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Research Article

RESEARCH ARTICLE

PUFA-induced cell death is mediated by Yca1p-dependent and -independent pathways, and is reduced by vitamin C in yeast

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One sentence summary: PUFA production in *Saccharomyces cerevisiae* lead to shortened lifespan, oxidative stress and increased caspase activity; the lethal phenotype is reversed when treated with antioxidant vitamin C.

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ABSTRACT

Polyunsaturated fatty acids (PUFA) such as linoleic acid (LA, n-6, C18:2) and γ-linolenic acid (GLA, n-6, C18:3) are essential and must be obtained from the diet. There has been a growing interest in establishing a bio-sustainable production of PUFA in several microorganisms, e.g. in yeast *Saccharomyces cerevisiae*. However, PUFAs can also be toxic to cells because of their susceptibility to peroxidation. Here we investigated the negative effects of LA and GLA production on *S. cerevisiae* by characterizing a strain expressing active Δ6 and Δ12 desaturases from the fungus *Mucor rouxii*. Previously, we showed that the PUFA-producing strain has low viability, down-regulated genes for oxidative stress response, and decreased proteasome activity. Here we show that the PUFA strain accumulates high levels of reactive oxygen species (ROS) and lipid peroxides, and accumulates damaged proteins. The PUFA strain also showed great increase in metacaspase Yca1p activity, suggesting cells could die by caspase-mediated cell death. When treated with antioxidant vitamin C, ROS, lipid peroxidation and protein carbonylation were greatly reduced, and the activity of the metacaspase was significantly decreased too, ultimately doubling the lifespan of the PUFA strain. When deleting YCA1, the caspase-like activity and the oxidative stress decreased and although the lifespan was slightly prolonged, the phenotype could not be fully reversed, pointing that Yca1p was not the main executor of cell death.

Keywords: polyunsaturated fatty acids; oxidative stress; lipid peroxidation; protein carbonylation; caspase activation; *Saccharomyces cerevisiae*

INTRODUCTION

Polyunsaturated fatty acids (PUFA) have been in focus in the last few years because of their important roles in human health and nutrition (Marventano, Kolacz and Castellano 2015). Linoleic acid (LA, n-6, C18:2) and γ-linolenic acid (GLA, n-6, C18:3) are both essential fatty acids that must be obtained from the diet. Due to the importance of these fatty acids, there has been a growing interest in establishing a sustainable production of PUFA in several microorganisms, including in yeast *Saccharomyces cerevisiae* (Beaudoin *et al.* 2000; Domergue *et al.* 2002,

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2003). *Saccharomyces cerevisiae* is used as a production platform for many different chemicals, fuels and pharmaceuticals (Keasling 2010) as well as it is used as a model organism for molecular eukaryotic microbiology (Mirisola, Braun and Petranovic 2014), making it also attractive for production of PUFAs (Uemura 2012). However, *S. cerevisiae* has only one desaturase, Ole1p (delta-9 desaturase), which is required for the desaturation of saturated fatty acids to generate palmitoleic (C16:1) and oleic (C18:1) acids, which have only one double bond (Klug and Daum 2014). In our previous work, *S. cerevisiae* was engineered to express two heterologous desaturases from the fungus *Mucor rouxii* (Ruenwai *et al.* 2011). The expression of the Δ6 and Δ12 desaturases resulted in the production of LA and GLA, and it was shown that the PUFAs were incorporated in both storage lipids and phospholipids pools. Although production of PUFAs could be confirmed, their presence was shown to be damaging to cell viability. The PUFA-producing strain was shown to accumulate reactive oxygen species (ROS), has decreased proteasome activity and has a shorter lifespan (Ruenwai *et al.* 2011).

Like other aerobic organisms, *S. cerevisiae* has an effective defense system against ROS (Herrero *et al.* 2008; de la Torre-Ruiz, Pujol and Sundaran 2015) that can arise from electron 'leakage' from the electron transport chain (Herrero *et al.* 2008) and β-oxidation during the breakdown of fatty acids, (Kohlwein, Veenhuis and van der Klei 2013). Cells experience oxidative stress when the amount of ROS exceeds the capability of the antioxidant defense systems to detoxify. The resulting ROS, can react with (and cause damage to) biological macromolecules such as DNA, lipids and proteins. Yeast exposed to PUFAs (by endogenous/heterologous production or exogenous addition to the medium) is susceptible to lipid peroxidation (Priault *et al.* 2002; Cipak *et al.* 2006) because unsaturated double bonds in PUFAs are chemically more prone to oxidation, which results in lipid hydroperoxides that can initiate chain reactions leading to the formation of different cytotoxic aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Ayala, Munoz and Arguelles 2014). Aldehydes are highly reactive and can bind to the side chains of proteins, causing irreversible protein carbonylation (Berlett and Stadtman 1997). Carbonylated proteins lose their function and need to be degraded, otherwise they can cause cross-linking aggregation of proteins and lead to cell death (Farrugia and Balzan 2012).

In this study, we have investigated the PUFA toxicity and cell damage that lead to induction of cell death in *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains, media, growth conditions

The experiments were carried out in four different *S. cerevisiae* strains; LM 003-D126 (PUFA strain) and LM 001-REF (REF strain) engineered by Ruenwai *et al* (2011), and LM 003-D126_YCA1Δ (PUFA YCA1Δ strain) and LM 001-REF_YCA1Δ (REF YCA1Δ strain). In brief, the strain background is CEN.PK113-9D (MAT**a**; *ura3-52; HIS3; LEU2; trp1-289; MAL2-8; SUC2*); REF contains the empty plasmids pESC-TRP and YEp356; PUFA contains the plasmids pE912-TRP and pYE96-URA, expressing the delta-12 and the delta-6 desaturases, respectively. Yeast cultures were grown in minimal synthetic defined SD medium supplemented with 6.9 g/L yeast nitrogen base without amino acids, 720 mg/L complete supplement dropout (-TRP, -URA) and 20 g/L glucose. The media was adjusted to pH 5.8. The cultures were grown in 30°C at 200 rpm.

Table 1. Oligonucleotide primers used for YCA1 gene deletion. Primer overhangs containing part of YCA1 up/down sequences are in bold.

Primer	Primer sequence (5′-3′)
Fw1	CCTGTTAACTGACTGGTTGCATT
Rev1	CTGCAGCGTACGAAGCTTCAG**GATTGTAAATCTA GTCGGTC**
Fw2_kanmx	**GACCGACTAGATTTACAATC**CTGAAGCTTCGTAC GCTGCAG
Rev2_kanmx	**CCAACGTACACATTCATATATTT**CACTAGTGGAT CTGATATCAC
Fw3	GTGATATCAGATCCACTAGTG**AAATATATGAATG TGTACGTTGG**
Rev3	CCGAATTAGACGTAACTGTCCC
Rev_YCA1ctrl	CGCATCCTTGACCAACCATTGC

YCA1 gene deletion

The metacaspase MCA1/YCA1 is encoded by the YCA1 gene that was deleted in order to create the YCA1Δ strains. The gene was replaced by using the *loxP-kanMX-loxP* cassette acquired by PCR from the plasmid pUG6 (Wach *et al.* 1994) and a bipartite strategy (Erdeniz, Mortensen and Rothstein 1997). We used primers with upstream and downstream homologous regions of YCA1 for that purpose (Table 1). The PCR products were used for transformation of the PUFA and REF strains, and the transformed cells were selected in G418-YPD medium. The correct incorporation of the fragment disrupting the YCA1 coding sequence was tested by PCR.

Growth and viability assays

Yeast colonies were picked from agar plates and grown overnight after which the cultures were measured for OD_{600}. A total of 0.1 OD_{600} cells were centrifuged (12 000 × *g*) and resuspended in 20 mL SD-Trp-Ura media. The cultures were grown for five consecutive days, with sampling for the various assays taking place during exponential phase (day 0), days 2 and 4.

Specific growth rate was determined by measuring OD_{600} every 90 min for 12 h. Optical densities were then plotted in Excel and specific growth rates calculated as the natural logarithm (ln).

The viability was determined by spot test. A total of 4×10^6 cells ($OD_{600} = 0.2$) were sampled, centrifuged for 5 min at 6000 rpm, then resuspended in sterile Milli-Q water (18.2 MΩ, Merck Millipore), and then diluted in 10-fold series (10^{-1}, 10^{-2}, 10^{-3}). Five microliter were spotted on an SD-Trp-Ura agar plate, the plate was then placed in 30°C for 3 days before a snapshot of the colonies were taken.

Supplementation of vitamin C

Vitamin C, or L(+)-ascorbic acid (AppliChem), was supplied in a final concentration of 10 mM in the SD-Trp-Ura medium at the starting point (t = 0). The medium with ascorbic acid was buffered to pH 6.0 with K_2HPO_4 and KH_2PO_4 for a final concentration of 40 and 70 mM, respectively.

Induction of oxidative stress with H_2O_2

Oxidative stress was induced by the addition of the oxidizing agent 0.3 mM H_2O_2 when the optical density reached mid-exponential phase (OD_{600} ~0.8). For the viability test, 2.5 mM H_2O_2 was added to the medium. After the addition, the cultures were grown for two more hours before being spotted on SD-Trp-Ura plates.

Detection of ROS using the dihydrorhodamine 123 assay

Dihydrorhodamine 123 (DHR 123) is a fluorogenic probe that is used for the detection of ROS such as peroxide and peroxynitrite. It is not fluorescent until oxidized by ROS to form rhodamine 123. The method was performed as described previously (Madeo *et al.* 1999) with the following alterations. Cells ($OD_{600} = 1.0$) were harvested by centrifugation at 12 000 × *g*. After centrifugation, the cells were resuspended in 1 mL 50 mM sodium citrate buffer and 50 μM DHR (Life Technologies). The cells were incubated in darkness for 30 min at room temperature. After incubation the cells were centrifuged and resuspended in 40 μL sodium citrate buffer and viewed in an inverted fluorescent microscope (the Leica AF 6000 with a HCX PL APO CS 100.0×1.40 OIL objective was used for all staining protocols in the study) and images were captured with a DFC 360 FX camera and the Leica Application Suite software. For the quantification of the cells, at least 400 cells per sample were analyzed from three individual experiments (the quantification was repeated in the same way for the other fluorescent assays in the paper). Cells were viewed with the differential interference contrast filter and the YFP filter (excitation wavelength 500 nm, emission wavelength 536 nm).

Detection of ROS using the BODIPY C11 581/591 assay

C11-BODIPY (581/591) is a fluorescent probe for lipid peroxidation in model membrane systems and living cells. The BODIPY C11 assay was done as previously described in Hill *et al.* (2012). Cells ($OD_{600} = 1.0$) were harvested by centrifugation (6000 × *g* for 5 min) and resuspended in 0.5 mL phosphate buffered saline (PBS, pH 7.4) together with 2 μL of 2 mM BODIPY C11 (Molecular Probes) for 30 min at room temperature continuously shaking on a shaker. The cells were centrifuged (6000 × *g* for 5 min) and the pellet was resuspended in 0.5 mL PBS. The cells were inspected by fluorescent microscopy by using excitation wavelength 581 nm and emission wavelength 591 nm.

Detection of protein carbonylation using 2,4-dinitrophenylhydrazine derivatization and western blot assay

Protein carbonyl groups are commonly detected after derivatization with 2,4-dinitrophenylhydrazine (DNPH) and this is widely used as a measure of protein oxidation (Suzuki, Carini and Butterfield 2010). The method was performed as previously described in Levine *et al.* (1994), with the following alterations. The equivalent of 1 OD_{600} cells were prepared according to the following steps: the cells were pelleted and washed once with PBS (pH 7.4) then resuspended in 50 μL 12% SDS. Glass beads were added to the cells and then vortexed for 2×30 s. The cells were then boiled for 5 min and centrifuged for 1 min at 13 500 rpm. The supernatant (30 μL) was mixed with 60 μL DNPH and then left for 10 min at room temperature. The sample was then neutralized with 30 μL 2 M Tris containing 30% glycerol. The DNPH treated proteins (5 μg protein/sample) were run on a PAGE gel (Bio-Rad), transferred to an Immun-Blot PVDF membrane (Bio-Rad), and then incubated overnight at 4°C with an anti-DNP antibody (Sigma, D956). After the incubation, the membrane was washed in PBS-T 4×10 min with slow rotation. The membrane was then incubated with the secondary antibody (goat anti-rabbit igG-HRP, Santa Cruz Biotechnology) and blocking buffer for 1 h, then washed twice in PBS-T after which the carbonylated proteins were detected with a Gel Doc (Molecular Imager Gel Doc XR System 170–8170, Bio-Rad).

Detection of caspase-like activity using CaspACE FITC-VAD-FMK assay

The activation of metacaspase Yca1p can be detected by the caspase inhibitor FITC-VAD-FMK (CaspACE, Promega), which irreversibly binds to activated caspases. The assay was performed as previously described by Wysocki and Kron (2004). Cells (5×10^6) were harvested by centrifugation (6000 × *g* for 5 min), then washed once in 1 mL PBS. The pellets were then resuspended in 200 μL PBS containing 10 μM FITC-VAD-FMK (CaspACE, Promega). The cells were incubated in darkness for 20 min at room temperature on a shaker with low agitation. After the incubation, the cells were washed twice with PBS, and then detected with excitation at 488 nm and emission at 505 nm.

Detection of DNA using the 4′,6-diamidino-2-phenylindole assay

The 4′,6-diamidino-2-phenylindole (DAPI) assay is used to investigate chromatin morphology, it binds to AT-rich regions of the minor groove of DNA and emits blue fluorescence. The basic protocol for DAPI staining was used (Streiblova 1988). Cells (2×10^7, $OD_{600} = 1.0$) were harvested by centrifugation at 12 000 × *g*, and then resuspended in 50 μL sterile water. One milliliter EtOH (100%) and 1 μg mL^{-1} DAPI (1.1 μl of 1 mg mL^{-1} stock solution, Sigma-Aldrich) was added to the sample, and then incubated in darkness for 5 min at room temperature. After the incubation, the samples were washed twice with 1 mL water. The cells were visualized with fluorescence microscopy at excitation and emission wavelengths 358 and 461 nm, respectively.

Assessment of cell integrity using the propidium iodide assay

Propidium iodide (PI) is used to analyze cell integrity, which can be used as a viability marker. PI binds to DNA and emits red fluorescence, but since it is membrane impermeable it can only enter cells with ruptured membranes. The assay was performed as previously described in Weinberger, Ramachandran and Feng (2005). The equivalent of 1 OD_{600} cells were harvested by centrifugation (6000 rpm for 4 min), then washed once in 1 mL PBS. The pellets were resuspended in 1 mL PBS and 0.5 μL (1 mg mL^{-1}) PI (Life Technologies) and incubated for 20 min in darkness at room temperature. After the incubation, the samples were centrifuged (6000 rpm for 4 min) and the pellet was resuspended in 10 μL PBS. The cells were visualized with fluorescence microscopy at excitation and emission wavelengths 493 and 617 nm, respectively.

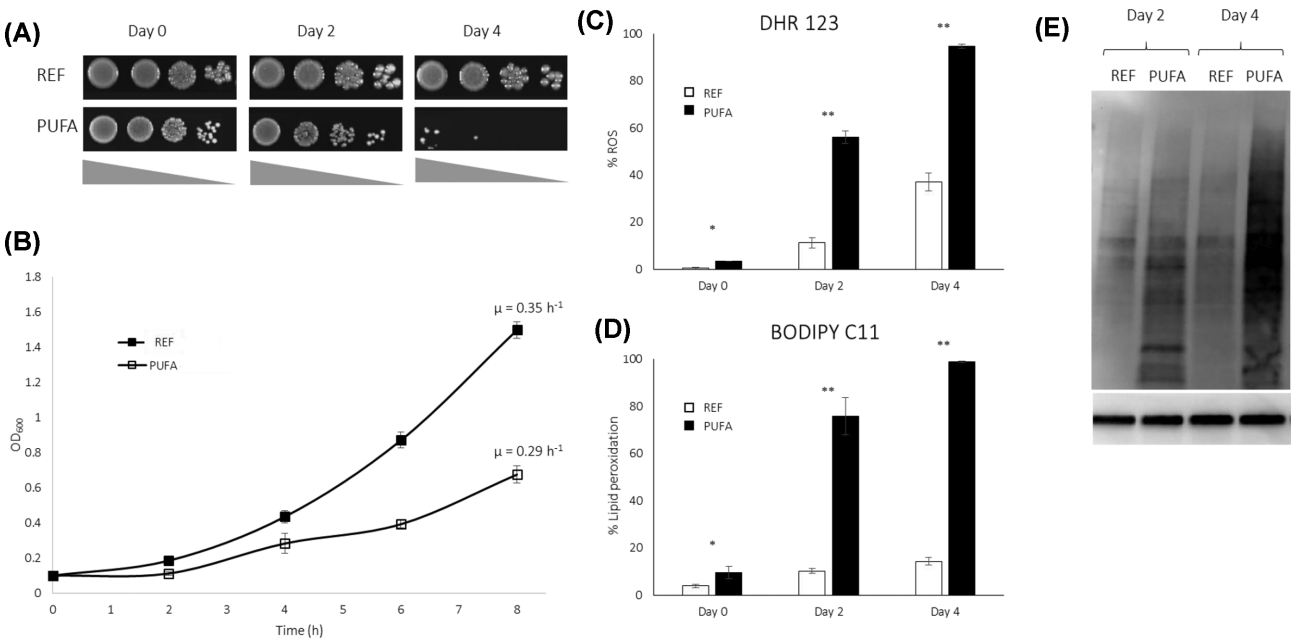

Figure 1. 'Reduced viability and growth rate, and higher levels of oxidative stress in the PUFA strain.' **(A)** Spot assay was performed with 10× serial dilutions on SD-Trp-Ura plates, with samples on days 0, 2 and 4. **(B)** Growth rates (μ) were calculated based on exponential growth on glucose in liquid medium in shake-flasks. **(C)** Accumulation of ROS during growth and stationary phase was measured with DHR 123 staining. **(D)** Accumulation of lipid peroxides during growth and stationary phase was measured with BODIPY C11. **(E)** Accumulation of carbonylated proteins during growth and stationary phase was measured with DNPH-derivatization and western blot. The loading control is GAPDH. (N = 3, ± SD, * = $P \leq 0.05$, ** = $P \leq 0.001$, Student's *t*-test, two-tailed, two-sample equal variance between PUFA and REF).

Data analysis

Data were analyzed using Student's *t*-test. Values are reported as means ± standard deviation (SD).

RESULTS

Reduced growth and decreased viability in the PUFA strain

It has been shown previously that the PUFA strain (LM 003-D126 strain, 'PUFA' in the text and figures) was producing LA and GLA and that they were found in phospholipids pools and storage lipids (Ruenwai *et al.* 2011). We studied the viability of the PUFA strain by cultivating the strain in liquid SD-Trp-Ura medium, over 5 days, and taking samples during exponential phase and, after 2 and 4 days. The samples were used for spot assay on SD-Trp-Ura plates. While the reference control (LM 001-REF, containing two empty plasmids 'REF' in the text and figures) showed no significant decrease in viability after 4 days (Fig. 1A), the PUFA strain showed a decrease in capacity to form colonies from exponential phase, and by day 4 viability was severely impaired.

To test the growth in exponential phase we calculated the specific growth rate and it was shown that the PUFA strain had ∼17% lower growth rate, when compared to the reference (Fig. 1B).

Increased ROS in the PUFA strain

The accumulation of ROS was investigated with the fluorogenic probe DHR 123. DHR 123 passively diffuses across membranes and is oxidized into its fluorescent form rhodamine 123 by H_2O_2 in the presence of peroxidases, cytochrome c or Fe^{2+} (Wardman 2007). The two strains were continuously grown for 5 days and samples for staining were taken during exponential phase (day 0), on day 2 and 4 (Fig. 1C). There were almost no detectable ROS in the REF strain during day 0. On day 2, there was ∼11% and on day 4 there were ∼37% of the cells that tested positive for ROS. The PUFA strain had also low number of ROS positive cells on day 0 (∼3%), but on day 2 it has increased to almost 56%, and on day 4 almost ∼95% of the cells tested positive for ROS.

We also tested ROS accumulation by adding low amounts (0.3 mM) of H_2O_2 to the medium for 2 h during exponential phase, which resulted in an increase in ROS from 1,1% to 4,4% in the REF strain and from 1,7% to ∼19% in PUFA (Fig. S1A, Supporting Information). The viability was also more impaired in the PUFA strain, when exposed to H_2O_2; the exponentially growing cells were subjected for 2 h to 2.5 mM H_2O_2 and spotted on SD-Trp-Ura plate (Fig. S1B, Supporting Information).

Increased lipid peroxidation in the PUFA strain

It has previously been shown in yeast that n-6 PUFAs are highly susceptible to peroxidation in the presence of ROS (Cipak *et al.* 2006; Ferreira, de Moraes and Campos 2011). We measured lipid peroxidation with the fluorescent lipid peroxidation sensor BODIPY C11 581/591 in the PUFA strain and control, during exponential phase (day 0), and on days 2 and 4. BODIPY C11 581/591 is a lipophilic fluorescent fatty acid analog that incorporates into membranes. It shifts fluorescence from red to green when it is oxidized (Foulks *et al.* 2008). The PUFA strain was shown to be more prone to lipid peroxidation; on day 0, in the LM 003-D126 strain 9.46% of cells are positive for lipid peroxidation, compared to 3.83% for the REF strain. During day 2 and 4 the PUFA strain had 75.9% and 98.87% lipid peroxidation positive cells, respectively (In comparison, for the REF strain it was 10.17% and 14.30% on days 2 and 4, respectively; Fig. 1D).

Increased protein oxidation in the PUFA strain

It has been found previously that oxidation of PUFA can lead to the generation of oxidized proteins due to production of reactive end products such as 4-hydroxynonenal and malondialdehyde that can react with amino acid side chains and the polypeptide backbone (Suzuki, Carini and Butterfield 2010). Protein extracts were derivatized with DNPH, which reacts with the oxidized side chains on the proteins. For each sample, 5 μg of protein was loaded on the SDS-PAGE and carbonylated proteins were detected by western blot with an anti-DNP antibody. There was no significant difference in samples from exponential phase (day 0, data not shown) but there was a significant difference in amount of carbonylated proteins in the PUFA strain, on days 2 and 4 (Fig. 1E). There was also a slight increase in the amount of damaged proteins in the control strain, in day 4 when compared to day 2 or the exponential phase. The PUFA strain showed also a much higher increase of oxidized proteins between days 2 and 4.

Earlier onset and higher level of caspase activation in PUFA strain

The yeast metacaspase Yca1p (also Mca1p) is involved in the induction of cell death upon certain different stimuli, e.g. oxidative stress, acetic acid stress, hyperosmotic stress and aging (Mazzoni and Falcone 2008). During exponential phase (day 0), we found that there was a subpopulation of cells with activated caspase in both strains (~11% in PUFA and ~8% in REF). After 2 days, the difference was larger: PUFA strain had ~65% of positive cells, compared to ~18% in REF. After 4 days, almost all PUFA cells (98.5%) were positive for caspase activation, while around 35% of REF cells were positive (Fig. 2A).

More dead cells in PUFA strain

PI is used to identify dead cells: the intact membrane is impermeable so if the cells tests positive for the dye it means that the membrane integrity has been compromised, and hence can be assumed dead. There are essentially very few dead cells during logarithmic phase and this was also found to be the case in the PUFA strain and the REF. After 2 days, the PUFA strain had more than 50% of dead cells while the reference strain had about 9.5%. On the fourth day, 80% of the PUFA cells were dead while the reference strain had 22% of dead cells (Fig. 2B).

More DNA damage in PUFA strain

The fluorescent stain DAPI was used to detect nuclei with condensed and fragmented chromatin, indicating DNA damage. On day 0, there were ~35% of cells in the PUFA strain with morphologically changed chromatin, compared to the REF strain (5%). During day 2 and 4, there was more than 90% of cells in the PUFA strain with fragmented and/or condensed chromatin, including crescent-shaped chromatin, as compared to the REF (on day 2 ~11%, and day 4 ~27%, Fig. 2C).

Deletion of the metacaspase YCA1 results in prolonged lifespan and decreased levels of oxidative stress in the PUFA strain

We measured higher levels and earlier onset of the caspase, but the assay is not very specific and other proteases can affect the result. Therefore, we deleted YCA1 in the PUFA and REF strains, generating the PUFA_YCA1Δ and REF_YCA1Δ strains in order to find out if the caspase was the main executor of cell death.

We found that the PUFA_YCA1Δ strain had an increased ability to form colonies in the spot assay, showing even a few

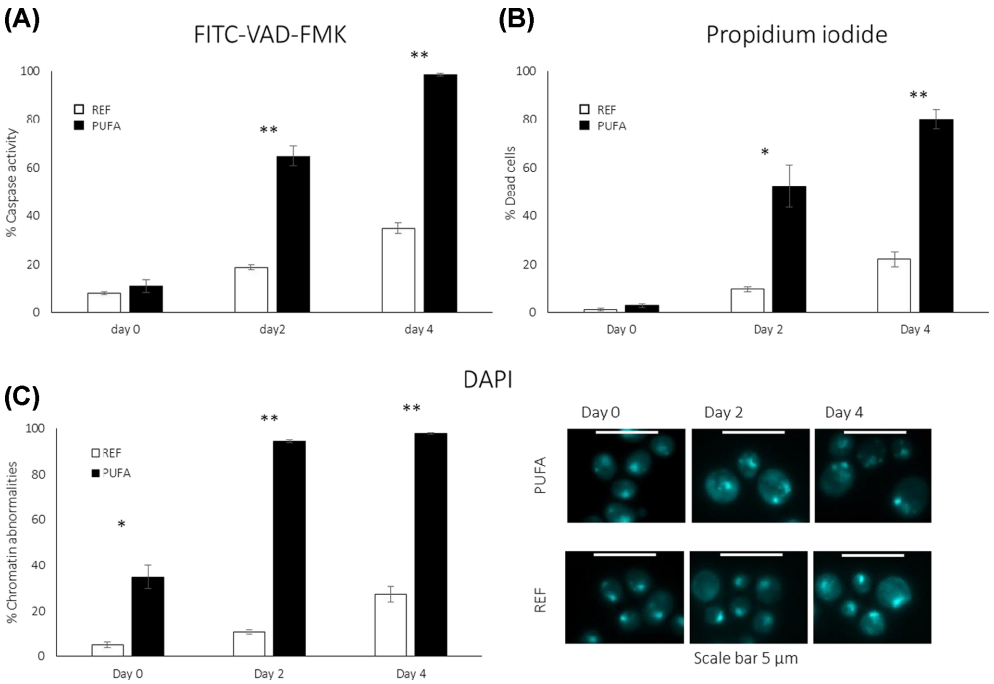

Figure 2. 'Increased caspase-like activity, DNA damage and cell death in the PUFA strain.' The strains were grown continuously for five consecutive days, and sampled on days 0, 2 and 4. **(A)** The non-specific caspase activity was measured with CaspACE FITC-VAD-FMK. **(B)** The number of dead cells was estimated by using PI that stains cells with compromised plasma membrane. **(C)** DNA damage was estimated by visualizing chromatin condensation and/or fragmentation using DAPI that stains the DNA. (N = 3, ± SD, * = $P \leq 0.05$, ** = $P \leq 0.001$, Student's *t*-test, two-tailed, two-sample equal variance between PUFA and REF).

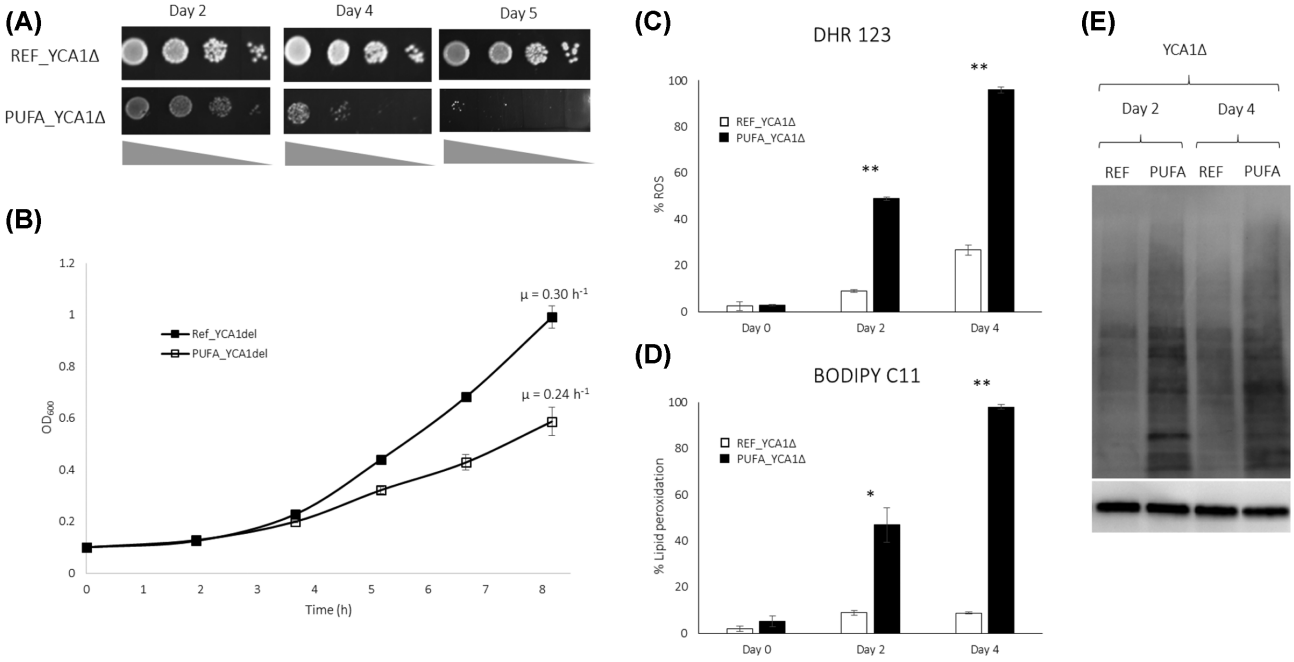

Figure 3. 'Partially increased viability and decreased oxidative stress upon deletion of YCA1.' **(A)** Spot assay was performed with 10× serial dilutions on SD-Trp-Ura plates, with samples on days 0, 2, 4 and 5. **(B)** Growth rates (μ) were calculated based on exponential growth on glucose in liquid medium in shake-flasks. **(C)** Accumulation of ROS during growth and stationary phase was measured with DHR 123 staining. **(D)** Accumulation of lipid peroxides during growth and stationary phase was measured with BODIPY C11. **(E)** Accumulation of carbonylated proteins during growth and stationary phase was measured with DNPH-derivatization and western blot. The loading control is GAPDH. (N = 3, ± SD, * = $P \leq 0.05$, ** = $P \leq 0.001$, Student's *t*-test, two-tailed, two-sample equal variance between PUFA_YCA1Δ and REF_YCA1Δ).

colonies on day 5 (Fig. 3A, day 0 not shown as it is identical to day 2). The REF_YCA1Δ show no difference in colony forming ability compared to the REF strain. Both the PUFA_YCA1Δ and the REF_YCA1Δ strains grow slower compared to the PUFA and REF strains (∼17% and ∼14% slower, respectively) (Fig. 3B).

The REF_YCA1Δ exhibited lower levels of ROS and lipid peroxidation compared to the REF strain on all days and the overall amount of ROS and lipid peroxidation was decreased in the PUFA_YCA1Δ strain, in exponential phase and especially in day 2 (Fig. 3C and D).

The amount of carbonylated proteins were measured on days 2 and 4. There were more carbonylated proteins in the PUFA_YCA1Δ compared to the REF_YCA1Δ (Fig. 3E) on both days, but there is much less protein carbonylation in the PUFA_YCA1Δ strain compared to the PUFA strain, on day 4 (compare to Fig 1E).

Caspase-like activity and membrane integrity in the YCA1Δ strains

On days 0 and 2, the PUFA_YCA1Δ show lower levels of caspase-like activity by FITC-VAD-FMK compared to the PUFA strain that has a functioning Yca1p (Fig. 4A compared to Fig 2A). By day 4, there is no difference between the strains. The REF_YCA1Δ show lower levels of caspase-like activity on all days.

Compromised plasma membrane integrity (PI staining) of the PUFA_YCA1Δ strain was not significantly different on days 0 and 2, respectively, compared to the PUFA strain. On day 4, the PUFA_YCA1Δ showed ∼97% PI positive cells, compared to ∼80% in the PUFA strain (Fig. 4B).

Supplementation of vitamin C extends chronological lifespan considerably and greatly decreases levels of oxidative stress

In order to investigate if the accumulation of ROS was the main culprit for the lifespan reduction in the PUFA strain we used the antioxidant vitamin C, which is a scavenger that neutralizes different radicals.

Treating the PUFA strain with buffered 10 mM vitamin C from the start of the culture increased dramatically the lifespan, when compared with the untreated PUFA strain (Fig. 5A, compare to Fig. 1A). Interestingly, the specific growth rate in exponential phase was not improved (Fig. 5B).

The PUFA strain treated with vitamin C showed a great reduction in ROS, lipid peroxidation and protein carbonyls on all days (Fig. 5C, D, E) compared to the non-treated PUFA strain (Fig. 1C, D, E). The vitamin C treated PUFA strain even showed lower ROS and lipid peroxidation levels than the levels of the non-treated REF strain (on any day).

Caspase-like activity and cell death are reduced in the vitamin C-treated strains.

When we treated the PUFA and REF strains with 10 mM vitamin C, we found a substantial decrease in caspase-like activity compared to the non-treated strains on all 3 days (Fig. 6A). The vitamin C treated cells were also tested for plasma membrane integrity, which is a non-specific marker of cell death and we found that cells treated with vitamin C have much higher viability, in any day (Fig. 6B, compare to Fig. 2B). The PUFA strain still has higher number of dead cells than the REF, in the same conditions, especially on day 4.

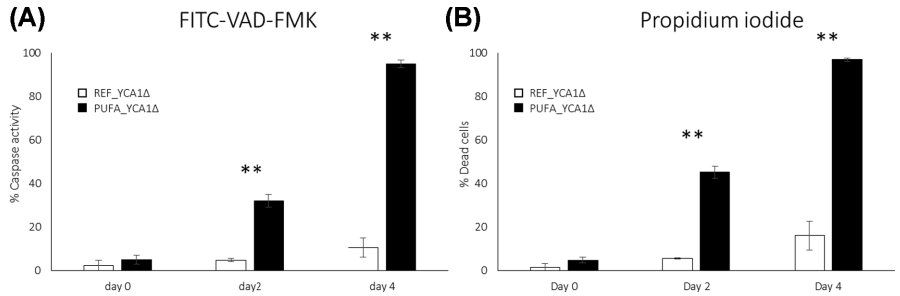

Figure 4. 'Partially decreased caspase-like activity and cell death upon deletion of YCA1.' The strains were grown continuously for five consecutive days, and sampled on days 0, 2 and 4. **(A)** The non-specific caspase activity was measured with CaspACE FITC-VAD-FMK. **(B)** The number of dead cells was estimated by using PI that stains cells with compromised plasma membrane. (N = 3, ± SD, * = $P \leq 0.05$, ** = $P \leq 0.001$, Student's *t*-test, two-tailed, two-sample equal variance between PUFA_YCA1Δ and REF_YCA1Δ).

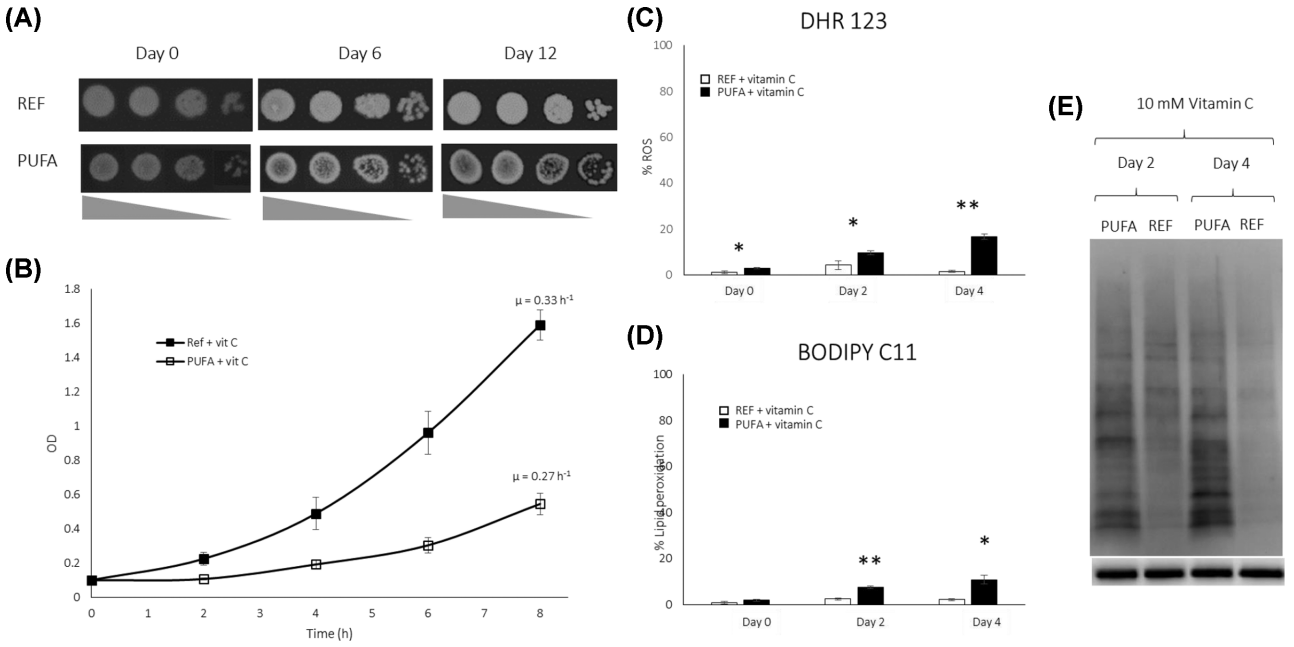

Figure 5. 'Increased lifespan and decreased oxidative stress upon vitamin C addition.' The PUFA and REF strains were grown in media supplemented with vitamin C for five consecutive days. **(A)** Spot assay was performed with 10× serial dilutions on SD-Trp-Ura plates, with samples on days 0, 2, 4 and 5. **(B)** Growth rates (μ) were calculated based on exponential growth on glucose in liquid medium in shake-flasks. **(C)** Accumulation of ROS during growth and stationary phase was measured with DHR 123 staining. **(D)** Accumulation of lipid peroxides during growth and stationary phase was measured with BODIPY C11. **(E)** Accumulation of carbonylated proteins during growth and stationary phase was measured with DNPH-derivatization and western blot. The loading control is GAPDH. (N = 3, ± SD, * = $P \leq 0.05$, ** = $P \leq 0.001$, Student's *t*-test, two-tailed, two-sample equal variance between PUFA and REF).

DISCUSSION

The production of PUFAs was previously shown to have negative effects on the strain (Ruenwai *et al.* 2011). The objective of this study was to investigate the nature of the toxic effect of heterologously produced LA and GLA.

It has been shown before that *S. cerevisiae* strains accumulating and/or incorporating PUFAs in their membranes are sensitive to oxidative stress (Cipak *et al.* 2006; Rockenfeller *et al.* 2010; Ferreira, de Moraes and Campos 2011). In this study, we showed that there was an increase in ROS, lipid peroxides and carbonylated proteins in the PUFA strain compared to the REF strain, detectable in exponential phase but especially during chronological aging. Additionally, when the strains were challenged with H_2O_2 the PUFA strain was more sensitive in terms of specific growth rate and viability by spot test (Fig. S1, Supporting Information).

The risk of lipid peroxidation increases with the level of fatty chain desaturation (Howlett and Avery 1997), which in turn can generate different aldehydes such as HNE and MDA that are known to react with specific amino acid side chains (arginine, histidine, lysine and proline) of proteins, as well as cleaving the protein backbone at specific amino acid residues (proline, glutamate and aspartate) generating protein carbonyls (Costa, Quintanilha and Morades-Ferreira 2007; Grimsrud *et al.* 2008). Carbonylated proteins need to be degraded rapidly since they otherwise can form aggregates that cannot be degraded by normal proteolytic pathways (Costa, Quintanilha and Morades-Ferreira 2007). We used the DNPH-assay and found an increase in protein carbonylation in the PUFA strain on days 2 and 4. We showed previously (Ruenwai *et al.* 2011) that the proteasome activity was decreased in the PUFA strain, *in vivo* and also at the transcriptional level. Additionally, several genes related to proteasome (BLM10, RPN4), autophagy (e.g. ATG1, ATG3, ATG4) and

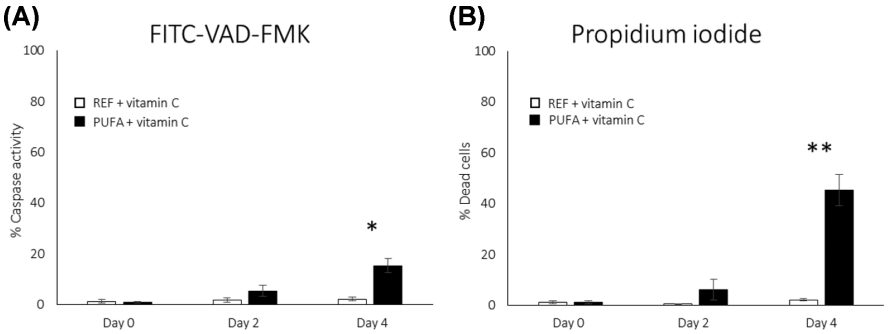

Figure 6. 'Decreased caspase-like activity and cell death upon vitamin C addition.' The strains were grown continuously for five consecutive days, and sampled on days 0, 2 and 4. **(A)** The non-specific caspase activity was measured with CaspACE FITC-VAD-FMK. **(B)** The number of dead cells was estimated by using PI that stains cells with compromised plasma membrane. ($N = 3$, ± SD, $^{*} = P \leq 0.05$, $^{**} = P \leq 0.001$, Student's t-test, two-tailed, two-sample equal variance between PUFA and REF).

chaperones (e.g. HSP42, LHS1) were also downregulated, indicating that the cellular systems responsible for handling damaged and oxidized proteins were reduced in the PUFA strain. Coupled with the high levels of ROS and lipid peroxidation, we expected to find increased protein carbonylation.

It has been shown that yeast cells can undergo caspase-mediated cell death upon induction of oxidative stress and by accumulating carbonylated proteins (Khan, Chock and Stadtman 2005). The same study showed that *S. cerevisiae* cells having the YCA1 deletion and exposed to H_2O_2 had increased levels of ROS and protein carbonyls without the induction of cell death. In the present study, caspase activation was shown to increase with increasing levels of ROS and carbonylated proteins in the PUFA strain, indicating that the PUFA strain's premature death might be caspase related. To investigate this hypothesis further, we deleted YCA1. Deletion of YCA1 has previously been shown to increase the chronological lifespan (Madeo *et al.* 2002). The lifespan of the PUFA_YCA1Δ strain has also been extended, although the specific growth rate has not been improved (Fig. 3B). The REF_YCA1Δ strain also grew slightly slower and it has been shown before that the deletion of YCA1 had a negative effect on growth (Lee *et al.* 2008). The YCA1 deletion showed that although the lack of Yca1p led to slightly extended lifespan and lower levels of oxidative stress, the low viability of the PUFA strain was not only caspase dependent. Other mechanisms should be investigated to define which pathway contributes to cell death, and to which extent.

One of the main features of the PUFA strain is its high level of ROS. In order to reverse the toxic effect of PUFA, we used vitamin C and found a major decrease in ROS and lipid peroxidation, as well as protein carbonylation, especially during chronological aging.

In conclusion, we found that *S. cerevisiae* cells producing LA and GLA have higher amount of ROS, lipid peroxidation and consequently protein carbonylation, than the REF strain. This type of damage is present from exponential phase and becomes more pronounced in stationary and post-stationary phases. The reduction of ROS by treatment with vitamin C has a clear positive effect by reducing lipid and protein oxidation thereby keeping the caspase activity low as well, and ultimately extending the lifespan of the cells. Even though the deletion of YCA1 had positive effects on the oxidative stress and caspase activity, it was not enough to significantly reverse the toxic effect of PUFA.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

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