shanklin J, Whittle E, Somerville C (1998) Catalytic plasticity of fatty acid modification enzymes underlying chemical diversity of plant lipids. *Science* 282: 1315–1317.
- Meesapyodsuk D, Reed DW, Savile CK, Buist PH, Ambrose SJ, et al. (2000) Characterization of the regiochemistry and cryptoregiochemistry of a *Caenorhabditis elegans* fatty acid desaturase (FAT-1) expressed in *Saccharomyces cerevisiae*. *Biochemistry* 39: 11948–11954.
- Oura T, Kajiwara S (2008) Substrate Specificity and Regioselectivity of  $\Delta 12$  and  $\omega 3$  Fatty Acid Desaturases from *Saccharomyces kluyveri*. *Biosci Biotechnol Biochem* 72: 3174–3179.

53. Kajiwara S, Shirai A, Fujii T, Toguri T, Nakamura K, et al. (1996) Polyunsaturated fatty acid biosynthesis in *Saccharomyces cerevisiae*: Expression of ethanol tolerance and the FAD2 gene from *Arabidopsis thaliana*. *Appl Environ Microbiol* 62: 4309–4313.
54. Rodríguez-Vargas S, Sánchez-García A, Martínez-Rivas JM, Prieto JA, Rande-Gil F (2007) Fluidization of membrane lipids enhances the tolerance of *Saccharomyces cerevisiae* to freezing and salt stress. *Appl Environ Microbiol* 73: 110–116.
55. Yazawa H, Iwahashi H, Kamisaka Y, Kimura K, Uemura H (2009) Production of polyunsaturated fatty acids in yeast *Saccharomyces cerevisiae* and its relation to alkaline pH tolerance. *Yeast* 26: 167–184.