FEMS Yeast Research, 19, 2019, foz020

doi: 10.1093/femsyr/foz020
Advance Access Publication Date: 27 February 2019
Research Article

RESEARCH ARTICLE

The hypoxic transcription factor *Kl*Mga2 mediates the response to oxidative stress and influences longevity in the yeast *Kluyveromyces lactis*

Rosa Santomartino[1,†], Ilaria Camponeschi[1], Germano Polo[1], Alessio Immesi[1], Teresa Rinaldi[1], Cristina Mazzoni[1,‡], Luca Brambilla[2] and Michele M. Bianchi[1,*]

[1]Department Biology and Biotechnology C. Darwin, University of Roma Sapienza, Piazzale Aldo Moro 5, 00185 Roma, Italy and [2]Department Biotechnology and Biosciences, University of Milano Bicocca, Piazza della Scienza 2, 20126 Milano, Italy

[*]**Corresponding author:** Department Biology and Biotechnology C. Darwin, Piazzale Aldo Moro 5, Roma 00185, Italy. Tel: +39 0649912215; E-mail: michele.bianchi@uniroma1.it

[†]Present address: UK Centre for Astrobiology, School of Physics and Astronomy, University of Edinburgh, Edinburgh (UK)

One sentence summary: A network of connections among hypoxia, respiration, growth and ageing, mediated by a single protein regulating lipid homeostasis, has been established in yeast.

Editor: Ian Dawes

[‡]Cristina Mazzoni, http://orcid.org/0000-0002-1504-6189

ABSTRACT

Hypoxia is defined as the decline of oxygen availability, depending on environmental supply and cellular consumption rate. The decrease in O_2 results in reduction of available energy in facultative aerobes. The response and/or adaptation to hypoxia and other changing environmental conditions can influence the properties and functions of membranes by modifying lipid composition. In the yeast *Kluyveromyces lactis*, the *Kl*Mga2 gene is a hypoxic regulatory factor for lipid biosynthesis—fatty acids and sterols—and is also involved in glucose signaling, glucose catabolism and is generally important for cellular fitness.

In this work we show that, in addition to the above defects, the absence of the *KlMGA2* gene caused increased resistance to oxidative stress and extended lifespan of the yeast, associated with increased expression levels of catalase and SOD genes. We propose that *Kl*Mga2 might also act as a mediator of the oxidative stress response/adaptation, thus revealing connections among hypoxia, glucose signaling, fatty acid biosynthesis and ROS metabolism in *K. lactis*.

Keywords: fatty acids; ROS; life span; catalase; superoxide dismutase; lipid droplets

INTRODUCTION

The yeast *Kluyveromyces lactis* has been widely used in industrial applications and basic research (Rodicio and Heinisch 2013). Its preference for respiration is the main regulatory trait that differentiates it from *Saccharomyces cerevisiae*. Indeed, *K. lactis* is a Crabtree-negative organism, having weak or absent glucose repression and the balance between respiration and fermentation depends on oxygen availability.

Received: 15 October 2018; **Accepted:** 26 February 2019

Fatty acid desaturases (FAD) are redox enzymes that generate carbon–carbon double bonds in fatty acids, they require oxygen as an electron acceptor and differ in substrate specificity, in the position of the double bond they generate and in cellular localization (Los and Murata 1998). In yeasts, FAD enzymes are differently distributed among strains and clades (Santomartino, Riego-Ruiz and Bianchi 2017). In *S. cerevisiae*, only the ubiquitous stearoyl-CoA desaturase (SCD) Δ9 Ole1 is present (Stukey, McDonough and Martin 1989) and the modulation of membrane properties is achieved by synthesis of the mono-unsaturated fatty acids (MUFAs) oleate (C18) and palmitoleate (C16). In *K. lactis* the Δ12 and Δ15 desaturases (Fad2 and Fad3, respectively) are also present (Kainou *et al.* 2006; Micolonghi *et al.* 2012), allowing the synthesis of the poly-unsaturated (PUFA) linoleic and linolenic fatty acids (ω6 and ω3, respectively). In humans, PUFAs are essential FAs since they cannot be synthesized and they need to be introduced by the diet (Di Pasquale 2009). In particular, shortage of ω3 has been related to severe diseases like cardiovascular disorders and Alzheimer's disease (Jicha and Markesbery 2010). The deletion of *FAD2* and *FAD3* genes in *K. lactis* did not create appreciable phenotypes, except a slow growth rate at very low temperature (De Angelis *et al.* 2016), suggesting that MUFAs are sufficient to achieve a functional membrane composition, as has also been suggested for *S. cerevisiae* (Stukey, McDonough and Martin 1989). Nevertheless, the presence of a larger family of unsaturated fatty acids (PUFAs) in *K. lactis* should permit a fine tuning of FAs synthesis and a change in membrane composition in response and/or adaptation to a large variety of environmental conditions.

Yeast desaturase genes are regulated at the transcription level: factors that affect this regulation are saturated FAs, UFAs, low temperature, hypoxia and, occasionally, ethanol and glucose (see Santomartino, Riego-Ruiz and Bianchi 2017, for a review). Promoter sequences involved in UFA repression and hypoxic induction of *OLE1* have been characterized in *S. cerevisiae* (Bossie and Martin 1989; McDonough, Stukey and Martin 1992; Choi *et al.* 1996; Kwast *et al.* 1999; Nakagawa *et al.* 2001;Vasconcelles *et al.* 2001). The best characterized transcription regulatory proteins of desaturases are *S. cerevisiae* Mga2 and Spt23, regulators of *OLE1* gene. Mga2 and Spt23 are homologous and inactive ER proteins, activated by proteasomal proteolysis (Hoppe *et al.* 2000); they are involved in regulation mediated by UFAs, low temperature and low oxygen (Chellappa *et al.* 2001; Jiang *et al.* 2002). The transcriptional control of these proteins acts on the induction/repression of mRNA synthesis and/or mRNA stability (Jiang *et al.* 2001; Kandasamy *et al.* 2004). A low-oxygen response element (LORE), interacting with Mga2, has been characterized in the *OLE1* promoter (Jiang *et al.* 2001). Single deletion of *MGA2* or *SPT23* does not exhibit a growth phenotype, while the double deleted strain is auxotrophic for unsaturated FAs, similarly to the *ole1* mutant strain (Zhang, Skalsky and Garfinkel 1999; Chellappa *et al.* 2001). The Whole Genome Duplication occurred in *S. cerevisiae* after the evolutionary separation from *K. lactis* (Wolfe and Shields 1997) and consequently the *SPT23* gene is absent in *K. lactis*. *KlMGA2*, which is the orthologous to *S. cerevisiae MGA2*, is involved in the transcriptional regulation of hypoxic genes, in particular of lipid biosynthetic genes (Micolonghi *et al.* 2012). KlMga2 controls *ACC1*, *FAS1* and *FAD2* expression at low temperature and under hypoxia (Micolonghi *et al.* 2012; Ottaviano *et al.* 2015).

The *KlMGA2* deleted strains of *K. lactis* display many phenotypes: they have a slow growth rate, they are Rag$^-$ strains (Resistance to Antimycin A on high Glucose concentration), they are multi-budded with partially collapsed mitochondria, and they have a low oxygen-consumption rate. All these phenotypes are reversed by the addition of UFAs, indicating that restoration of membrane fluidity is sufficient to recover wild type behavior (Ottaviano *et al.* 2015). Interestingly, the Rag$^-$ phenotype is usually associated to glycolytic/fermentative defects, while oxygen-consumption rate and mitochondrial morphology defects are correlated to the respiratory pathway, suggesting the involvement of KlMga2 in both metabolisms. Moreover, the *Klmga2*Δ strain has altered membrane content and composition that, together with defective respiration and altered mitochondria, might be associated to altered permeability and resistance to hydrogen peroxide (Hermann and Shaw 1998; Branco *et al.*, 2004; Tafforeau *et al.*, 2006; Matias *et al.*, 2007) or to reactive oxygen species (ROS) accumulation and consequently impaired response to oxidative stress. Consistent with this hypothesis, adaptation to oxidative stress induced by hydrogen peroxide has been reported to involve transcription factors in *S. cerevisiae,* including Mga2, with correlations on fatty acids and ergosterol metabolism (Kelley and Ideker 2009). The involvement of Mga2 in response to the oxidative stress has also been reported in *Candida glabrata* (Huang and Kao 2018).

Despite the evolutionary conservation between *K. lactis* and *S. cerevisiae*, the mechanisms of ROS response and adaptation in *K. lactis* are far from being understood and to date observations indicated they could differ from those of *S. cerevisiae* (Blanco *et al.* 2007; González-Siso *et al.* 2009). Our present results demonstrate the involvement of the transcription factor KlMga2 and of unsaturated fatty acids in regulation of ROS metabolism and longevity.

MATERIALS AND METHODS

Strains and media

The wild type strain GDK (MAT**a**, *ura3*, *leu2*, *metA1–1*, *lac4::ura3*, *promKlPDC1::promKlPDC1-LacZ-LEU2*) and mutant strain GDK/*Klmga2*Δ (MAT**a**, *ura3*, *leu2*, *metA1–1*, *lac4::ura3*, *promKlPDC1::promKlPDC1-LacZ-LEU2*, *Klmga2::kanMX4*), derived from parental strains of the University of Lyon (France) collection, have been previously described (Micolonghi, Wésolowski-Louvel and Bianchi 2011; Micolonghi *et al.* 2012). YPD medium was composed of 1% Yeast Extract (Becton Dickinson and Company, Sparks, MD, USA), 1% Peptone (Becton, Dickinson and Company, Sparks, MD, USA) and 2% glucose. Synthetic medium contained 0.67% Yeast Nitrogen Base w/o aminoacids (Becton, Dickinson and Company, Sparks, MD, USA) and 2% glucose, supplemented with auxotrophic requirements (SD medium). Solid media contained 2% Bacto-Agar (Becton, Dickinson and Company, Sparks, MD, USA). Tween80 (Sigma-Aldrich, St. Luis, MO, USA) was added to media at 0.1% as oleate source.

ROS determination

The relative H_2O_2 level in living cells was measured with the profluorescent dye dihydrorhodamine 123 (DHR, Fluka; Madeo *et al.* 1999). Briefly, 2×10^6 cells were harvested by centrifugation and suspended in 3 ml of 5 μM dye in PBS (10 mM sodium phosphate and 150 mM NaCl, pH 7.0). Samples were treated with 3, 6, 9 and 12 mM H_2O_2 (final concentrations), respectively, and were incubated for 75 min at 30°C with agitation in the dark. Concentrations were chosen to encompass the inhibitory concentration for growth (5 mM; Nikolaou *et al.* 2009). Growth was affected but not inhibited at 3 mM H_2O_2 on YPD medium plates (not shown). Prior to analysis cells were washed twice and diluted to 10^6 cells/ml in PBS. Samples were then analyzed using a CYTOMICS

FC 500 flow cytometer (Beckman Coulter) equipped with a diode laser (excitation wavelength 488 nm). The fluorescence emission was measured through a 530 nm long pass filter (FL1 parameter). The sample flow rate during analysis did not exceed 600–700 cells/s. Threshold settings were adjusted so that the cell debris was excluded from the data acquisition; 25 000 cells were measured for each sample. Data analysis was performed with WinMDI 2.8 software (Purdue University, Cytometry Laboratories [http://facs.scripps.edu/software.html]). In order to account for difference in autofluorescence between the strains, fluorescence evolution was normalized to the value for cells not challenged with H_2O_2. The rate of survival to H_2O_2 treatment was assayed by plating cells on YPD plates before and after hydrogen peroxide incubation and measuring the colony forming units (CFU). About 250 to 2250 colonies per sample were counted.

Catalase and Superoxide dismutase activity

About 3 to 4 OD_{600} units of cells were collected from cultures and extracts were prepared by glass bead crushing in lysis buffer (50 mM Tris-HCl pH 6.8; 100 mM NaCl) or in phosphate buffer, as described in Landi *et al.* (2015). Protein content in samples was determined photometrically (Nanodrop 1000) or by Coomassie Plus Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) using bovine serum albumin as a standard. To determine catalase activity, 1–1.5 μl aliquots of samples were added to 0.5 ml of 11 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0), 1 μM EDTA. H_2O_2 decomposition was monitored at 25°C using a spectrophotometer at 240 nm ($\varepsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$). One Unit of catalase activity was defined as the amount of enzyme that catalyzed the degradation of 1 μmole min^{-1} of H_2O_2. Superoxide dismutase activity (SOD) was determined by measuring the rate of WST1-Formazan formation, using the SOD Assay kit-WST, as suggested by the supplier (Sigma-Aldrich, St. Luis, MO, USA). A calibration curve was produced using a commercial SOD enzyme (Sigma-Aldrich, St. Luis, MO, USA), in order to define the linearity range between product formation and enzyme concentration. Activity measurements have been performed in two to four biological repetitions, each of them in technical triplicates. Standard deviations and statistical significances (*P*-values) have been determined.

RNA analysis

Transcript analysis was performed by northern blotting. Total RNAs were prepared by the hot phenol procedure as described by Köhrer and Domdey (1991). Northern blotting was performed by electrophoresis of RNAs in agarose/formaldehyde gels followed by transfer to membranes and hybridization with probes following a standard procedure as described by Bianchi *et al.* (1996). Probes were obtained by PCR using wild type GDK genomic DNA as a template and primes as follows. CTA1-fw: 5′-GCGTCGTGTAGGTAAGATGGTT-3′; CTA1-rv: 5′-CTTGGATTTCAGGGCATGCACT-3′; CTT1-fw: 5′-CGGTACCATTACCCTAACCCAT-3′; CTT1-rv: 5′-GACTGGAGATGCACCATTGACA-3′; SOD1-fw: 5′-CAGTTGCAGTTTTGAAGGGTT-3′; SOD1-rv: 5′-TACCAATAACACCAC-3′; SOD2-fw: 5′-ATTGGACTGGGATTT-3′; SOD2-rv: 5′-CTTAGCAGCTTCCTTCCA-3′. [α-^{32}P] ATP labeling of the probes was performed using the Random Primed Labelling Kit (Roche/Sigma-Aldrich, St. Luis, MO, USA), following the instructions provided by the manufacturer. Quantification of signals was performed by pixel counting with Phoretics 1D plus (Non-linear Dynamics, Newcastle upon Tyne, UK) using rRNAs as loading references.

Longevity

Cells were inoculated in flasks containing SD medium and grown with orbital shaking (175 rpm) at 28°C. SD medium supplemented with 0.1% Tween80 (Sigma-Aldrich, St. Luis, MO, USA) was also used for semi-quantitative analysis. At each time point, OD_{600} was measured and dilutions were analyzed. Quantitative Chronological Life Span (CLS) was determined by measuring the percentage of cells capable of reproduction and forming microcolonies on YPD medium, observed and counted under light microscope at 40x (Palermo, Falcone and Mazzoni 2007; Carmona-Gutierrez *et al.* 2018). About 500 counts (dead cells plus microcolonies) per sample were registered after 1–2 days (wild type) or 2–3 days (mutant strain) on YPD medium. Semi-quantitative CLS was determined by 10-fold serial dilutions of the cultures in micro-titer plates followed by transfer with a 48-pin replicator onto YPD plates and incubation for 3–4 days at 28°C (Smith *et al.* 2007; Carmona-Gutierrez *et al.* 2018).

Lipid droplets analysis

Cultures of wild type and mutant strains were grown in YPD and collected at early and late exponential phase and at stationary phase (0.7, 2 and 8 OD_{600}, respectively). About 25 μl of DMSO were added to 250 μl of culture to allow an optimal stain solubilization. DMSO at this concentration does not interfere with yeast growth (Cirigliano *et al.* 2016). Nile Red (Sigma-Aldrich, St. Luis, MO, USA) was added to samples at the final concentration of 5 μg/ml (Sitepu *et al.* 2012). Stained cells were then observed by fluorescence microscopy with the microscope Zeiss Axio Imager Z1 Fluorescence Microscope (AxioVision 4.8 Digital Image Processing System and objective lens 63 × Oil): excitation wavelength 515–560 nm; emission wavelength > 590 nm. Quantitative analysis of lipid droplets-containing cells was carried out by observing 1 to 6 hundred cells for each sample.

RESULTS

ROS content in the *Klmga2*Δ strain

The respiratory and mitochondrial phenotypes associated with *KlMGA2* deletion (Ottaviano *et al.* 2015) suggested the occurrence of an altered ROS metabolism. In order to investigate this issue, we characterized ROS content in exponentially growing cultures ($OD_{600} = 0.5$) stained with 123-DHR by cytofluorimetric assay. Fluorescence levels of the mutant cells (not shown) were lower than the wild type cells. Following the exposure to hydrogen peroxide (H_2O_2), a treatment that elicits ROS production, cellular ROS formation was measured in the wild type and *Klmga2*Δ strains. Cells in early exponential phase ($OD_{600} = 0.5$) were transferred into fresh medium with progressively higher concentrations of hydrogen peroxide (up to 12 mM) in presence of 123-DHR. After 75 min incubation, cells were collected and fluorescence was measured. Results of the normalized fluorescence are reported in Fig. 1A. We observed an increase in fluorescence detection dependent on the concentration of hydrogen peroxide, suggesting that the amount of peroxide used was not saturating the response of the cells. Interestingly, developed fluorescence was weaker in mutant cells, suggesting that *KlMGA2* absence led to a more efficient ability in ROS inactivation in exponential phase.When the same experiment was performed with cells

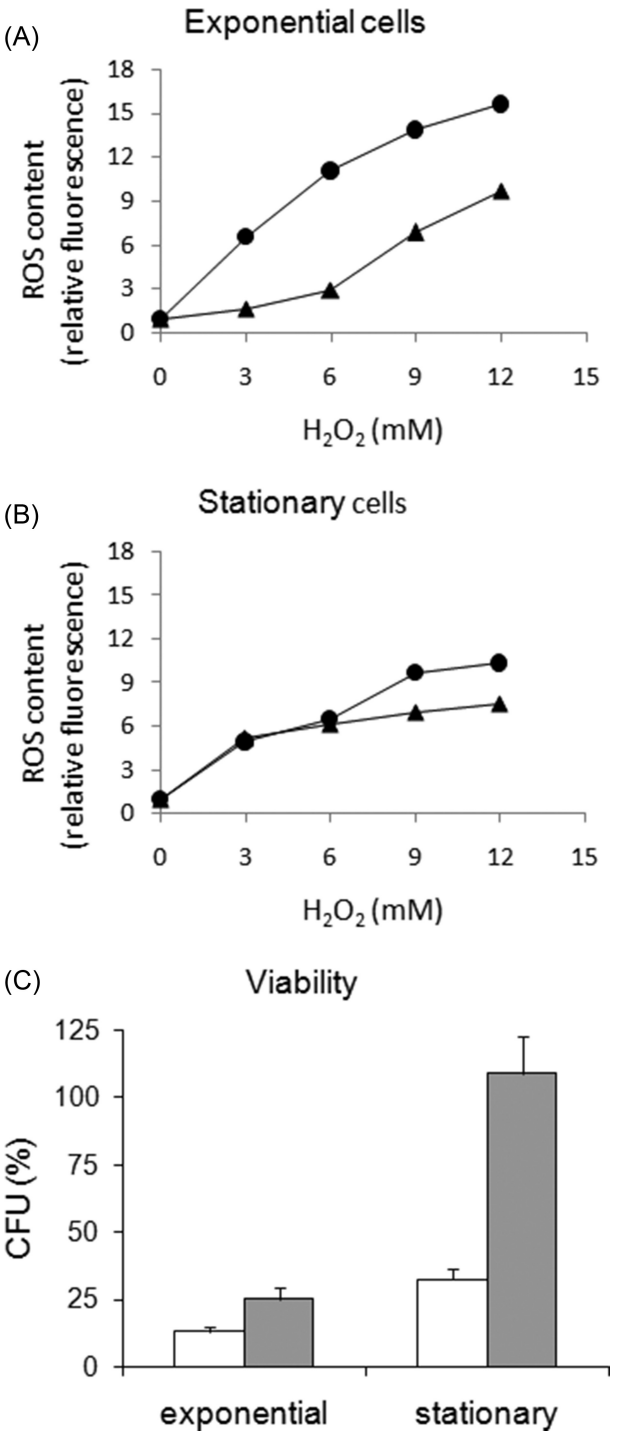

Figure 1. ROS content of wild type and *Klmga2*Δcells. **(A)** and **(B)**, show the ROS content, revealed by fluorescence after staining with DHR, in exponential (A; $OD_{600} = 0.5$) and stationary (B; $OD_{600} = 10$) wild type (circles) and mutant (triangles) cells incubated (75 min) with increasing concentrations of hydrogen peroxide. Fluorescence values have been normalized by untreated ($H_2O_2 = 0$) cell fluorescence. **(C)**, reports the viability (percentage of cells able to form colonies; CFU) of exponentially growing cells ($OD_{600} = 0.5$) and stationary cells ($OD_{600} = 10$) after treatment with12 mM hydrogen peroxide. About 250 to 2250 colonies per sample were counted. White blocks indicate wild type and grey blocks indicate mutant cells.

at $OD_{600} = 10$ (stationary phase), ROS content was similar in the two strains (Fig. 1B) with differences appearing only after exposure to the higher concentrations of hydrogen peroxide. Again, mutant cells accumulated ROS to a lesser extent.

In order to assay how induced ROS accumulation affected cell viability, the wild type and the deleted strains were plated on YPD medium to allow colony formation. This was performed before and after treatment. Considering that 12 mM of H_2O_2 gave the higher difference in ROS production between two strains, this concentration was used for this experiment and results are shown in Fig. 1C. After hydrogen peroxide treatment, only 13% of wild type cells in exponential growth phase were able to form colonies. The fraction of viable cells increased to 32% in the stationary phase($OD_{600} = 10$). Mutant cells showed an increased viability compared to the wild type in exponential phase and were not affected at all in stationary phase by this treatment.

Chronological life span of the *Klmga2*Δ strain

ROS can elicit mechanisms of cell defense, including modulation of life span, programmed cell death, autophagy and mitophagy, and mitochondria are strongly involved in this process as major producers of ROS (González Siso and Cerdán 2012).The role of oxidative stress on aging and longevity has been widely established and several molecular mechanisms underlying cellular aging are conserved in eukaryotes (Kainz *et al.* 2017).Yeast cells are an excellent model system in this type of studies. We wondered if the increased protection against ROS, together with lower growth rate (Ottaviano *et al*, 2015), could lead to differences in lifespan in the deleted mutant strain. To this aim, we performed a quantitative assay of viability of the wild type and mutant strains (Chronological Life Span, CLS) by measuring the percentage of cells capable to form microcolonies (μCFU) under microscopic observation in samples collected at different time points from cultures grown in synthetic SD medium (Carmona-Gutierrez *et al.* 2018). Results are shown in Fig. 2. Cell population had the highest viability in wild type and mutant cultures after 1 day and values were normalized to Day 1 measurement. In wild type cultures viability decreased progressively and reached 0.2% after 14 days. In contrast, the viability of the mutant strain decreased slowly to 10% over 19 days and then more rapidly to about 0.2% after 24 days. These results demonstrated an extended CLS of the mutant strain. The extended viability could be dependent on the activity of ROS metabolic enzymes, protecting mutant cells from cumulative damage caused by ROS generation with better efficiency than wild type cells.

Transcription analysis of *KlCTA1*, *KlCTT1*, *KlSOD1* and *KlSOD2* genes

The observed reduced level of ROS and extended cell survival of the mutant strain could be the result of an increased activity of catalase (Cat) and superoxide dismutase (SOD) enzymes. In *S. cerevisiae*, these enzymes are coded by *CTA1*, *CTT1*, *SOD1* and *SOD2* genes. The first two are the peroxisomal and the cytosolic catalases (Cohen *et al.* 1985), respectively. The latter two are the Cu-Zn (cytosolic) and Mn (mitochondrial) containing superoxide dismutases, respectively (Gralla and Kosman 1992). Catalase genes *CTA1* and *CTT1* (*KLLA0D11660g* and *KLLA0D14685g*, respectively) have been identified in *K. lactis* by Blanco *et al.* (2007). We have identified the homologous *SOD1* and *SOD2* genes in the GRYC database (http://www.gryc.inra.fr) as *KLLA0E05567g*

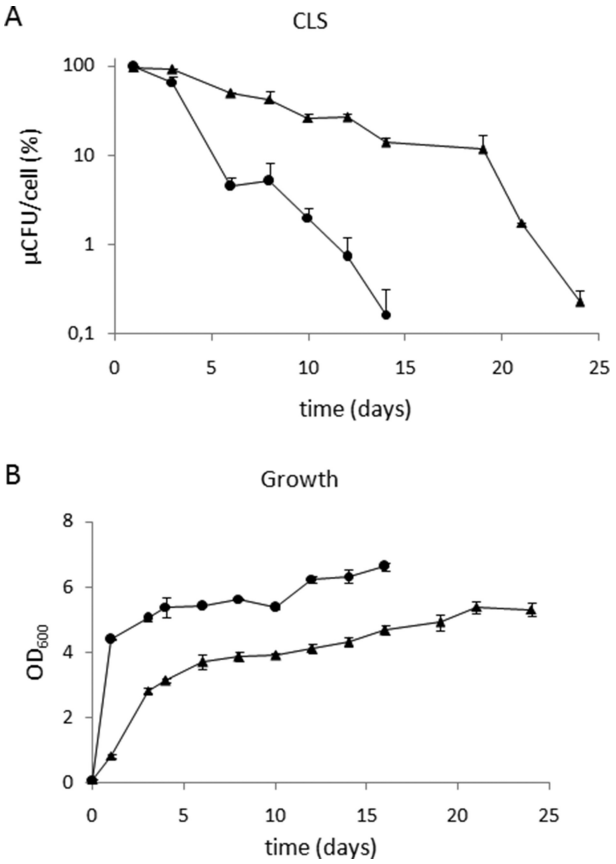

Figure 2. Chronological Life Span (CLS) in wild type and *Klmga2*Δ strains. CLS was determined by viability, calculated at each time point by measuring the percentage of cells capable to undergo cell divisions and to form microcolonies (µCFU/cell). Samples at different time points (days of growth) were plated on YPD and observed for microcolonies formation **(A)**. 500 counts per sample were registered. OD_{600} was also measured for each time point **(B)**. Averages and standard deviations of 3 to 4 independent cultures are reported. Circles are wild type strain values and triangles are mutant strain values.

and *KLLA0E03609g*, respectively. The *K. lactis* enzymes were 68%–80% identical to the *S. cerevisiae* enzymes and sequence analysis (http://smart.embl-heidelberg.de/) showed the presence of catalase and superoxide dismutase functional domains. No other gene encoding for highly similar Cat or SOD enzymes was found in the *K. lactis* genome. These genes were named *KlCTA1*, *KlCTT1*, *KlSOD1* and *KlSOD2*.The *KlSOD1* gene of *K. lactis* has been previously characterized in heterologous protein production studies (Raimondi *et al.* 2008).

Exponential and stationary phase cells of *K. lactis* behaved differently in the wild type and in the mutant strains in relation to ROS metabolism. We thus assayed the transcription of *KlCTA1*, *KlCTT1*, *KlSOD1* and *KlSOD2* genes by northern blot analysis. Strains were cultivated in flask on YPD medium and cells were collected at different growth phases. Results, together with the corresponding histogram for signal quantification respect to ribosomal RNAs, are reported in Fig. 3. In the wild type strain, transcript levels of *KlCTA1*, *KlCTT1*, *KlSOD1* and *KlSOD2* genes showed a progressive increase from early growth phase (OD_{600} = 0.7) to late stationary phase (OD_{600} = 12). The levels of transcription of the four genes were higher across the exponential phase in the mutant strain. Accordingly, the high expression of catalase and SOD genes in the mutant, already occurring in early exponential phase, could explain the more efficient response to H_2O_2 addition shown in Fig. 1.

Activity of catalase and superoxide dismutase enzymes

To assess if the higher transcription rates of the catalase (Cat) and SOD genes in the mutant strain corresponded to increased enzymatic activities, Cat and SOD activities were measured in cells sampled between OD_{600} = 0.7 to OD_{600} = 8 in YPD cultures. Results are reported in Fig. 4. Cat activity (Fig. 4A) in the wild type strain increased progressively from less than 5 U/mg in exponential cells to 28 U/mg in stationary cells. Cat activity was constant during late stationary phase in the wild type strain (OD_{600} > 12; not shown). The mutant strain showed a different pattern of Cat activity: it was consistently high (30 U/mg) in exponential cells up to OD_{600} = 4, then increased abruptly at OD_{600} = 8 (> 60 U/mg).

SOD activity in the wild type strain (Fig. 4B) showed a progressive increase from exponential to stationary cells: 3.4 U/mg at OD_{600} = 0.7 and 6.4 U/mg at OD_{600} = 8. Similar to the catalases, SOD enzymes were highly expressed (> 15 U/mg) in mutant cells during the exponential phase (OD_{600} = 0.7 and 2) with values significantly higher than the wild type up to OD_{600} = 4. However, when approaching to the stationary phase (OD_{600} = 4 and 8), cells showed a significantly decreased SOD activity with respect to cells in exponential phase at OD_{600} = 0.7 (P = 0.02 and 0.007, respectively).

The overall data of Cat and SOD enzymes showed high and constant values in the mutant strain at the exponential phase while a progressive increase of enzymatic activity was present in the wild type strain. Notably, the enzymatic activities correlated well with the transcription profiles reported above (Fig. 3), although total protein extraction could not distinguish the contribution of the two isoforms to the measured enzyme activity. These findings were consistent with a constitutively activated ROS metabolism in the absence of *Kl*Mga2, independently of the growth phase.

Effects of unsaturated fatty acids on ROS metabolism and longevity

It has been previously shown that addition of oleic and/or palmitoleic unsaturated fatty acids suppress *Klmga2*Δ mutant phenotypes (Ottaviano *et al.* 2015). In order to assay the effects of UFAs on ROS metabolism, wild type and mutant strains were cultivated in YPD medium in the presence of a source of oleate (Tween80). Figure 4 shows results of Cat and SOD activity assays performed using cell grown at different growth phases. In wild type cells, Cat activity values significantly increased during growth phases, but no differences were observed with oleate addition (Fig. 4A). As observed for YPD grown cells, Cat activity in the mutant strain on Tween80-supplemented medium was much higher than in the wild type, and progressively increased along growth phases. The SOD activity profile of wild type cells didn't change in the presence of Tween80. Interestingly, Tween80 added in mutant cell cultures (Fig. 4B) caused a significant decrease of SOD activity, especially in the exponential phase (OD = 0.7 and 2) leading to a flat profile with values comparable to that of the wild type. In summary, the addition of an oleate source did not affect Cat and SOD activities in growing wild type cells, while we observed a different effect of oleate on Cat and

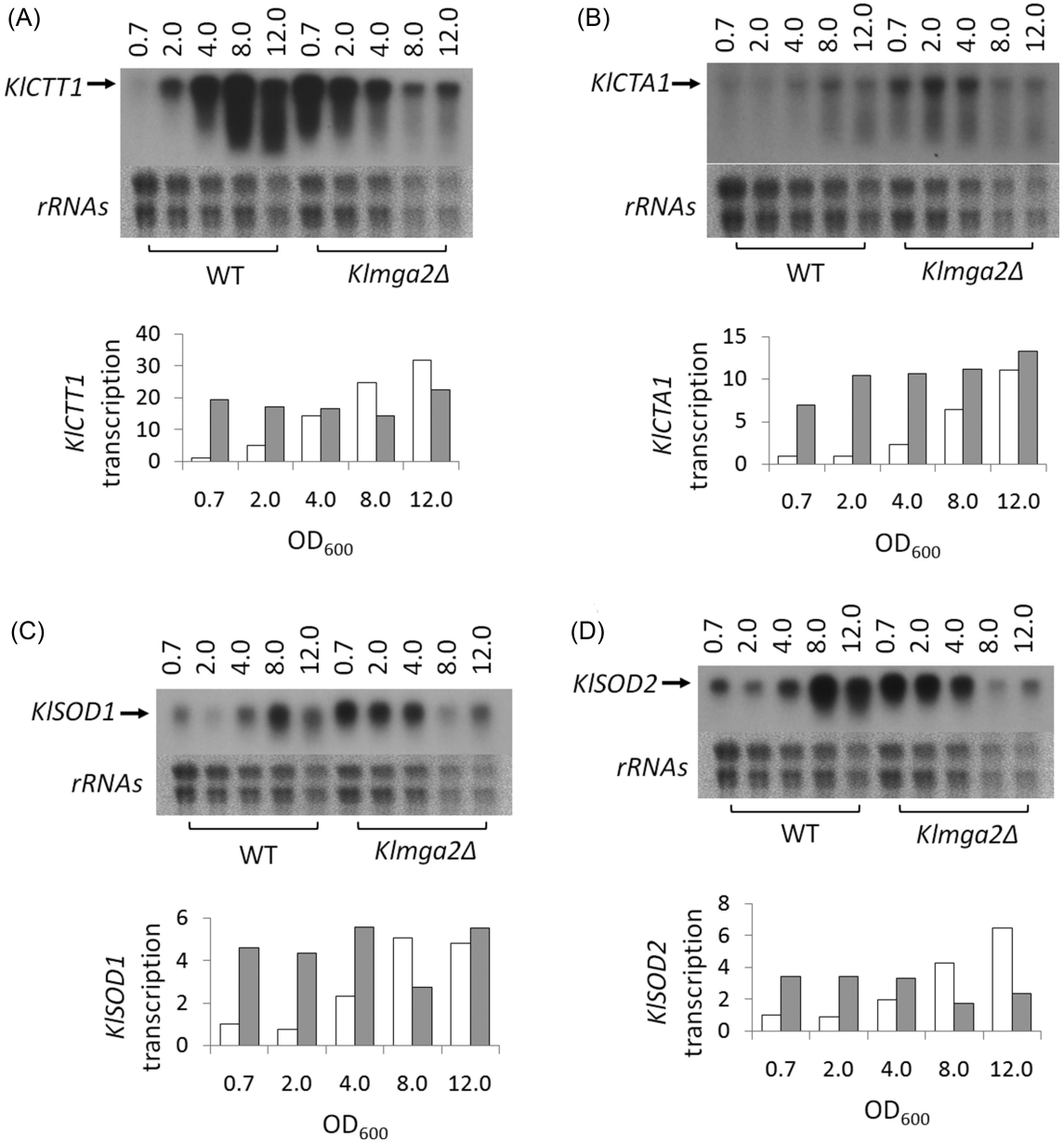

Figure 3. Transcription of *KlCTT1*, *KlCAT1*, *KlSOD1* and *KlSOD2* genes. Results of northern blot analysis of catalase and superoxide dismutase genes in wild type (WT) and mutant (*Klmga2*Δ) strains grown in YPD flask cultures to the indicated OD_{600} are shown. Ribosomal RNAs (*rRNAs*) have been used as loading control. Signals have been quantified by pixel counting and normalized by *rRNAs* pixel values (Phoretics 2D, Non-linear Dynamics). Wild type value at $OD_{600} = 0.7$ has been used as unit value and obtained data are reported as histogram below each corresponding northern blot image. White blocks are wild type values and grey blocks are the mutant values. **(A)**, **(B)**, **(C)** and **(D)** report *KlCTT1*, *KlCTA1*, *KlSOD1* and *KlSOD2* transcription analysis, respectively. Duplicated experiment sets gave the same results.

SOD activities in the mutant strain: Cat activity was maintained higher than the wild type while SOD activity was reduced.

We tested if growth in the presence of oleate could also reverse the extended CLS of the *Klmga2*Δ strain. The wild type and mutant were grown in SD medium with or without addition of 0.1% Tween80 in duplicate cultures. CLS was determined then semi-quantitatively: at various time points (days) cells were collected and serial dilutions were grown on YPD plates (Fig. 5). Viable wild type cells could be detected up to day 5 and 7 while viability of the mutant strain was extended at least to day 21. Surprisingly, CLS of the wild type was slightly increased to day 7–11 by the addition of Tween80, while the viability in the mutant strain was shortened to day 9 in the same growth medium. This finding further confirmed that UFAs could affect phenotypes of *Klmga2*Δ mutant strain.

Lipid droplets

Lipid droplets (LDs) are ubiquitous cellular organelles specialized in storing and supplying lipids. Despite they were first thought to be only storage organelles, they mediate the main functions related to lipid metabolism (Pol, Gross and Parton 2014); they accumulate during oxidative stress (Lee *et al.* 2015) and have been related to longevity (Goldberg *et al.* 2009). Considering the role of KlMga2 in lipid biosynthesis and the impairment of FAs/UFAs cell content when *KlMGA2* is deleted (Micolonghi *et*

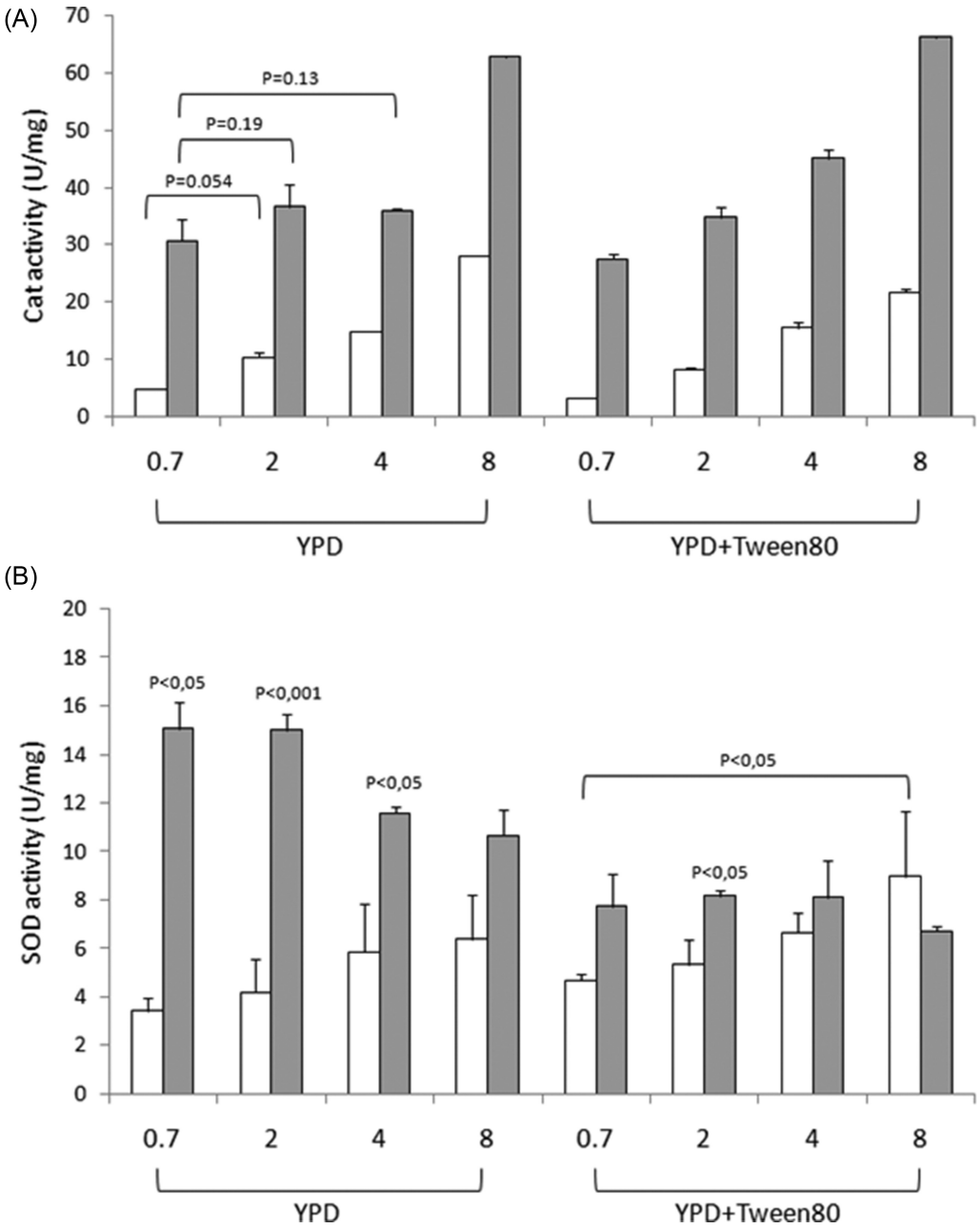

Figure 4. Activity of catalases (Cat) and SOD enzymes. Total extracts from wild type and *Klmga2*Δ strains grown to the indicated OD_{600} (x axis) were assayed for catalase and superoxide dismutase activities as described in the Materials and Methods section. Results are reported as histograms: white blocks are wild type values and grey blocs are mutant values. **(A)**, shows Cat activities: on the left sides, activities of YPD cultures are reported; on the right sides, activities of YPD cultures supplemented with 0.1% Tween80 are reported. Activity values are averages of two to four independent determinations, each measured by three technical repetitions, with standard deviations reported. Differences between wild type and mutant and between cultures of the same strain at different OD_{600} were all significant ($P < 0.05$) except those indicated. Other statistics are reported in text. **(B)**, shows SOD activities: activities of YPD cultures are reported on the left; activities of YPD cultures supplemented with 0.1% Tween80, as a source of UFAs, are reported on the right. Activity values are averages of three independent determinations, each measured by three technical repetitions, with indicated standard deviations. P-values between wild type and mutant and between cultures of the same strain at different OD_{600} are reported. Other P-values are reported in text.

al. 2012; Ottaviano *et al.* 2015), LD content in the wild-type and mutant cells has been investigated. Cultures were analyzed by fluorescence microscopy at different growth phases after Nile Red staining (Greenspan, Mayer and Fowler 1985) and representative images of the analyzed samples are reported in Fig. 6. Quantification of LD content showed that all wild type cells contained LDs, independent on the growth phase. Conversely, as few as 3% of the mutant cells contained LDs at the exponential phase ($OD_{600} = 0.7$). This percentage increased to 16% at $OD_{600} = 2$ and 57% at $OD_{600} = 8$, respectively. These results suggested a low fatty acid storage in *Klmga2*Δ strain.

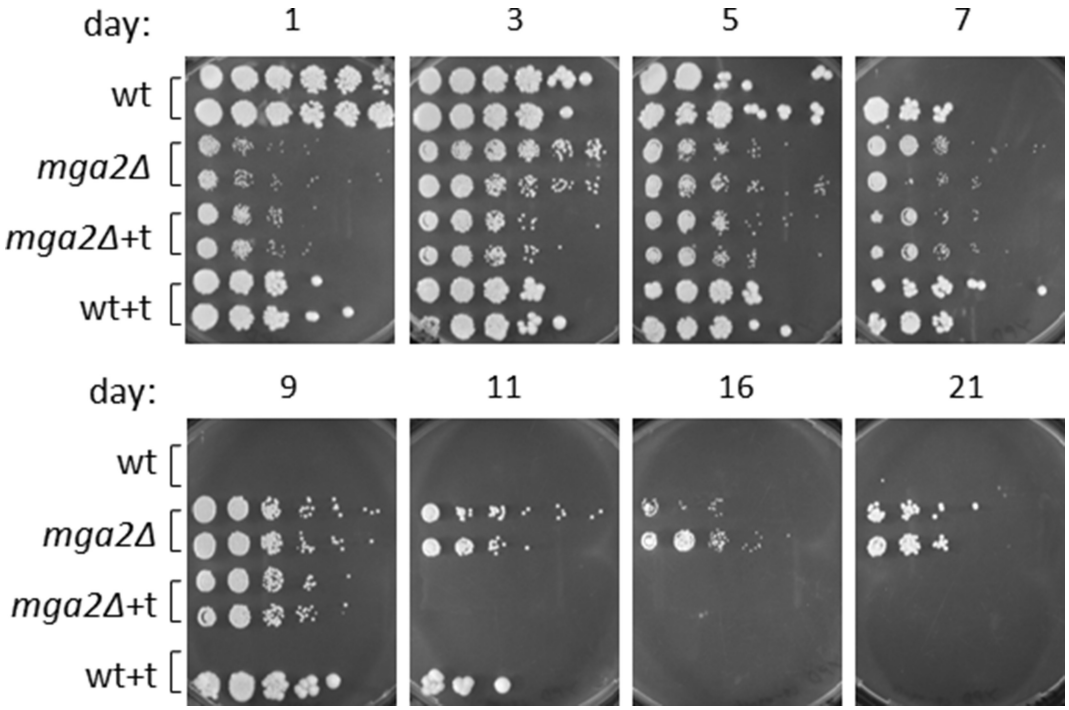

Figure 5. Viability of wild type and *Klmga2*Δ strains on oleate. Strains were cultured on SD (wt and mga2Δ rows) and SD supplemented with Tween80 (+ t rows). Two independent cultures (two rows) were performed for each strain/condition. Viability was determined as the capacity to form colonies on YPD plates after 10-fold serial dilutions in microtiter plates. Samples were collected at various time points (day). Plates of the last day showing colony growth at the first dilution of each sample are shown. Viability of *Klmga2*Δ strain was maintained up to day 24 in SD (plate not shown).

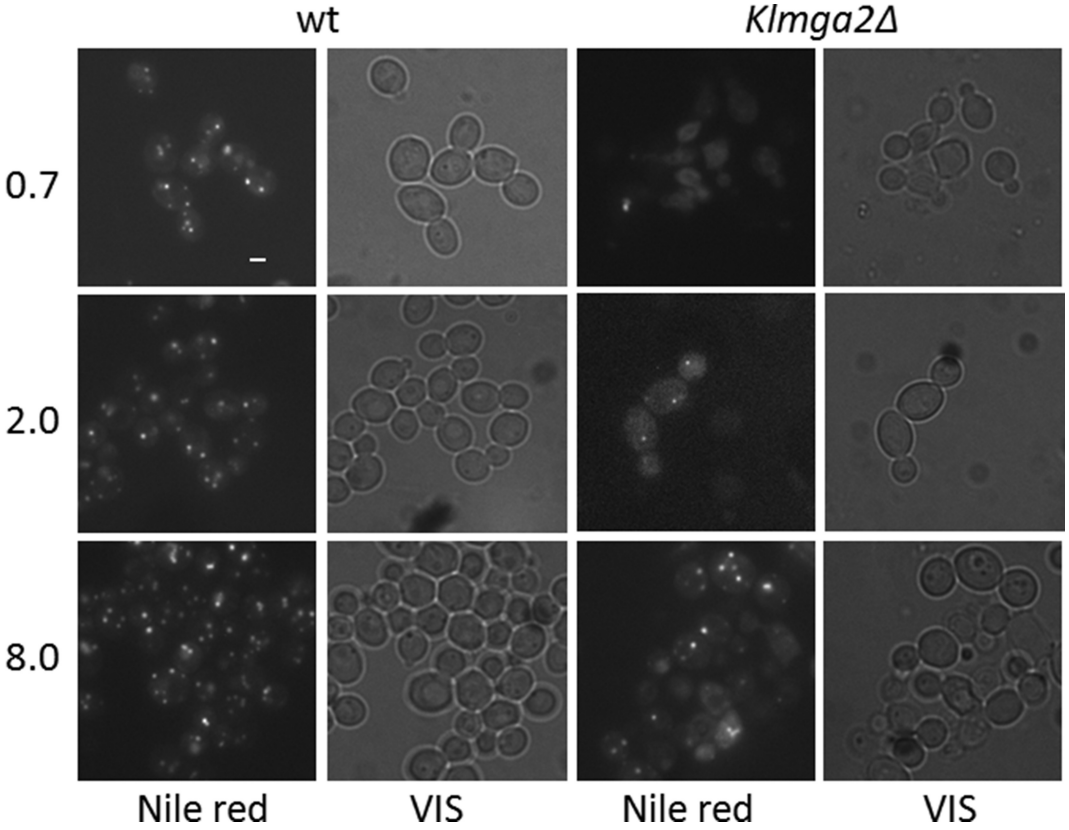

Figure 6. Lipid droplets in wild type and mutant strains. Cells were grown in YPD, collected at different growth phases (OD_{600} = 0.7, 0.2 and 8) and observed after Nile Red staining. Wild type cells -left columns (fluorescence and visible visualizations); mutant cells—right columns. Scale bar: 1 μm. Quantification of LDs has been performed by counting the number of LD-containing cells in 1 to 6 hundred cells per sample. LD-containing cells were 98% (OD_{600} = 0.7), 100% (OD_{600} = 2) and 100% (OD_{600} = 8) in the wild type strain and 3% (OD_{600} = 0.7), 16% (OD_{600} = 2) and 57% (OD_{600} = 8) in the mutant strain, respectively.

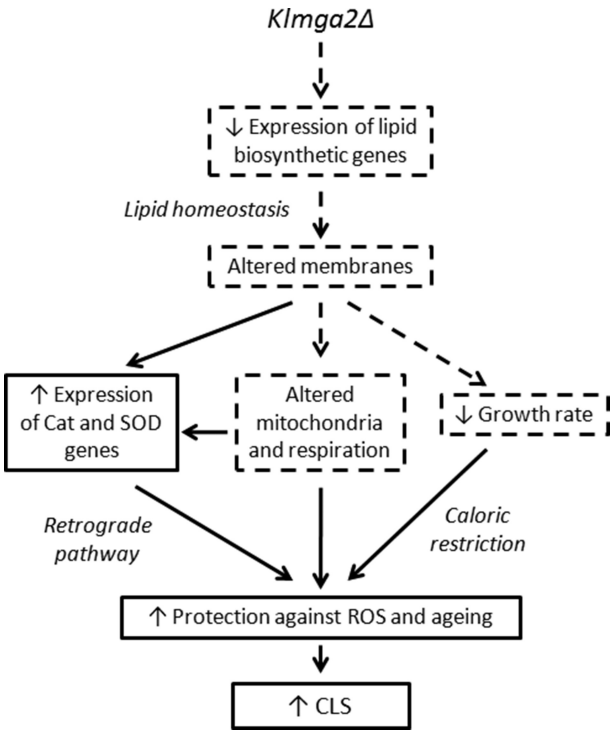

Figure 7. Scheme of possible connections among the functions affected by deletion of *KlMGA2*. Features considered as 'positive' in terms of cell fitness are marked with full lines. Features considered as 'negative' are marked with dashed lines. Mechanisms possibly involved, are indicated (see text for details).

DISCUSSION

We previously reported that deletion of *KlMGA2* produces pleiotropic phenotypes that can be ascribed to defects in lipid biosynthesis, glucose catabolism (fermentation) and/or mitochondrial functions (respiration) (Micolonghi *et al.* 2012; Ottaviano *et al.* 2015). In this work, we report new phenotypes of the *Klmga2*Δ strain, which are related to ROS metabolism and altered expression of Cat and SOD genes, extended life span and lipid storage, as summarized in Fig. 7. Phenotypes of the mutant strain could be explained by the fact KlMga2 is a transcription regulator of FA biosynthesis and its absence affects the proportion of UFAs and the unsaturation index (Micolonghi *et al.* 2012). The finding that most of mutant's phenotypes are suppressed by UFAs suggests that defects derive from altered membrane composition and, consequently, from affected cell functions associated with membranes. The importance of lipid composition in cell homeostasis has been well established in all organisms, from bacteria to humans (see Zhang and Rock 2009; Santos and Riezman 2012; Holthuis and Menon 2014 for review).

The results presented here indicate that membrane functions affected by KlMga2 deletion are involved in the regulation of the expression of ROS protective enzymes, suggesting a link between a fine tuning of lipid metabolism and response to the oxidative stress.

To investigate this hypothesis, we should take into account also the respiratory and mitochondrial defects of the *Klmga2*Δ strain (Ottaviano *et al.* 2015) since they might be directly correlated to the onset of oxidative stress. Notably, KlMga2 has mitochondrial related functions not reported to date for its counterpart Mga2 in *S. cerevisiae*. Retrograde response, defined as mitochondria-mediated signaling from damaged or stressed mitochondria to the nucleus (Liu and Butow 2006), could be involved in this mechanism. Such communication triggers changes in nuclear gene expression in order to repair the mitochondrial defects. In particular, the retrograde pathway could induce signals to protect cells from ROS accumulation due to damaged mitochondria, including transcription of Cat and SOD genes (Espinosa-Diez *et al.* 2015). When oxidative response enzymes were already functional during early exponential phase, as in the case of *Klmga2* mutant, ROS accumulation during culture lifetime could be reduced, thus protecting cells against ROS damage and aging and consequently expanding lifespan.

Besides the indirect effect of *KlMGA2* deletion through membrane-associated functions, the solubility of molecular oxygen and ROS also depends on membrane composition (Steels, Learmonth and Watson 1994). Changes in the ratio between FAs and UFAs in membranes could cause local changes in the redox properties of cell membranes, elicit an oxidative stress response and activate the expression ROS metabolic enzymes. Furthermore, the down-regulation of *FAD2* in the *Klmga2*Δ strain reduces the content of linoleic acid (De Angelis *et al.* 2016) which is a potential oxidant precursor because it can be oxidized to the highly toxic 13-hydroperoxylinoleic acid (Levine, 2002; Alic *et al.* 2004; Kelley and Ideker, 2009). This situation might alter the ROS response in the mutant strain. Indeed, when a heterologous Δ12 desaturase from plant has been expressed in *S. cerevisiae*, it resulted in an enhanced sensitivity to oxidative stress (Cipak *et al.* 2006). Deletion of *KlMGA2* also expands CLS. In addition to the early activation of ROS response, this behavior might also be the consequence of the reduced growth rate of the mutant strain a feature that could be assimilated to caloric restriction which is, in turn, a condition known to expand longevity (Kyryakov *et al.* 2012). The *Klmga2* mutant strain showed a reduced lipid droplet content consistent with caloric restriction (limiting carbon source consumption rate) and with the reduced FA content (Micolonghi *et al.* 2012).

Finally, we cannot exclude that phenotypes of the mutant strain might derive from the deregulated transcription of Cat and SOD genes, both in timing (early logarithmic growth phase) and quantity (increased level of transcription), due to a direct role of KlMga2 in phase-dependent repression of transcription of Cat and SOD genes or indirectly in regulating the expression of Cat and SOD genes regulators.

In conclusion, analysis of the phenotypes resulting from *KlMGA2* deletion demonstrated that KlMga2 ensures optimal growth rate, respiration and fitness, counter-balanced by reduced longevity and ROS protection. The results presented here on the hypoxic factor KlMga2 open new interesting fields to study the connection between unsaturated fatty acids, oxidative stress and longevity in *K. lactis*.

ACKNOWLEDGMENTS

We would like to thank Adam Stevens, from the University of Edinburgh, for helpful comments and review on the manuscript.

Funding

This work was supported by Sapienza Università di Roma and Ministero Affari Esteri e Cooperazione Internazionale [grant number MX14MO08, PGR00746].

Conflicts of interest. None declared.

REFERENCES

Alic N, Felder T, Temple MD *et al*. Genome-wide transcriptional responses to a lipid hydroperoxide: adaptation occurs without induction of oxidant defenses. *Free Radic Biol Med*. 2004;**37**:23–35.

Bianchi MM, Tizzani L, Destruelle M *et al*. The "petite-negative" yeast *Kluyveromyces lactis* has a single gene expressing pyruvate decarboxylase activity. *Mol Microbiol* 1996;**19**:27–36.

Blanco M, Núñez L, Tarrío N *et al*. An approach to the hypoxic and oxidative stress responses in *Kluyveromyces lactis* by analysis of mRNA levels. *FEMS Yeast Res* 2007;**7**:702–14.

Bossie MA, Martin CE. Nutritional regulation of yeast Δ-9 fatty acid desaturase activity. *J Bacteriol* 1989;**171**:6409–13.

Branco MR, Marinho HS, Cyrne L *et al*. Decrease of H_2O_2 plasma membrane permeability during adaptation to H_2O_2 in *Saccharomyces cerevisiae*. *J Biol Chem* 2004;**279**:6501–6.

Carmona-Gutierrez D, Bauer MA, Zimmermann A *et al*. Guidelines and recommendations on yeast cell death nomenclature. *Microb cell* 2018;**5**:4–31.

Chellappa R, Kandasamy P, Oh CS *et al*. The membrane proteins, Spt23p and Mga2p, play distinct roles in the activation of *Saccharomyces cerevisiae OLE1* gene expression. Fatty acid-mediated regulation of Mga2p activity is independent of its proteolytic processing into a soluble transcription activator. *J Biol Chem* 2001;**276**:43548–56.

Choi JY, Stukey J, Hwang SY *et al*. Regulatory elements that control transcription activation and unsaturated fatty acid-mediated repression of the *Saccharomyces cerevisiae OLE1* gene. *J Biol Chem* 1996;**271**:3581–9.

Cipak A, Hasslacher M, Tehlivets O *et al*. *Saccharomyces cerevisiae* strain expressing a plant fatty acid desaturase produces polyunsaturated fatty acids and is susceptible to oxidative stress induced by lipid peroxidation. *Free Radic Biol Med* 2006;**40**:897–906.

Cirigliano A, Stirpe A, Menta S *et al*. Yeast as a tool to select inhibitors of the cullin deneddylating enzyme Csn5. *J Enzyme Inhib Med Chem* 2016;**31**:1632–7.

Cohen G, Fessl F, Traczyk A *et al*. Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the *cta1* mutation. *Mol Gen Genet* 1985;**200**:74–9.

De Angelis L, Rinaldi T, Cirigliano A *et al*. Functional roles of the fatty acid desaturases encoded by *KlOLE1*, *FAD2* and *FAD3* in the yeast *Kluyveromyces lactis*. *Microbiology* 2016;**162**:1435–45.

Di Pasquale MG. The essentials of essential fatty acids. *J Diet Suppl* 2009;**6**:143–61.

Espinosa-Diez C, Miguel V, Mennerich D *et al*. Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biol* 2015;**6**:183–97.

Goldberg AA, Bourque SD, Kyryakov P *et al*. A novel function of lipid droplets in regulating longevity. *Biochem Soc Trans* 2009;**37**:1050–5.

González Siso MI, Cerdán ME. *Kluyveromyces lactis*: a suitable yeast model to study cellular defense mechanisms against hypoxia-induced oxidative stress. *Oxid Med Cell Longev* 2012;**2012**: 634674.

González-Siso MI, García-Leiro A, Tarrío N *et al*. Sugar metabolism, redox balance and oxidative stress response in the respiratory yeast *Kluyveromyces lactis*. *Microb Cell Fact* 2009;**8**:46.

Gralla EB, Kosman DJ. Molecular genetics of superoxide dismutases in yeasts and related fungi. *Adv Genet* 1992;**30**:251–319.

Greenspan P, Mayer EP, Fowler SD. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* 1985;**100**:965–73.

Hermann GJ, Shaw JM. Mitochondrial dynamics in yeast. *Annu Rev Cell Dev Biol* 1998;**14**:265–303.

Holthuis JCM, Menon AK. Lipid landscapes and pipelines in membrane homeostasis. *Nature* 2014;**510**:48–57.

Hoppe T, Matuschewski K, Rape M *et al*. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* 2000;**102**:577–86.

Huang M, Kao KC. Identifying novel genetic determinants for oxidative stress tolerance in *Candida glabrata* via adaptive laboratory evolution. *Yeast* 2018;**35**:605–618.

Jiang Y, Vasconcelles MJ, Wretzel S *et al*. *MGA2* is involved in the low-oxygen response element-dependent hypoxic induction of genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* 2001;**21**:6161–9.

Jiang Y, Vasconcelles MJ, Wretzel S *et al*. Mga2p processing by hypoxia and unsaturated fatty acids in *Saccharomyces cerevisiae*: impact on LORE-dependent gene expression. *Eukaryot Cell* 2002;**1**:481–90.

Jicha GA, Markesbery WR. Omega-3 fatty acids: potential role in the management of early Alzheimer's disease. *Clin Interv Aging* 2010;**5**:45–61.

Kainou K, Kamisaka Y, Kimura K *et al*. Isolation of Δ12 and ω3-fatty acid desaturase genes from the yeast *Kluyveromyces lactis* and their heterologous expression to produce linoleic and α-linolenic acids in *Saccharomyces cerevisiae*. *Yeast* 2006;**23**:605–12.

Kainz K, Tadic J, Zimmermann A *et al*. Methods to assess autophagy and chronological aging in yeast. *Methods Enzymol* 2017;**588**:367–94.

Kandasamy P, Vemula M, Oh CS *et al*. Regulation of unsaturated fatty acid biosynthesis in *Saccharomyces*: the endoplasmic reticulum membrane protein, Mga2p, a transcription activator of the *OLE1* gene, regulates the stability of the *OLE1* mRNA through exosome-mediated mechanisms. *J Biol Chem* 2004;**279**:36586–92.

Kelley R, Ideker T. Genome-wide fitness and expression profiling implicate Mga2 in adaptation to hydrogen peroxide. *PLoS Genet* 2009;**5**:e1000488.

Köhrer K, Domdey H. Preparation of high molecular weight RNA. *Methods Enzymol* 1991;**194**:398–405.

Kwast KE, Burke P V, Staahl BT *et al*. Oxygen sensing in yeast: evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. *Proc Natl Acad Sci USA* 1999;**96**:5446–51.

Kyryakov P, Beach A, Richard VR *et al*. Caloric restriction extends yeast chronological lifespan by altering a pattern of age-related changes in trehalose concentration. *Front Physiol* 2012;**3**:256.

Landi C, Paciello L, de Alteriis E *et al*. High cell density culture with *S. cerevisiae* CEN.PK113-5D for IL-1β production: optimization, modeling, and physiological aspects. *Bioprocess Biosyst Eng* 2015;**38**:251–61.

Lee J, Homma T, Kurahashi T *et al*. Oxidative stress triggers lipid droplet accumulation in primary cultured hepatocytes by activating fatty acid synthesis. *Biochem Biophys Res Commun* 2015;**464**:229–35.

Levine RL. Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radic BiolMed* 2002;**32**:790–6.

Liu Z, Butow RA. Mitochondrial retrograde signaling. *Annu Rev Genet* 2006;**40**:159–85.

Los DA, Murata N. Structure and expression of fatty acid desaturases. *Biochim Biophys Acta* 1998;**1394**:3–15.

Madeo F, Fröhlich E, Ligr M *et al*. Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol* 1999;**145**:757–67.

Matias AC, Pedroso N, Teodoro N *et al*. Down-regulation of fatty acid synthase increases the resistance of *Saccharomyces cerevisiae* cells to H_2O_2. *Free Radic Biol Med* 2007;**43**:1458–65.

McDonough VM, Stukey JE, Martin CE. Specificity of unsaturated fatty acid-regulated expression of the *Saccharomyces cerevisiae OLE1* gene. *J Biol Chem* 1992;**267**:5931–6.

Micolonghi C, Wésolowski-Louvel M, Bianchi MM. The Rag4 glucose sensor is involved in the hypoxic induction of *KlPDC1* gene expression in the yeast *Kluyveromyceslactis*. 2011. *EukaryotCell* 2011;**10**:146–8.

Micolonghi C, Ottaviano D, Di Silvio E *et al*. A dual signalling pathway for the hypoxic expression of lipid genes, dependent on the glucose sensor Rag4, is revealed by the analysis of the *KlMGA2* gene in *Kluyveromyces lactis*. *Microbiology* 2012;**158**:1734–44.

Nakagawa Y, Sugioka S, Kaneko Y *et al*. O2R, a novel regulatory element mediating Rox1p-independent O(2) and unsaturated fatty acid repression of *OLE1* in *Saccharomyces cerevisiae*. *J Bacteriol* 2001;**183**:745–51.

Nikolaou E, Agriafioti I, Stumpf M *et al*. Phylogenetic diversity of stress signaling pathways in fungi. *BMC Evol Biol* 2009;**9**:44.

Ottaviano D, Montanari A, De Angelis L *et al*. Unsaturated fatty acids-dependent linkage between respiration and fermentation revealed by deletion of hypoxic regulatory *KlMGA2* gene in the facultative anaerobe-respiratory yeast *Kluyveromyces lactis*. *FEMS Yeast Res* 2015;**15**:1–12.

Palermo V, Falcone C, Mazzoni C. Apoptosis and aging in mitochondrial morphology mutants of *S. cerevisiae*. *Folia Microbiol (Praha)* 2007;**52**:479–83.

Pol A, Gross SP, Parton RG. Review: biogenesis of the multifunctional lipid droplet: lipids, proteins, and sites. *J Cell Biol* 2014;**204**:635–46.

Raimondi S, Zanni E, Talora C *et al*. *SOD1*, a new *Kluyveromyces lactis* helper gene for heterologous protein secretion. *Appl Environ Microbiol* 2008;**74**:7130–7.

Rodicio R, Heinisch JJ. Yeast on the milkyway: genetics, physiology and biotechnology of *Kluyveromyces lactis*. *Yeast* 2013;**30**:165–77.

Santomartino R, Riego-Ruiz L, Bianchi MM. Three, two, one yeast fatty acid desaturases: regulation and function. *World J Microbiol Biotechnol* 2017;**33**:1–12.

Santos AXS, Riezman H. Yeast as a model system for studying lipid homeostasis and function. *FEBS Lett* 2012;**586**:2858–67.

Sitepu IR, Ignatia L, Franz AK *et al*. An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *J Microbiol Methods* 2012;**91**:321–8.

Smith DL, Jr, McClure JM, Matecic M *et al*. Caloric restriction extends the chronological life span of *Saccharomyces cerevisiae* independently on the Sirtuins. *Aging Cell* 2007;**6**:649–62.

Steels EL, Learmonth RP, Watson K. Stress tolerance and membrane lipid unsaturation in *Saccharomyces cerevisiae* grown aerobically or anaerobically. *Microbiology* 1994;**140**:569–76.

Stukey JE, McDonough VM, Martin CE. Isolation and characterization of OLE1, a gene affecting fatty acid desaturation from *Saccharomyces cerevisiae*. *J Biol Chem* 1989;**264**:16537–44.

Tafforeau L, Le Blastier S, Bamps S *et al*. Repression of ergosterol level during oxidative stress by fission yeast F-box protein Pof14 independently of SCF. *EMBO J* 2006;**25**:4547–56.

Vasconcelles MJ, Jiang Y, McDaid K *et al*. Identification and characterization of a low oxygen response element involved in the hypoxic induction of a family of *Saccharomyces cerevisiae* genes: implications for the conservation of oxygen sensing in eukaryotes. *J Biol Chem* 2001;**276**:14374–84.

Wolfe KH, Shields DC. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* 1997;**387**:708–13.

Zhang S, Skalsky Y, Garfinkel DJ. *MGA2* or *SP T23* is required for transcription of the delta9 fatty acid desaturase gene, *OLE1*, and nuclear membrane integrity in *Saccharomyces cerevisiae*. *Genetics* 1999;**151**:473–83.

Zhang Y-M, Rock CO. Transcriptional regulation in bacterial membrane lipid synthesis. *J Lipid Res* 2009;**50**:S115–9.