Sapienza University of Rome

Ph.D. Course in Cell and Developmental Biology

Cycle XXXIII

Ph.D. student: Ilaria Camponeschi

Supervisor: Prof. Michele M. Bianchi

External reviewers:

Prof. Maurizio Ruzzi
Department for Innovation in Biological, Agro-food and Forest systems (DIBAF)
University of Tuscia, Viterbo, Italy

Dr. Sergio Giannattasio
Institute of Biomembranes, Bioenergetics and Molecular Biotechnologies (IBIOM)
National Research Council of Italy (CNR), Bari, Italy

TITLE: The role of the hypoxic transcription factor gene *MGA2* in *Kluyveromyces lactis* fatty acids metabolism and cell fitness

Index

1. General introduction

The yeast *Kluyveromyces lactis* is one of the few yeasts that can uptake lactose and use this sugar as a carbon and energy source (Chang and Dickson 1988). It is an alternative eukaryotic model organism phylogenetically correlated to *Saccharomyces cerevisiae*. Unlike the latter, *K. lactis* did not undergo the whole genome duplication (WGD) event (Wolfe and Shields 1997), resulting in a lower redundancy of key genes as compared to *S. cerevisiae* and thus facilitating some kind of studies and analysis. One of the main differences is that *S. cerevisiae* is a typically fermentative yeast, while *K. lactis* has a predominantly respiratory metabolism, which is not inhibited even in the presence of high glucose concentrations under normoxic conditions (Crabtree negative yeast; De Deken, 1966; Rodicio and Heinisch, 2013). However, *K. lactis* is able to produce ethanol under oxygen-limited conditions or in presence of the respiratory drug Antimycin A (Rag^+ phenotype; Wèsolowski-Louvel *et al.*, 1992). Thus, respiratory and fermentative metabolic pathways can proceed in parallel in *K. lactis* (respiro-fermentative metabolism; González-Siso *et al.*, 2000). The key metabolic step in the balance between fermentative and respiratory metabolism is pyruvate channelling. The pyruvate can be oxidized into acetyl-CoA by the mitochondrial pyruvate dehydrogenase (PDH) complex and directed into the respiratory metabolism; otherwise, pyruvate can be converted into acetaldehyde by the cytoplasmic pyruvate decarboxylase (PDC) and then reduced to ethanol. In addition, cytosolic acetyl-CoA can be formed by the so-called PDH bypass pathway, because different enzymes from PDH convert pyruvate into acetaldehyde, acetate and finally into acetyl-CoA (Breunig *et al.* 2000). Each molecule of pyruvate that is decarboxylated and reduced to ethanol is not available for channelization into the tricarboxylic acid (TCA) cycle. Thus, adenosine triphosphate (ATP) and biomass yield is higher in Crabtree-negative yeasts, such as *K. lactis*, than Crabtree–positive yeasts, and this represents one of the advantages for the biotechnological and industrial applications of *K. lactis* (Spohner *et al.* 2016). Carbon metabolism and activity of metabolic enzymes are precisely regulated to respond and adapt to the various physiological or pathological/stress conditions. The biology of stress responses has been very well studied in eukaryotic microorganisms, such as *S. cerevisiae*, *Neurospora crassa* and *Aspergillus nidulan*s (Rensing, Monnerjahn and Meyer, 1998; Fillinger *et al.*, 2001; Rangel, 2011; Taymaz-Nikerel, Cankorur-Cetinkaya and Kirdar 2016; Saini *et al.* 2018), but little is known on the respiratory yeast *K. lactis*.

In our laboratory we are studying hypoxic role of *KlMGA2*, the orthologue of *S. cerevisiae MGA2/SPT23*, and the linkage between low oxygen concentration and glucose metabolism (Micolonghi *et al.* 2012; Ottaviano *et al.* 2015). Mga2 is a transcription factor constitutively expressed as inactive form bound into endoplasmic reticulum (ER). It is activated in hypoxic conditions by proteasome cleavage (Figure 1) (Hoppe *et al.* 2000). Mga2 forms homodimers and the transmembrane domain maintains a certain freedom of rotation within the ER membrane bilayer. Changes in conformation of Mga2 dimer allow its activation. The cytosolic domains of Mga2 adopt an orientation that makes them accessible for Rsp5-mediated ubiquitination. Ubiquitinated Mga2 is processed by cytosolic proteasome which induces a cleavage on one of the polypeptides of the homodimer and generates a 90 kDa fragment that remains attached to the unprocessed binding partner of the dimer (Goder, Alanis-Dominguez and Bustamante-Sequeiros 2020). Finally, the 90 kDa fragment is mobilized by the AAA-ATPase Cdc48 and its co-factors Npl4, Ufd1, and Ubx2 (Surma *et al.* 2013). Then the soluble N- terminal fragment moves into the nucleus where it induces the expression of low-oxygen responsive genes, like the desaturase gene *OLE1* (Zhang, Skalsky and Garfinkel, 1999; Jiang *et al.*, 2001; Chellappa *et al.* 2001).

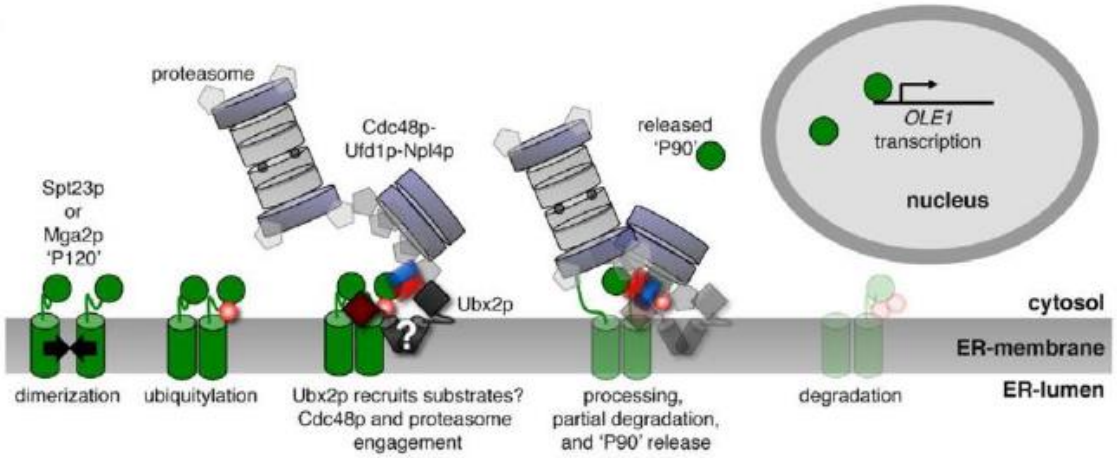


Figure 1 – Schematic model of transcription factors Mga2/Spt23 maturation in *S. cerevisiae*. After protein dimerization and ubiquitylation, proteasome engagement induces partial protein degradation with release of 90 kDa fragment (p90). The fragment is mobilized by the AAA-ATPase Cdc48 and its co-factors Npl4, Ufd1, and Ubx2 might assist this reaction. P90 translocates into the nucleus to activate *OLE1* transcription (Surma *et al.* 2013).

We have shown that also in *K. lactis Kl*Mga2 is a transcription factor involved in the regulation of lipid synthesis but, differently from *S. cerevisiae*, is also involved in respiratory functions and in the general cellular fitness (Micolonghi *et al.* 2012; Ottaviano *et al.* 2015; Santomartino *et al.* 2017).
Studies on lipid metabolism have been manly conducted on *S. cerevisiae* (Natter and Kohlwein 2013): in this yeast, fatty acids (FAs) biosynthesis is restricted to saturated and monounsaturated FAs (MUFAs) and essentially accomplished by acetyl-CoA carboxylase (Acc1) and FA synthase (Fas1) enzymes and by the unique Δ9 desaturase enzyme (Ole1) that produces only the monounsaturated palmitoleic and oleic acids (Santomartino *et al.* 2017). In *K. lactis* the composition of FAs is enriched with the polyunsaturated linoleic and α-linolenic acids generated by the Δ12 (Fad2) and ω3 (Fad3) desaturases respectively (Kainou *et al.* 2006; Micolonghi *et al.* 2012). Therefore, the environmental conditions might have effects on the synthesis and abundance of different (mono- and poly-) unsaturated FA molecules (Rossi *et al.* 2009), as compared to *S. cerevisiae*. In *K. lactis FAD2* gene is a hypoxic target of *Kl*Mga2. Interestingly, desaturase level and the resultant lipid unsaturation index were both light-dependent in cold stress in plants (Yuan *et al.* 2012). Although yeast is an organism devoid of photoreceptors, it is known that *S. cerevisiae* could respond to blue light stimulation (Bodvard *et al.* 2011, 2013, 2017) suggesting that light can influence cellular mechanisms also in non-photosynthetic organism. This work includes studies about the role of the yeast multi-functional mediator *Kl*Mga2 deletion in the oxidative stress response, longevity and general fitness. Effects of light on oxidative stress and lipid biosynthesis were also studied.

2. Aims of the work

According to our previous works, the aim of this Thesis is to investigate the effects of the absence of *Kl*Mga2 transcription factor on oxidative stress response, fatty acid metabolism and cell fitness in *Kluyveromyces lactis*.

For this reason, in the first part, we focused on observing ROS metabolism and on the expression of the main genes involved in oxidative stress response (Catalases and Superoxide dismutase enzymes) in correlation to hypoxic mediator *Kl*Mga2. Results allowed to establish that the absence of *Kl*Mga2 leads to protection against stress conditions and to expanding lifespan of the mutant strain (*).

Despite the lack in yeast of specialized photosensory proteins, we wanted to investigate *Kl*Mga2 role in presence of white light, as a source of environmental stress. In *S. cerevisiae* the light impulse is converted into H_2O_2. This molecule is a second messenger that triggers Msn2 nuclear accumulation, a typical stress- transcription factor in yeast. For this reason, in the second part of the Thesis, we studied the response to light and darkness in absence of *Kl*Mga2 and the role of Msn2 in the oxidative stress response activation (**).

*= the first part of the Thesis is an extract of our publication (Santomartino *et al.* 2019).

**= the second part of the Thesis is our manuscript in preparation for submission.

3. Part I: The hypoxic transcription factor *Kl*Mga2 mediates the response to oxidative stress in the yeast *Kluyveromyces lactis.*

3.1 Sommario

K. lactis è un lievito non convenzionale ampiamente utilizzato sia nelle applicazioni industriali che nella ricerca di base. La principale caratteristica che lo differenzia dal lievito modello *S. cerevisiae* è la sua preferenza per la respirazione. Infatti, *K. lactis* è un organismo negativo al Crabtree, per cui l'equilibrio tra respirazione e fermentazione dipende principalmente dalla disponibilità di ossigeno e non dalla concentrazione di glucosio. Nel nostro laboratorio stiamo studiando il gene *KlMGA2*, che codifica per un modulatore della risposta all'ipossia. In lavori precedenti, abbiamo mostrato come la delezione del gene *KlMGA2* in *K. lactis* generi un ceppo vitale, ma con difetti di crescita cellulare, alterazione in quantità e composizione degli acidi grassi, respirazione difettosa e morfologia mitocondriale alterata. Tutti questi difetti vengono ripristinati con l'aggiunta nel terreno di acidi grassi insaturi. Con questo lavoro mostriamo come l'assenza del gene *KlMGA2* causi, oltre ai fenotipi sopra citati, una maggiore resistenza allo stress ossidativo ed una longevità molto estesa, accompagnati da un aumento dell'espressione genica delle catalasi e delle superossido dismutasi. Questo potrebbe suggerire un coinvolgimento di *Kl*Mga2 come mediatore diretto non solo della risposta ipossica, ma anche nella risposta allo stress ossidativo, ipotizzando una correlazione tra ipossia, regolazione del glucosio, biosintesi degli acidi grassi e metabolismo dei ROS.

3.2 Abstract

The yeast *K. lactis* has been widely used in both industrial applications and basic research. A main regulatory trait that differentiates it from the conventional yeast *S. cerevisiae* is its preference for respiration. Indeed, *K. lactis* is a Crabtree-negative organism, having weak or absent glucose repression and the balance between respiration and fermentation dependent on oxygen availability and not glucose concentration. We previously demonstrated that deletion of the *KlMGA2*, coding for a hypoxic mediator in *K. lactis*, generated a viable strain, although suffering of several deficiencies, all restored by addition of UFAs (Unsaturated Fatty Acids) to the medium. We also showed that glucose signaling and glucose catabolism were involved in *Kl*Mga2 regulation. In this work, we show that, in addition to these defects, the deletion of *KlMGA2* also caused increased resistance to oxidative stress and extremely extended lifespan. These phenotypes are associated with increased expression levels of catalase and superoxide dismutase genes. We propose that *Kl*Mga2 might act as a direct mediator not only of hypoxic response, but also of oxidative stress response/adaptation, thus revealing connections between hypoxia, glucose signaling, fatty acid biosynthesis and ROS metabolism.

3.3 Introduction

Environmental oxygen is a fundamental component for living organisms, especially aerobic organisms that use molecular oxygen for energy and essential metabolic reactions. For this reason, they have generally developed systems for oxygen sensing and developed responses to hypoxic conditions. Hypoxia is defined as the decline of oxygen availability under a certain threshold, depending on environmental supply and cellular consumption rate. The decrease in O_2 concentration results in reduction of available energy in facultative aerobes, because anaerobic metabolism is less efficient in terms of ATP synthesis.

Fatty acid desaturases 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). As reported above, 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. 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 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).

The *Kl*MGA2 deleted strains of *K. lactis* display many phenotypes: reduced growth rate, sensitivity to the mitochondrial drug antimycin A on high (5%) glucose medium (rag- phenotype), multi-budded cell morphology with altered mitochondrial morphology (collapsed morphology) and respiration 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).

The altered membrane composition observed in *Klmga2*Δ strain, together with defective respiration and the mitochondrial morphology changes, might be associated to altered permeability and resistance to hydrogen peroxide (Matias *et al.* 2007) or to reactive oxygen species (ROS) accumulation and consequently impaired response to oxidative stress (Larosa and Remacle 2018). 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 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). In this work, we investigated the effects of deletion of *Kl*Mga2 on the regulation of ROS metabolism and longevity. Results indicate the involvement of this transcription factor and of unsaturated fatty acids in these mechanisms.

3.4 Results

ROS content in the *Klmga2Δ* 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. 2A. 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 at OD_{600} = 10 (stationary phase), ROS content resulted similar in the two strains (Fig. 2B) with differences appearing only after exposure to the higher concentrations of H_2O_2. 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 12mM 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. 2C. 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.

Catalase and superoxide dismutase genes in *K. lactis*– 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 peroxisomal and the cytosolic catalases (Cohen *et al.* 1985), 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 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 the typical 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*. *KlSOD1* gene of *K. lactis* has been previously characterized in heterologous protein production studies (Raimondi *et al.* 2008).

Transcription analysis of *KlCTA1, KlCTT1, KlSOD1* and *KlSOD2* genes – 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 are reported in Fig. 3, together with the corresponding histogram for signal quantification respect to ribosomal RNAs. In the wild-type strain, transcript levels of the four 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 these 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 showed in Figure 2.

Activity of catalase and superoxide dismutase enzymes – To assess if the higher transcription rate of the Catalase (Cat) and SOD genes 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 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 at the exponential phase 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). These findings were consistent with a constitutively activated ROS metabolism in the absence of *Kl*Mga2, independently of the growth phase.

Chronological life span of the *Klmga2Δ* strain – ROS can elicit mechanisms of cell defense, including modulation of life span, programmed cell dead, 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 these studies. We wondered if the increased protection against ROS, together with the low 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. Results are shown in Fig. 5. 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. Differently, the viability of the mutant strain decreased slowly to 10%

over 19 days and then more rapidly to about 0.2% after 24 days. OD_{600} of the cultures of each time point is represented in panel B of figure 5. These results demonstrated an extended CLS of the mutant. The extended viability could be dependent on the activity of the ROS metabolic enzymes highly expressed since the exponential growth phase (Fig. 3 and 4). The activity of Cat and SOD enzymes could protect mutant cells from cumulative damages by ROS generation with better efficiency than wild type cells.

Effects of unsaturated fatty acids (oleate) on ROS metabolism and longevity– It has been previously shown (Ottaviano *et al.* 2015), addition of unsaturated fatty acids oleic and/or palmitoleic suppresses phenotypes of the *Klmga2Δ* mutant strain. 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 0.1% (w/v) Tween80, a source of oleate. Cells were collected for Cat and SOD activity assays at the usual OD_{600} values. Results are reported in Fig. 4. In wild type cells cultivated in Tween80-containing YPD medium, Cat activity did not change substantially (Fig. 4A). In fact, activity values significantly increased along the growth phases as in standard YPD medium: activities at OD_{600} = 0.7 and 8 were only slightly (3.2 and 22 U/mg instead of 4.6 and 28 U/mg, respectively) but significantly reduced (P = 0.0024 and 0.0017, respectively). Cat activity in the mutant strain on Tween80-supplemented medium was again much higher than in the wild type, especially at the exponential growth phases, similarly to the experiment carried out in standard medium. However, Cat activities in the mutant cells progressively and significantly increased along growth phases as the wild type. The presence of Tween80 had no effect on SOD activity profile of wild type cells: only a small but significant (P = 0.04) increase of activity at OD = 0.7 (Fig. 4B). On the contrary, Tween80 addition in mutant cell cultures caused a significant decrease of SOD activity, especially in the exponential phase (OD = 0.7 and 2). Only at OD = 4 the decrease was not statistically significant (P = 0.055). SOD activity was constant in the mutant cells at all OD_{600} tested and comparable to the wild type. In summary, the addition of an oleate source did not affect Cat and SOD activity in growing wild type cells. In the mutant cells, the effect of oleate was different on Cat and SOD: Cat activity was maintained higher than the wild type while SOD activity was reduced. Also the dependence on growth phases was differently influenced by oleate in the two strains.

We tested if growth in the presence of oleate could also reverse the extended CLS of the *Klmga2Δ* 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 semi-quantitatively by assaying growth on YPD plates of serial dilutions of culture samples withdrawn at various time points (days) after inoculum. Growth results at selected days are reported in Fig. 6. Viable wild type cells could be detected up to day 5 and 7 while, similarly to the experiment reported in Fig. 5, viability of the mutant strain was extended at least to day 21. Surprisingly, CLS of the wild type was slightly increased to 7-11 days by Tween80 while the viability in the mutant strain was strongly shortened to day 9 in the same growth medium. This finding further confirms that UFAs could suppress the large variety of *Klmga2Δ* mutant phenotypes.

3.5 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) (Ottaviano *et al.* 2015). 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 results presented here indicate that membrane functions affected by *Kl*Mga2 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 these hypotheses, 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, *Kl*Mga2 has mitochondrial related functions not reported 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 catalases 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-hydroperoxy linoleic 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).

In conclusion, analysis of the phenotypes resulting from *KlMGA2* deletion demonstrated that *Kl*Mga2 ensures optimal growth rate, respiration and fitness, counter-balanced by reduced longevity and ROS protection. The results presented on the hypoxic factor *Kl*Mga2 open new interesting fields to study the connection between unsaturated fatty acids, oxidative stress and longevity in *K. lactis.*

3.6 Tables

Table 1. List of *K. lactis* strains used in this work.

Name	Description	Reference
Strains		
GDK	MATa ura3 leu2 metA1-1 lac4::ura3 promKlPDC1:: lacZ::LEU2	Micolonghi et al, 2012
GDK/Klmga2Δ	MATa ura3 leu2 metA1-1 lac4::ura3, promKlPDC1:: lacZ::LEU2 Klmga2 ::kanMX4	Micolonghi et al, 2012

Table 2. List of oligonucleotides used in this work.

N	Name	Sequence	Use
1	CTA1-fw	GCGTCGTGTAGGTAAGATGGTT	Northern blot probe
2	CTA1-rv	CTTGGATTTCAGGGCATGCACT	Northern blot probe
3	CTT1-fw	CGGTACCATTACCCTAACCCAT	Northern blot probe
4	CTT1-rv	GATTGGAGATGCACCATTGACA	Northern blot probe
5	SOD1-fw	CAGTTGCAGTTTTGAAGGGTC	Northern blot probe
6	SOD1-rv	TACCAATAACACCACAG	Northern blot probe
7	SOD2-fw	ATTGGACTGGGATTTCGATG	Northern blot probe
8	SOD2-rv	CTTAGCAGCTTCCTTCCAGT	Northern blot probe

3.7 Figures

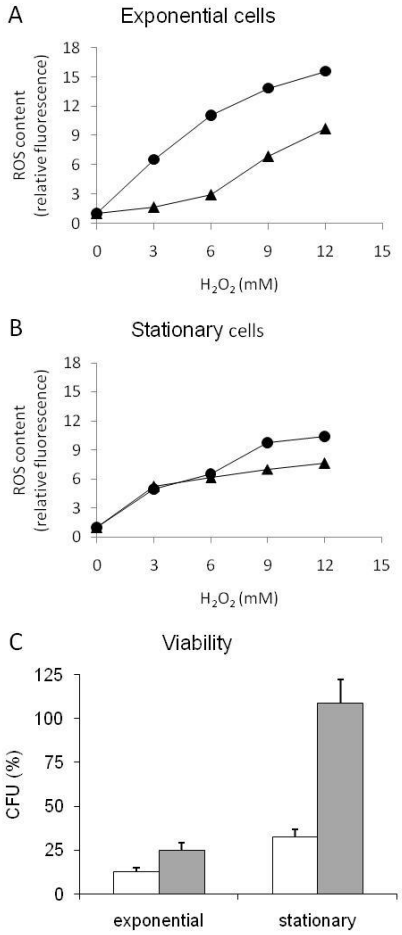


Figure 2 – ROS content of wild type and *Klmga2*Δ cells. 2A shows the ROS content, revealed by fluorescence after staining with DHR, in exponential (OD_{600}=0.5) wild type (circles) and mutant (triangles) cells incubated (75 min) with increasing amount of hydrogen peroxide. Fluorescence values have been normalized by untreated (H_2O_2=0) cell fluorescence. 2B shows the ROS content of stationary (OD_{600}=10) wild type (circles) and mutant (triangles) cells incubated with increasing amount of hydrogen peroxide. Fluorescence values have been normalized by untreated (H_2O_2=0) cell fluorescence. 2C 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. White bars indicate wild type and gray bars indicate mutant cells.

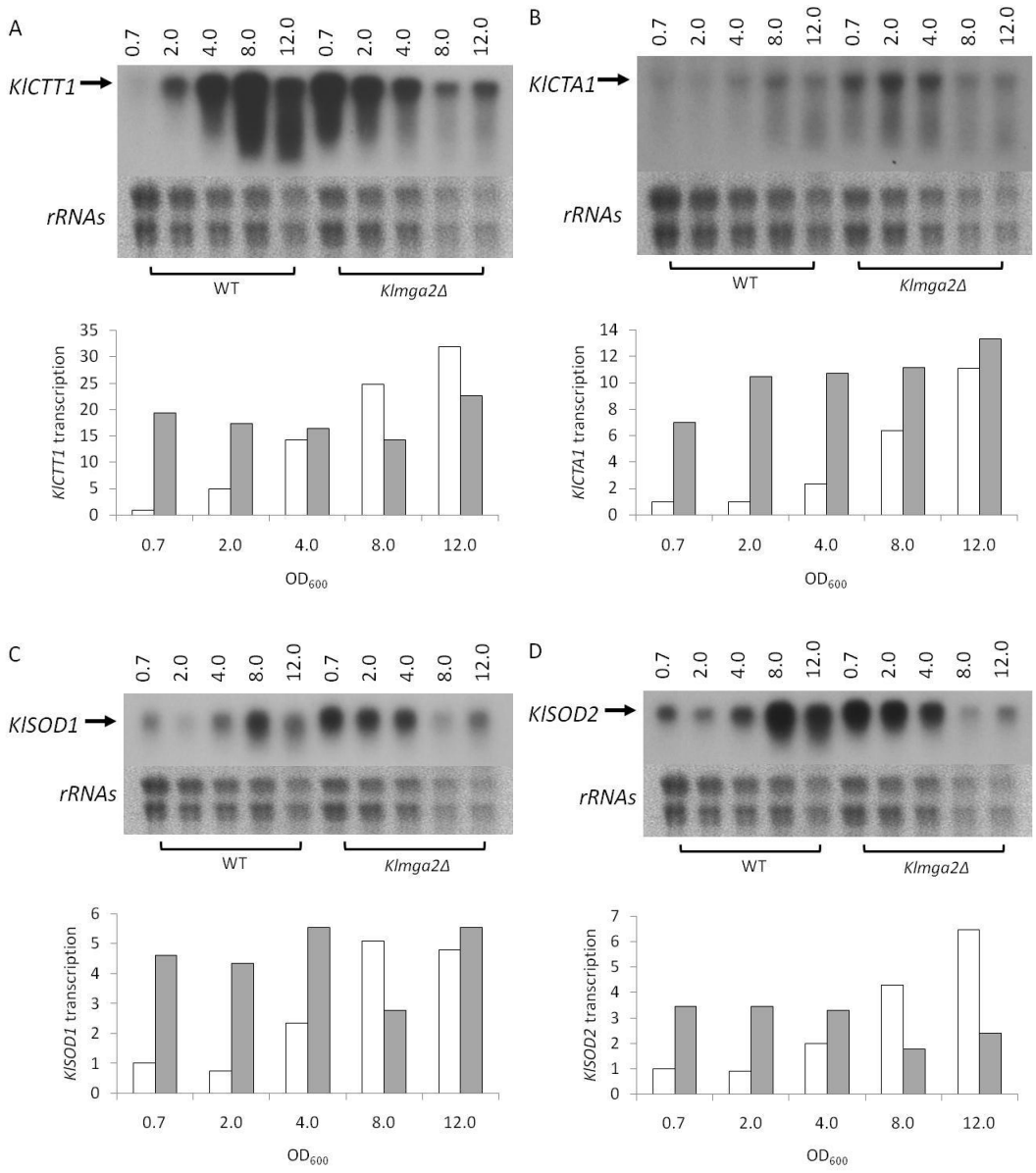


Figure 3– Transcription of *KlCTT1, KlCAT1, KlSOD1* and *KlSOD2* genes. Results of Northern blot analysis of Catalase (Cat) and Superoxide dismutase (SOD) genes in wild type (WT) and mutant (*Klmga2Δ*) strains grown in YPD flask cultures to the indicated OD_{600} are shown. Ribosomal RNAs (*rRNAs*) have been used as loading control. Signals have been quantified by pixel counting and normalized by *rRNAs* pixel values (Phoretix 2D, Nonlinear 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 bars are wild type values and grey bars are the mutant values. 3A, 3B, 3C and 3D report *KlCTT1*, *KlCTA1*, *KlSOD1* and *KlSOD2* transcription analysis, respectively. Duplicated experiment sets gave the same results.

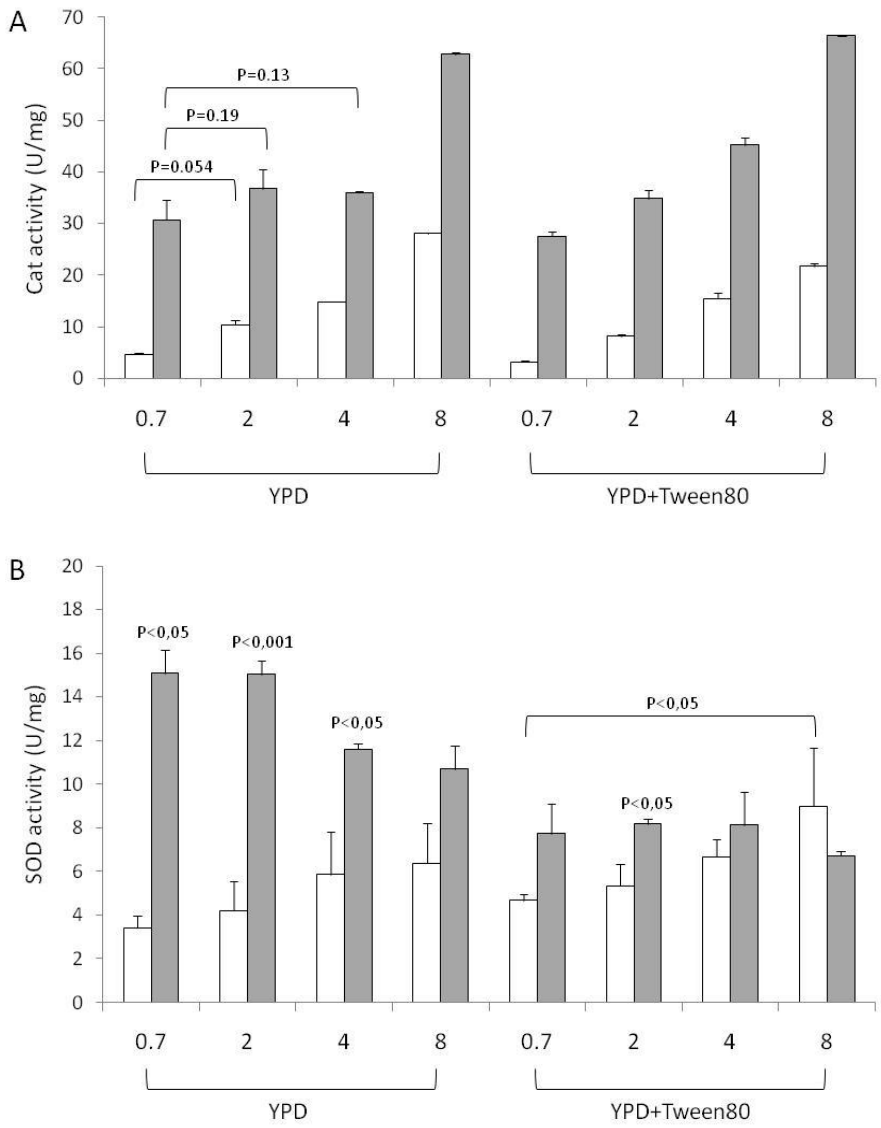


Figure 4 – Activity of catalases (Cat) and SOD enzymes. Total extracts from wild type and *Klmga2*Δ strains grown to the indicated OD_{600} (reported on x axis) were assayed for catalase and superoxide dismutase activities as described in the Materials and Methods section. Results are reported as histograms: white bars are wild type values and grey bars are mutant values. 4A 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. 4B 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 sides. Activity values are averages of three independent determinations, each measured by three technical repetitions, with 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 indicated in text.

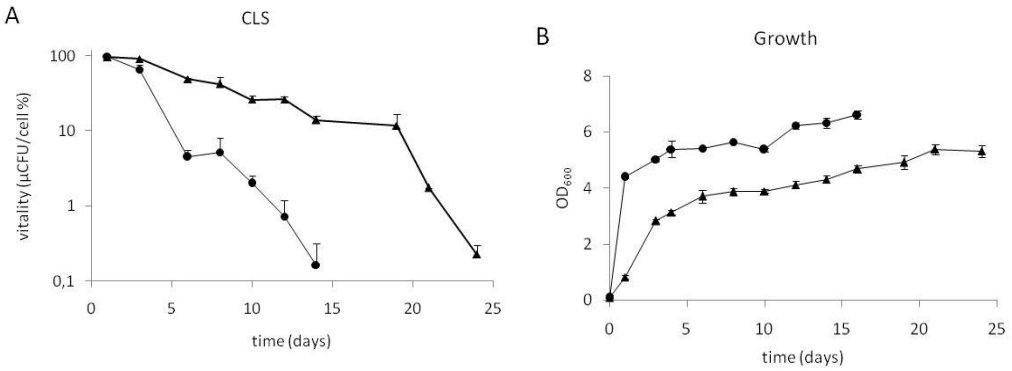


Figure 5– Chronological Life Span (CLS) in wild type and *Klmga2Δ* strains. CLS was determined by viability, calculated at each time point by measuring the percentage of cells capable to undergo cell divisions and to form microcolonies (µCFU/cell). Samples at different time points (days of growth) were plated on YPD and observed for microcolonies formation (5A). OD_{600} was also measured for each time point (5B). Averages and standard deviations of 3 to 4 independent cultures are reported. Circles are wild type strain values and triangles are mutant strain values.

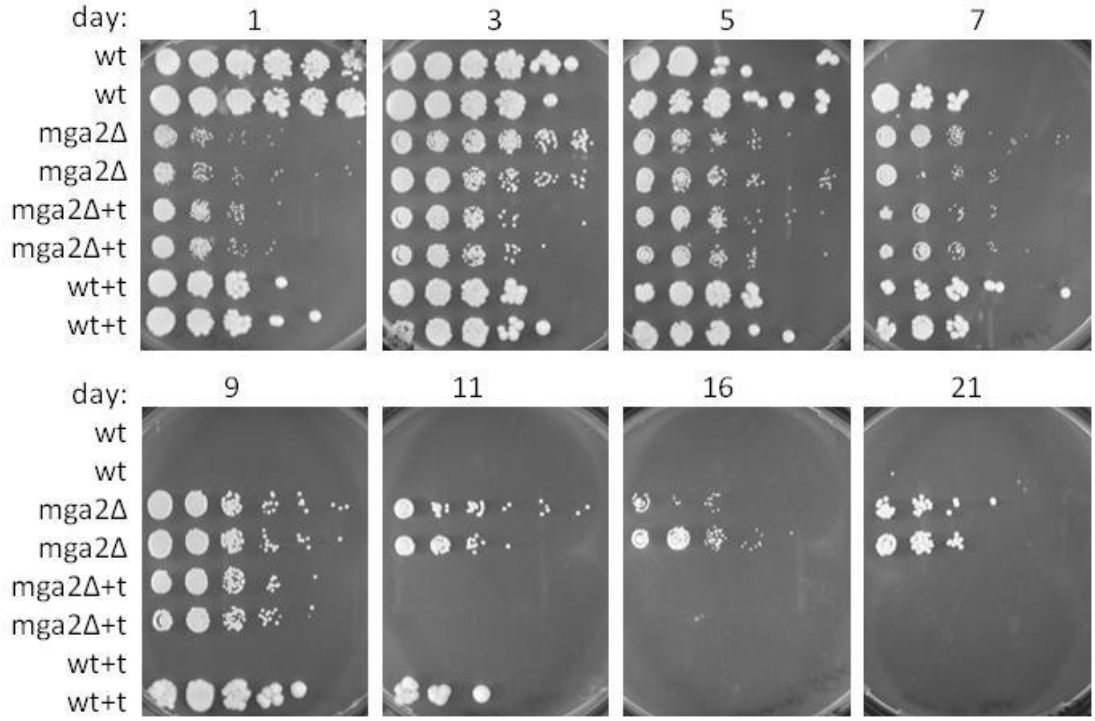


Figure 6- Viability of wild-type and *Klmga2Δ* strains on oleate. Strains were cultured on SD (wt and mga2Δ rows) and SD supplemented with Tween80 (+t rows). Two independent cultures (two rows) were performed for each strain/condition. Viability was determined as the capacity to form colonies on YPD 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 *Klmga2Δ* strain was maintained up to day 24 in SD (not shown).

3.8 Materials and methods

STAR★Methods

Key resources table

Reagent or resource	Source	Identifier
Yeast Extract	Becton Dickinson and Company	212750
Peptone	Becton Dickinson and Company	211677
Yeast Nitrogen Base w/o aminoacids	Becton Dickinson and Company	233520
Bacto-Agar	Becton Dickinson and Company	214010
Tween80	Sigma-Aldrich	P4780
Geneticin (G418)	Sigma-Aldrich	A1720
Coomassie Plus Assay kit	Thermo Scientific	23236
Hydrogen peroxide	Sigma-Aldrich	216763
Sod enzyme	Sigma-Aldrich	S2515
Critical Commercial Assay		
SOD Assay kit-WST	Sigma-Aldrich	19160-1KT-F
Random Primed Labelling Kit	Roche	11004760001
Software and Algorithms		
WinMDI 2.8 software	Purdue University, Cytometry Laboratories	N/A
Phoretics 1D plus	Nonlinear Dynamics	N/A

N/A = not available

Method Details

Strains and media – The yeast strains used are listed in Table 1.The wild type strain GDK and mutant strain GDK/*Klmga2*Δ have been previously described (Micolonghi *et al.* 2012). The YPD medium was composed of 1% Yeast Extract (Becton Dickinson and Company), 1% Peptone (Becton, Dickinson and Company) and 2% glucose (YPD medium). Synthetic medium contained 0.67% Yeast Nitrogen Base w/o aminoacids (Becton, Dickinson and Company) and 2% glucose, supplemented with auxotrophic requirements (SD medium). Solid media contained 2% Bacto-Agar (Becton, Dickinson and Company). Tween80 (Sigma-Aldrich) was added to media at 0.1% as oleate source. For positive selection of transformed cells, we used YPD solid medium supplemented with a final concentration of 100µ/mL Geneticin (G418; Sigma-Aldrich) or SD solid medium supplemented with aminoacids mix without uracil (Arg 1mg/mL, His 1mg/mL, Leu 6 mg/mL, Met 1mg/mL, Trp 2mg/mL, Lys 1mg/mL, Ade 2mg/mL; Sigma-Aldrich).

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, $2x10^6$ 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 choose 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 (data 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; 25000 cells were measured for every 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 values have been normalized to the value for cells not challenged with H_2O_2.

Catalase and Superoxide dismutase activity - 3 to 4 OD_{600} units of cells were collected from cultures and extracts were prepared by glass beads crushing in lysis buffer (50 mM Tris-HCl pH 6.8; 100 mM NaCl). Protein content in samples was determined photometrically (Nanodrop 1000) or by Coomassie Plus Assay kit (Thermo Scientific) 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 (Sigma-Aldrich) 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 (ε_{240}=39.4 M^{-1} cm^{-1}). One Unit of catalase activity is the amount of enzyme that catalyzed the degradation of 1 µmol of H_2O_2/min. Superoxide dismutase activity was determined by measuring the rate of WST1-Formazan formation, using the SOD Assay kit-WST (Sigma-Aldrich), as suggested by the supplier. Calibration curve was determined using a commercial Sod enzyme (Sigma-Aldrich). 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 from cultures grown to $OD_{600}\approx1$ 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 GDK genomic DNA as a template and primers 9-16 (Table 2). [α-^{32}P] ATP labeling of the probes was performed using the Random Primed Labelling Kit (Roche), following the instructions provided by the manufacturer. Quantification of signals was performed with Phoretics 1D plus (Nonlinear Dynamics) 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) were also used. At each time point, OD_{600} was measured and opportune dilutions were analyzed. Quantitative Chronological Life Span (CLS) was determined by measuring the percentage of cell capable to form microcolonies on YPD medium, observed and counted under light microscope at 40X (Fabrizio and Longo 2007; Palermo, Falcone and Mazzoni 2007). 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 3-4 days at 28°C (Carmona-Gutierrez *et al.* 2018).

4. Part II:

Effects of light and dark exposure on carbon metabolism in *Kluyveromyces lactis.*

4.1 Sommario

Il metabolismo del carbonio presenta precisi meccanismi regolativi che permettono alle cellule di adattarsi ai fattori ambientali e/o abiotici, sia essi fisiologici sia patologici o recanti stress. Difetti riguardanti tali meccanismi di regolazione sono spesso associati a patologie; pertanto, la comprensione delle vie metaboliche ha assunto un ruolo chiave nella ricerca biomedica. In tale contesto, il lievito respiratorio *K. lactis* rappresenterebbe un buon sistema modello per gli studi metabolici delle cellule umane. In particolar modo, con questo lavoro si è cercato di verificare una possibile risposta allo stress luminoso. Negli organismi unicellulari come il lievito, che non presentano tessuti specializzati per contrastare i cambiamenti dell'ambiente esterno, l'esistenza di meccanismi cellulari adattativi alle condizioni di stress risulta essere di fondamentale importanza. Precedenti studi, hanno evidenziato che in *S. cerevisiae* l'impulso luminoso rappresenta una fonte di stress, in quanto provoca l'aumento intracellulare di perossido di idrogeno, a sua volta coinvolto nella traslocazione nucleare di Msn2. Quest ultimo è un tipico fattore trascrizionale stress-dipendente ed è stato identificato anche in *K. lactis*. Visti i numerosi studi condotti dal nostro laboratorio riguardanti *Kl*Mga2 come mediatore di risposta allo stress ossidativo, potrebbe essere anche esso coinvolto nella trasduzione del segnale luminoso. Pertanto, tale studio permetterebbe di esaminare dei meccanismi foto-dipendenti anche in organismi apparentemente privi di domini proteici sensibili alla luce.

4.2 Abstract

Carbon metabolism is a fundamental biological system of the cells, precisely regulated to adapt to physiological or pathological/stress conditions and conserved in all organisms. Its dysfunction is related to multiple diseases; therefore, the understanding of these metabolic pathways has assumed a key role in biomedical research. In this context, the respiratory yeast *K. lactis*, could be a good simplified model to represent the metabolism in higher eukaryotes. In unicellular organisms like yeasts, that do not have specialized tissues for protection against environmental challenges, the presence of cellular mechanisms to respond and adapt to stress conditions is fundamental. In this work we aimed to investigate the response to environmental light in *K. lactis*. *S. cerevisiae* has been reported to respond to light by increasing hydrogen peroxide level and triggering nuclear translocation of Msn2. This is a stress-sensitive transcription factor also present in *K. lactis*. To investigate light response in this yeast, we analysed the different phenotypes generated by the deletion of the hypoxia responsive and lipid biosynthesis transcription factor *Kl*Mga2. Alterations in growth rate, mitochondrial morphology, ROS metabolism and fatty acid biosynthesis, provide evidence that light was a source of stress in *K. lactis* and that *Kl*Mga2 had a role in the light-stress response. The involvement of *Kl*Msn2 in light stress was also explored.

4.3 Introduction

Organisms respond to environmental stimuli as prerequisite for survival. The capability to respond to sunlight, that represents a common source of heat and energy, but also a source of stress (Bodvard *et*

al. 2017), is essential for biosynthetic organisms. Yeast, on the other hand, lacks specialized light sensing proteins such as phytochromes, opsins and cryptochromes (Idnurm, Verma and Corrochano 2010), but studies of light-induced stress conducted in *S. cerevisiae*, have shown a response to blue light (450-490 nm) via activation of the stress-regulated transcription factors Msn2 and Crz1 (Lazarova *et al.* 1994; Cai, Dalal and Elowitz, 2008; Bodvard *et al.*, 2011; Bodvard *et al.* 2013; Bodvard *et al.* 2017). Both factors control gene expression through nucleo-cytoplasmic translocations and the homologues of these two stress-sensitive transcription factors were identified also in *K. lactis* (Bussereau *et al.* 2006; Barsoum, Rajaei and Åström 2011). In *S. cerevisiae* Ca^{2+} signaling, mediated by the Ca^{2+}/calmodulin dependent phosphatase (calcineurin), is required for cell survival during environmental stresses. These conditions cause an increase in cytosolic Ca^{2+} that induces calcineurin activation (Cyert 2003). The phosphatase, in turn, dephosphorylates Crz1, which rapidly translocates from the cytosol to the nucleus, where activates the transcription of genes involved in adaptation to stress (Bodvard *et al.* 2013; Thewes 2014). The other stress-response transcription factor, Msn2, exhibits similar behaviors; it moves to the nucleus where it affects the expression of target genes with STREs (stress response elements) in their promoters (Estruch 2000). Several stresses are known to induce nucleocytoplasmic oscillations of Msn2, including high concentrations of extracellular Ca^{2+} (Cai, Dalal and Elowitz 2008), caloric restriction (low glucose levels 0.1%) (Medvedik *et al.* 2007), oxidative stress (Hao and O'Shea 2011), as well as light (Bodvard *et al.*, 2011; Bodvard *et al.*, 2013; Bodvard *et al.* 2017), but the overall activation pathway is less clear than Crz1. Nuclear localization of Msn2 is contrasted by cyclic AMP-controlled protein kinase A (PKA) and promoted by the phosphatases PP1 and PP2A (Görner *et al.*, 1998; Santhanam *et al.*, 2004). Another important feature is that light stimulates hydrogen peroxide (H_2O_2) production in cultured mouse, monkey and humans cells via photoreduction of flavin-containing oxidases (Hockberger *et al.* 1999). The intermediates and the mechanism by which PKA senses light remains unclear, but also it has been shown that in *S. cerevisiae* a conserved peroxisomal oxidase (Pox1) converts the light into a H_2O_2 signal which is sensed by the peroxiredoxin Tsa1 and then transduced to thioredoxin (Trx1) to inhibit PKA activity (Bodvard *et al.*, 2017; Fig. 7).

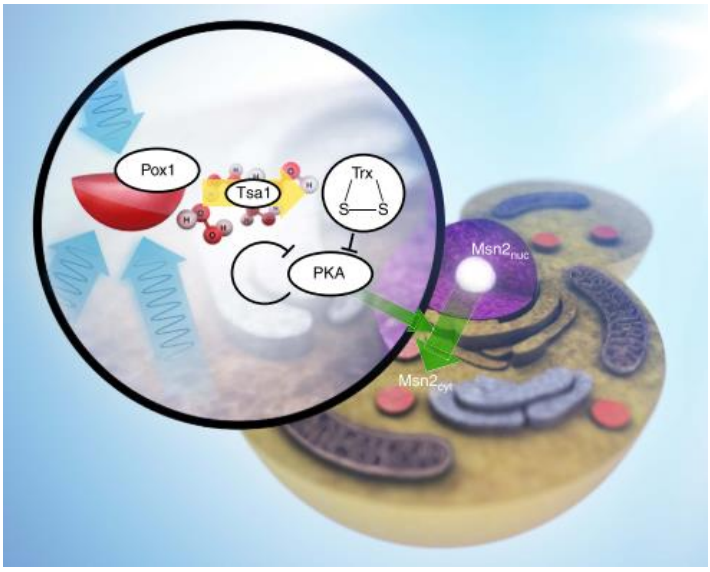


Figure 7 – Model of light-sensing in the absence of dedicated light receptors and light-induced Msn2 rhythms in *S. cerevisiae*. Light reduces flavin-containing peroxisomal acyl-CoA oxidase (Pox1), which produce H_2O_2 upon reoxidation by O_2. The H_2O_2 signal is sensed by peroxiredoxin Tsa1, which relays the signal to the cytosolic thioredoxins (Trx). Oxidized thioredoxins inhibit PKA-dependent phosphorylation allowing Msn2 to concentrate into the nucleus (Bodvard *et al.* 2017).

Therefore, as the light is converted into an oxidative stress signal, the oxidative stress mediator *Kl*Mga2, already studied in our laboratory, could also be involved in light response.
To our knowledge, studies on response to light have never been reported in *K. lactis* except the resonance response to light-dark 12+12 hours cycles generating an increase of phenotypic suppression in *KlMGA2* deletion mutant (Camponeschi *et al.*, 2020). Interestingly, the desaturase gene *FAD2* is a light-dependent gene in plants (Kargiotidou *et al.* 2008; Dar *et al.*, 2017) and its transcription is increased also at low temperatures (Byfield and Upchurch 2007). Desaturase level and lipid unsaturation index were both light-dependent in cold stress (Yuan *et al.* 2012). As reported above, in *K. lactis FAD2* gene is a hypoxic target of *Kl*Mga2, suggesting that it could be possible an overlap between light response and other conditions affecting the regulation of fatty acid biosynthetic metabolism, such as hypoxia, also in non-photosynthetic organism.

4.4 Results and discussion

Phenotypic analysis of *KlMGA2, KlCRZ1* and *KlMSN2* mutant strains in white light or dark conditions – In order to test the role of *Kl*Mga2, *Kl*Msn2 and *Kl*Crz1 in light-dependent response, we constructed deletion mutants into *K. lactis* MWL9S1 strain; indeed, this strain has the deletion of non-homologous end-joining (NHEJ) pathway, that in *K. lactis* greatly facilitates gene manipulations (Kegel *et al.* 2006; Wésolowski-Louvel 2011). The identification of *KlMGA2* has been described previously (Micolonghi *et al.* 2012). The homologues of *CRZ1* and *MSN2* were identified also in *K. lactis* genome using BLAST: *KlMSN2* (ORF KLLA0F26961g) had 24% of identity and 35% similarity with *MSN2*; *KlCRZ1* (ORF KLLA0E08713g) had 28% of identity and 40% similarity with *CRZ1* (Bussereau *et al.* 2006; Barsoum, Rajaei and Åström 2011). These mutant strains were named *Klmga2*Δ, *crz1*Δ and *msn2*Δ, respectively. Likewise, we generated an additional *Klmga2*Δ mutant strain harboring a truncated form of *MGA2* (*mga2*ΔTM strain): the latter contained *Kl*Mga2 lacking C-terminal transmembrane (TM) domain. Previous studies have shown that a C- terminal membrane-anchoring domains in *Sc*Mga2 represses protein activity (Chellappa *et al.* 2001; Huang and Kao 2018), therefore we have assumed that the *mga2*ΔTM strain codes for a truncated constitutively active *Kl*Mga2. Genotypes of these four mutant strains, obtained as described in the section 'Materials and Methods', are reported in Table 3. We assessed their growth in white light (4500K, 400 to 700 nm wavelength range, intensity of 134 $\mu M/s/m^2$) or dark condition with different carbon sources (glucose or glycerol) or respiration inhibitors (Antimycin A or Menadione). Results, reported in figure 8A, confirmed growth phenotypes previously reported in *KlMGA2* absence for strain GDK (Ottaviano *et al.* 2015; Camponeschi *et al.* 2020): *Klmga2*Δ strain in MWL9S1 background (outlined in red) showed a reduced growth respect to the wild-type and a rag^- phenotype, corresponded to strongly impaired growth in selective Antimycin A (GAA) medium. Interestingly, the mutant strain growth was partially recovered in the dark condition. This result suggests that light could act as stressing element respect to growth in *Klmga2*Δ strain. The other genes deletion (Fig. 8A) did not affect cells growth in both light or darkness, but in *KlMSN2* absence a slight reduction of growth onto GAA medium (outlined in blue) was observed in darkness. The rag^- phenotype is usually due to defects in the glycolytic and/or fermentative pathways or in their regulation (Wèsolowski-Louvel *et al.* 1992); the phenotype in *msn2*Δ strain only in darkness will require further investigation, but it could suggest an involvement of Msn2 in light/dark response. As reported above, an altered membrane composition of the *Klmga2*Δ strain is a direct consequence of the reduced expression of *FAD2*, however, the deletion of *FAD2* gene did not affect light growth, suggesting that the differences in growth between light and darkness observed in the *Klmga2*Δ strain should not be a direct consequence of the altered FAs

membrane composition that characterizes the mutant strain. The deletion of *KlCRZ1* did not caused evident phenotypes in the conditions explored in this work.
Expression of the *Kl*Mga2 truncated form in strain *mga2*ΔTM (Fig. 8B) allowed wild type growth in YPD medium, however growth was more similar to the *MGA2* deleted strain than wild type on the other media: YP, medium with glycerol as respiratory carbon source and onto GAA medium. In Figure 8C we tested growth of strains on YP supplemented with menadione, a compound that induces toxic oxidant stress associated with mitochondrial DNA damage and cell death (Loor *et al.* 2010). Also on YP media supplemented with menadione, we observed a better fitness of *Klmga2*Δ strain in darkness respect to light. Differently, light or darkness did not affect the growth of *mga2Δ*TM, that showed a sensibility to menadione like *Klmga2*Δ strain. These results indicated that the general fitness defects in the absence of *Kl*Mga2 were more evident in presence of light exposure, suggesting that *K. lactis* is able to sense light and that *Kl*Mga2 could play a role in light response.

Effects of light on *Kl*Mga2 activation – In order to assess the role of *Kl*Mga2 as a light-dependent mediator, we transformed the *Klmga2*Δ strain with a plasmid containing a modified *Kl*MGA2^{Flag} gene, encoding a N-terminus tagged protein to investigate the expression and maturation of *Kl*Mga2 protein under light or dark growth conditions. As reported above, in *S. cerevisiae* Mga2 is present in two forms: p120, an inactive 120-kDa precursor form that is anchored to the ER membrane by the C-terminus, and p90, a soluble 90-kDa N-terminal fragment which is the nuclear active form. Results, shown in figure 9, revealed that also *Kl*Mga2 was present in the two forms p120 and p90 and that both were present in light and dark conditions. As a control, we performed a western blot analysis with protein extract of *mga2Δ*TM strain that encodes a truncated p90 form of *Kl*Mga2 lacking transmembrane domain. These results suggested that light exposure did not control the *Klmga2* maturation process, because active and inactive forms were presented both in light or dark growth conditions.

Effects of light and dark exposure on growth rate of strain deleted for *KlMGA2* – We have previously shown (Ottaviano *et al.* 2015) that the deletion of *KlMGA2* causes a reduced growth rate compared to wild-type strain. We performed the growth experiment on standard glucose medium in light or darkness with MWL9S1 strain and the derived *Klmga2*Δ strain (Figure 10). According to our previous results, we observed that the deleted strain presented a longer lag phase and a slower growth rate as compared to wild type MWL9S1. Interestingly, incubation in dark or light conditions significantly affected the growth rate of mutant strain: in fact, the duplication time of deleted strain in the exponential phase shifted from about 6 hours in light to 4 hours in darkness, while the wild-type duplication time only changed from 2,7 hours in light to 2,2 in darkness condition. We also verified that light or dark did not interfere with growth rate of *msn2*Δ and *fad2*Δ strains (data shown on supplementary material, Figure S1).

Effects of light and dark exposure on respiration and mitochondrial morphology –We previously reported (Ottaviano *et al.* 2015) that *KlMGA2* deletion affected FAs biosynthesis, respiration rate and mitochondrial morphology. To establish the occurrence of a light-dependent connection between growth and respiration rates of *Klmga2*Δ strain, we measured oxygen consumption rates of exponentially growing MWL9S1 wild-type and *Klmga2*Δ mutant cells cultured in light or darkness using a Clark electrode, as described in the section 'Materials and Methods'. Results, reported in figure 11A, indicated that the wild type cells consumed oxygen (1,5 ± 0,05 nmol O_2 ml^{-1} sec^{-1}) faster

than deleted strain and consumption rate was independent on light or dark exposure. Differently, the deleted strains showed higher oxygen consumption rate when grown in darkness (0,92 ± 0,01 nmol O_2 ml^{-1} sec^{-1}) than in light condition (0,69 ± 0,03 nmol O_2 ml^{-1} sec^{-1}). Similarly to growth rate, the defective respiration rate of the deleted strain was exacerbated by light exposure while no effect of light could be recorded in the presence of *Kl*Mga2. Data reported in supplementary material (Figure S2) showed that light or dark did not affect the respiration rates of *msn2*Δ and *fad2*Δ strains.

In order to investigate mitochondrial morphology, cells were stained with the functional dye DASPMI that also allows to visualize mitochondrial membranes with an active potential by fluorescence microscopy (Bereiter-Hahn 1976). Results reported in figure 12A revealed that the mitochondrial membranes of the mutant strains, as well as those of the wild-type strain, were visualized by DASPMI staining that can suggest their functionality. Nevertheless, as previously reported (Ottaviano *et al.* 2015), mitochondrial network of *Klmga2*Δ strain frequently showed an irregular morphology under light condition; indeed as shown in Fig. 12A in some cells the mitochondrial network appeared as collapsed dots. However, when the *Klmga2*Δ cells were grown in the darkness, the percentage of cells bearing normal tubular mitochondria was significant increased compared to the wild-type values (Figure 12B). We observed a normal mitochondrial morphology in *msn2*Δ and *mga2Δ*TM strains (data not shown).

Effects of light on catalase and superoxide dismutase – Irregular mitochondria are often associated to ROS accumulation and impaired response to oxidative stress: we previously observed that GDK/*Klmga2*Δ strain showed a stronger protection against ROS by overexpressing ROS detoxification enzymes catalase (Cat) and superoxide dismutase (SOD) in the exponential growth phase as compared to wild type strain (Santomartino *et al.* 2019). Studies on *S. cerevisi*ae reported that light is converted to an oxidative stress signal inside the cell (Bodvard *et al.* 2017). To assess if light exposure activates an oxidative stress response in *K. lactis*, we assayed the expression of SOD and Cat enzymes by transcription analysis of *KlSOD1*, *KlSOD2*, *KlCAT1* and *KlCTT1* genes (Figure 13) and by measuring SOD and Cat enzyme activity (Fig. 14). Wild type and *Klmga2*Δ strains were cultivated in standard YPD medium in light or dark conditions to exponential growth phase. Results of transcription analysis, performed by RT-qPCR, showed that in the wild-type strain transcript levels of the four genes did not change between light and dark growth condition (Fig.13). On the contrary, the transcription levels of *KlSOD1* (cytosolic SOD) and *KlSOD2* (mitochondrial SOD) genes were higher in the mutant strain than the wild type when cells were grown in light (Fig. 13A and 13B). We also observed a ten-fold higher transcription of *KlCTT1* (Cytosolic Cat) gene in the mutant strain respect to the wild type cells in both light and dark condition (Fig. 13D). Differently, the transcription levels of *KlCAT1* (peroxisomal Cat) gene were not significant different between *Klmga2*Δ mutant and wild type cells (Fig. 13C).

To assess if increased transcription of the selected genes corresponded to increased enzyme activities, SOD and Cat activities were measured in cell extracts at exponential growth phase. Results are reported in figure 14. Both SOD and Cat activity levels in wild type strain resulted independent on light or dark exposure. On the contrary, SOD activity in *Klmga2*Δ mutant cells (Fig. 14A) resulted higher in light condition (10.9±0.73 U mg^{-1}), while in darkness it was very similar to wild type (6.4±0.04 U mg^{-1}). Differently, Cat activity (Fig. 14B) in *Klmga2*Δ mutant cells was higher both in dark (12.0±1.31 U mg^{-1}) and light (13.8±1.18 U mg^{-1}) growth conditions compared to wild type (4.8±1 and 4.7±0.33 U mg^{-1} in dark and light, respectively). We performed enzyme activities assay in the *msn2*Δ strain too, since in *S. cerevisiae* Msn2 upregulates cytosolic catalase gene *CTT1* (Hasan *et al.* 2002), which resulted strongly transcribed in *Klmga2*Δ mutant cells (Fig. 13D). In the

*msn2*Δ cells, we found a significantly decreased of Cat activity (3.2±0.38 U mg^{-1} and 3.5±0.14 U mg^{-1} in light and dark condition, respectively) compared to wild type cells, suggesting that also in *K. lactis* Msn2 upregulates the activity of catalases. No significant changes of SOD activity compared to wild type was found in the *msn2*Δ mutant.
The activity profiles of Cat and SOD enzymes confirmed the transcriptional profiles observed above (fig. 13). These findings were consistent with a general overactive ROS response in the absence of *KlMGA2* when the cells were exposed to light and suggest a possible role of *Kl*Mga2 as down-regulator of Msn2, because in the absence of *KlMGA2* we observed high Cat activity which is instead reduced in the absence of *MSN2*.

Effects of light on *FAD2* desaturase expression in wild-type and *Klmga2*Δ strains – Previous studies showed that FAs composition of membranes is modulated by hypoxia in *S. cerevisiae* (Vasconcelles *et al.*, 2001), by temperature and also light exposure, especially in plants and bacterial cells (Kis *et al.* 1998; Hernández *et al.* 2011), as a stress adaptation response. We reported that *K. lactis* strain GDK/*Kl*mga2Δ has an altered membrane composition and inhibited hypoxic induction of the desaturase gene *FAD2*, which was the main FA desaturation target gene of *Kl*Mga2 (Ottaviano *et al.* 2015). We aimed studying a possible light-regulation of desaturase expression in *K. lactis*, in detail, a *Kl*Mga2- dependent response to light. To this purpose, we first focused our study to the desaturase *FAD2* gene expression after light to dark and dark to light shift (15, 40 and 90 min). Results of RT-qPCR, reported in figure 15, showed that the transcription levels of *FAD2* in wild-type strain were influenced by light exposure. In detail, the shift from light (L) to darkness (D) caused a transient increase of transcription with a significant peak at 40 min from transition, while a progressive *FAD2* transcript reduction could be observed by shifting from darkness to light. Differently, the expression of *FAD2* in *Klmga2Δ* mutant strain resulted substantially unchanged during light/dark shifts. This result indicated that *Kl*Mga2 mediated the light-dependent regulation of *FAD2* observed in wild type cells.
The expression profile of *FAD2* in wild type strain was confirmed assaying Fad2 protein levels by western blot (Fig. 16). The shift from light to darkness caused a transient increase of Fad2 expression with a significant peak at 60 min from induction; from darkness to light, our results showed a Fad2 reduction after 120 min of light exposure.

Effects of light/darkness growth on FAs composition in wild-type and *Klmga2*Δ strains – Desaturases controls the synthesis of unsaturated FAs and, consequently, composition of membranes. We performed FA analysis of wild type and *Klmga2*Δ mutant cells grown overnight in light or darkness (Figure 17). In the wild-type strain, we observed a significant higher level of total fatty acids when cells were grown in light rather in darkness (figure 17A). We calculated the Unsaturation Index (UI), a parameter of membrane fluidity: a low UI indicates a low fluidity of the membrane lipids due to a reduced percentage of unsaturated carbon-carbon bonds in fatty acid molecules. Results in Figure 17B indicated a lower UI for the mutant strain with respect to the wild type, as also previously reported (Ottaviano *et al.* 2015), regardless on light (1.71±0.005 *vs* 1.93±0.03) or dark (1.65±0.004 *vs* 1.81±0.03) exposure. In addition, we have observed that UI was higher in both strains when cultivated in light condition as compared to darkness.
The detailed analysis of palmitic (C16:0), palmitoleic (C16:1*cis*), stearic (C18:0), oleic (C18:1Δ9*cis*), linoleic (C18:2Δ9,12*cis*,*cis*) and linolenic (C18:3Δ9,12,15*cis*,*cis*,*cis*) acids is reported in figure 17C. In the wild-type strain, the growth in darkness resulted in a very significant depletion of poly-unsaturated (PUFA) linoleic and linolenic acids. The most relevant differences between the wild type and the mutant strain were significant increase of palmitoleic acid in the mutant strain, both in light and

darkness growth, and the significant reduction of linoleic acid in the mutant strain, again in both light and darkness conditions.
This result supported our previous assumption that *Kl*Mga2 was involved in the light-dependent regulation of FAs biosynthesis, probably by upregulation of Fad2 (Δ12 fatty acid desaturase) that converts oleic acid in linoleic. In absence of *Kl*MGA2 we observed a reduction of Fad2 product linoleic acid; in wild type we observed a significant depletion of this fatty acid only in dark growth condition. To verify this hypothesis, we determined the amount of Fad2 by western blotting in wild type and in the *Klmga2*Δ mutant strain grown in overnight light or darkness. Results, reported in figure 18, showed a significant reduction of Fad2 in the mutant strain respect to the wild type, when grown under light stress, suggesting that, at least in light condition, the reduction of linoleic acid could be correlated to reduction of the corresponding biosynthetic enzyme.

4.5 Tables

Table 3. List of yeast plasmids and *K. lactis* strains used and obtained in this work.

Name	Description	Reference
Plasmids		
pFA6-KanMX4	Addgene plasmid from pFA6, containing *KanMX4* cassette	Wach et al., 1994
pYM27	Euroscarf, PCR template for C-terminal EGFP and $G418^R$	Jankeet al., 2004
pYM14	Euroscarf, PCR template for *3HA-KanMX4*	Jankeet al., 2004
Kp426[$Klmga2^{FLAG}$]	Plasmid Kp426 contains Flag tagged *KlMGA2*	Micolonghi *et al.*, 2012
pRS416	Stratagene, Yeast Centromere Plasmid (YCp), containing *URA3* marker	Stratagene
Strains		
MWL9S1	MATa, ura3, leu2, lysA1, trp1, metA1-1, ΔKlnej1::loxP	Hnatova et al., 2008
*crz1*Δ	MATa, ura3, leu2, lysA1, trp1, metA1-1, crz1Δ::kanMX4	This work
*msn2*Δ	MATa ura3, leu2, lysA1, trp1, metA1-1, msn2Δ::kanMX4	This work
LDA2	MATa, ura3, leu2, lysA1, trp1, metA1-1, fad2Δ::kanMX4	De Angelis et al., 2016
LD2G	MATa, ura3, leu2, lysA1, trp1, metA1-1, *FAD2*-GFP	De Angelis et al., 2016
Klmga2Δ	*MATa, lysA1, trp1, leu2, metA1-1, ura3,* ΔKlnej1::loxP, *Klmga2::URA3*	This work
*mga2Δ*TM	*MATa, lysA1, trp1, leu2, metA1-1, ura3,* ΔKlnej1::loxP, *mga2* (ΔTM)-*3HA*	This work
Kl$mga2^{FLAG}$	*MATa, lysA1, trp1, leu2, metA1-1, ura3, ΔKlnej1::loxP, Klmga2::URA3 with Kp426[Klmga2FLAG]*	This work
Klmga2Δ/Fad2-GFP	*MATa, lysA1, trp1, leu2, metA1-1, ura3, ΔKlnej1::loxP, Klmga2::URA3, FAD2*-GFP	This work

Table 4. List of oligonucleotides used in this work.

N	Name	Sequence	Use
1	CRZ1-DDK	ATGGATATTGACGATTATTTGAATGTCGATAGTCCC AGTGACATCGGTGCCAGCTGAAGCTTCGTACGCT	Crz1::kanMX4 cassette
2	CRZ1-UUK	GAATGGCATTTGTATTATTAGAGTAAGCGGACACT ATTGGAATGTCATATGCATAGGCCACTAGTGGATC	Crz1::kanMX4 cassette
3	MSN2-DDK	ATGGCTCTGGGTCGGTATGAGTCTGGAAACCGAGG CTCTTATACTTCAGACAGCTGAAGCTTCGTACGCT	msn2::kanMX4 cassette
4	MSN2-UUK	TGTATCTCATTTCCAATGTCACATCTGAAATATCGT AGATTATTTCAGTGGCATAGGCCACTAGTGGATC	msn2::kanMX4 cassette
5	MGA2-URA3 fw	GGGCAATTACTATTAGATAGGAATAGTCGTGCTTT GCTGAAGTCAAGGGAGAGTGCACCATACCACAG	Klmga2::URA3 cassette
6	MGA2-URA3 rv	GATTCGACTATATACGGGCTTTGCACATAGATAAA AGGTCGGTTCAAGGAGTTTAGTATACATGCATTTAC	Klmga2::URA3 cassette
7	MGA2ΔTM-fw	CTATCCAACTCTTAGTAGAATGTAAGGCCAACGTT ACAGCAAGGGCAGACTCTAGATACCCATACGATGT TC	*Kl*mga2ΔTm-3HA cassette
8	MGA2ΔTM-rv	GATTCGACTATATACGGGCTTTGCACATAGATAAA AGGTCGGTTCAAGCGAATCGACAGCAGTATAGC	*Kl*mga2ΔTm-3HA cassette
9	S3-fad2	TTCAGAAACGTGAACAACGTTGGTGTCGGAACTGG TAAGAAGAAGAACCGTACGCTGCAGGTCGAC	Fad2-GFP cassette
10	S2-fad2	TGAATTGATGGAAACTAAAGAAACCAAGTTAATTG TTAGCTAGTTCAATCGATGAATTCGAGCTCG	Fad2-GFP cassette
11	qCTA1-fw	GCTACGTGCAAGTGCATTTG	For RT-qPCR
12	qCTA1-rv	GGTAATTACCCTTGGCGATG	For RT-qPCR
13	qCTT1-fw	GCTATGTTCAGACGATGACC	For RT-qPCR
14	qCTT1-rv	GAGAGAAAGCAAGTTGCTCG	For RT-qPCR
15	qSOD1-fw	CCATGCATTCTCTGATGGAC	For RT-qPCR
16	qSOD1-rv	CGAACGGTGTGGATATACTG	For RT-qPCR
17	qSOD2-fw	GGGAAGCTTTCACCAAGATC	For RT-qPCR
18	qSOD2-rv	GGGTATGAATCTCTCATCGG	For RT-qPCR
19	qFAD2-Fw	CTGACCAATGGTCCTTCGC	For RT-qPCR
20	FAD2-R	TGAAGCAGGTCTAGCGTTGT	For RT-qPCR
21	18S-fw	CGGACTCCTTGATGATTCAT	For RT-qPCR
22	18S-rv	GATAGGGCAGAAATTTGAATG	For RT-qPCR

4.6 Figures

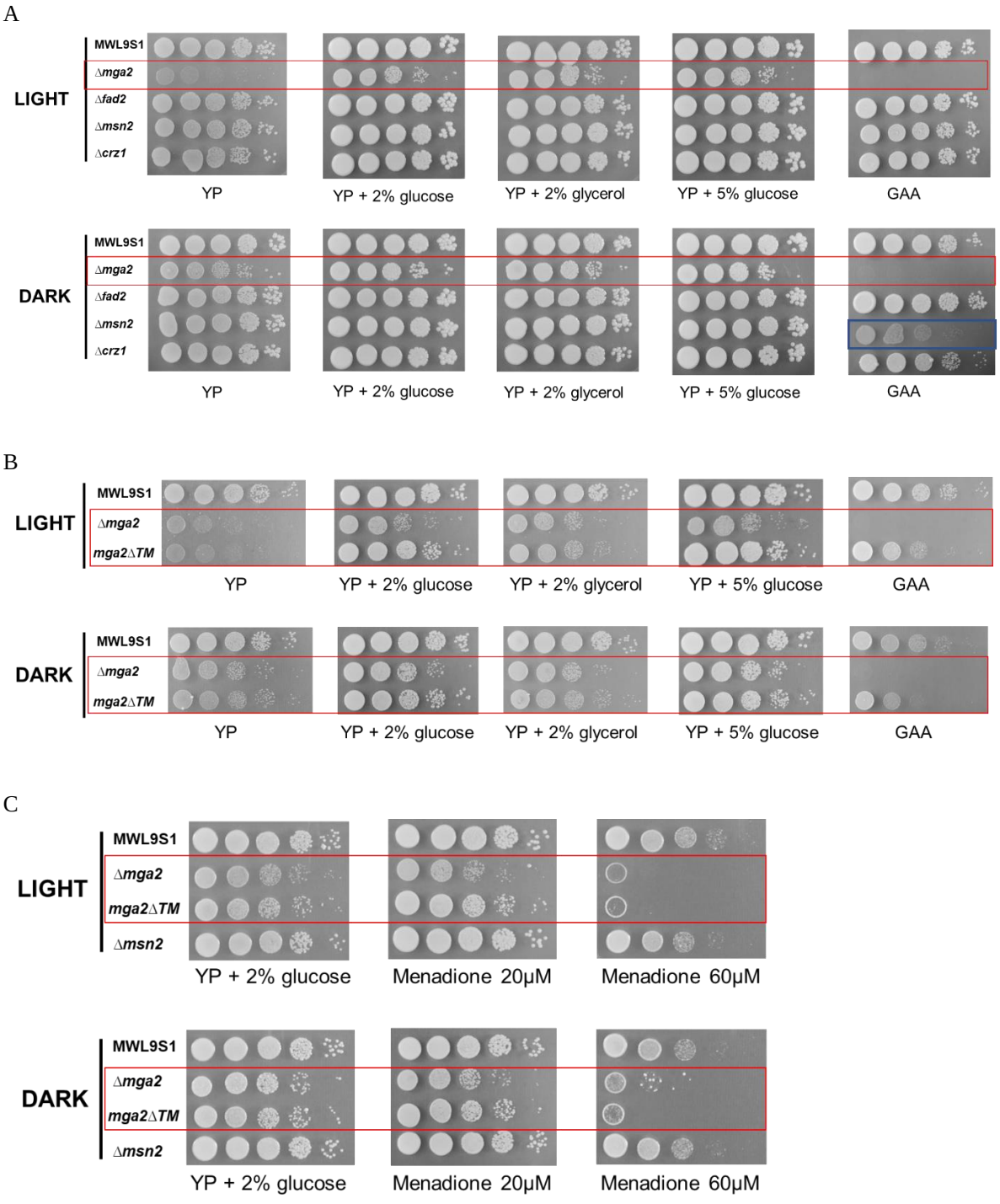


Figure 8 – Light/dark growth on different media of wild type MWL9S1 and *KlMGA2*, *KlCRZ1* and *KlMSN2* mutant strains. Cultures were grown overnight in white light (4500K, 400 to 700 nm range, intensity of 134 μM/s/m^2) or darkness in YPD medium, serially diluted and plated onto YP plates containing different carbon sources (2% glycerol, 2% or 5% glucose) and respiration inhibitors (Antimycin A in GAA medium or Menadione). Plates were incubated at 28°C under light or dark conditions for 3-4 days. Two or three independent cultures were performed for each strain/condition. Representative samples are shown. Panel A reports the comparison of the wild-type strain with different deleted strains. Panel B reports the comparison of the wild-type strain with the *KlMga2*Δ and *mga2*ΔTM strains. Panel C reports the effects of Menadione.

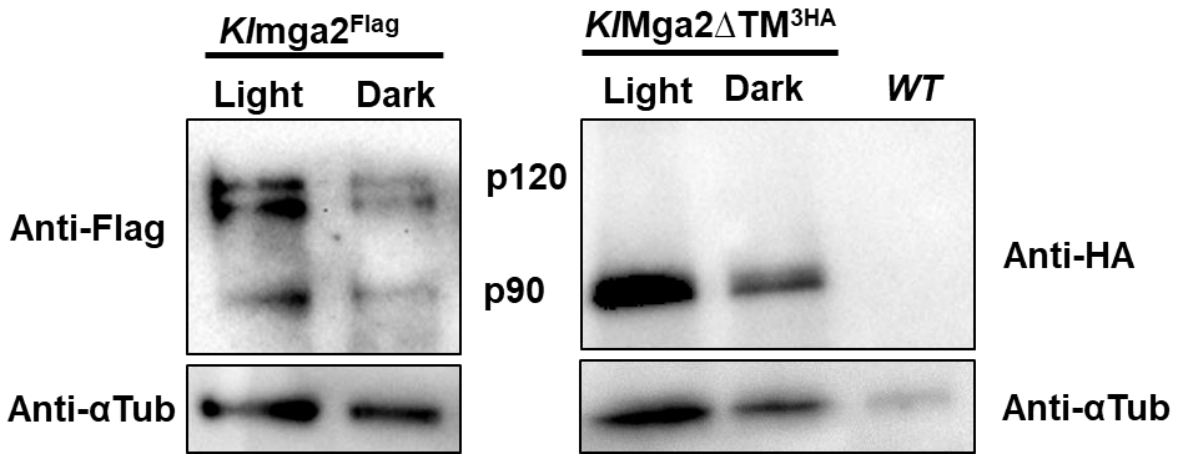


Figure 9 – *Kl*Mga2 expression in light and dark conditions. Western blot against *Kl*Mga2-Flag and *Kl*Mga2ΔTM-3HA, using extract from strains *Klmga2Δ* transformed with Kp426[Klmga2^{FLAG}] and strain *mga2*ΔTM respectively, is reported. Cells were grown in overnight white light or darkness in YPD medium. α-Tubulin detection was used as loading control. The positions of the p120 and p90 forms of *Kl*Mga2 are indicated.

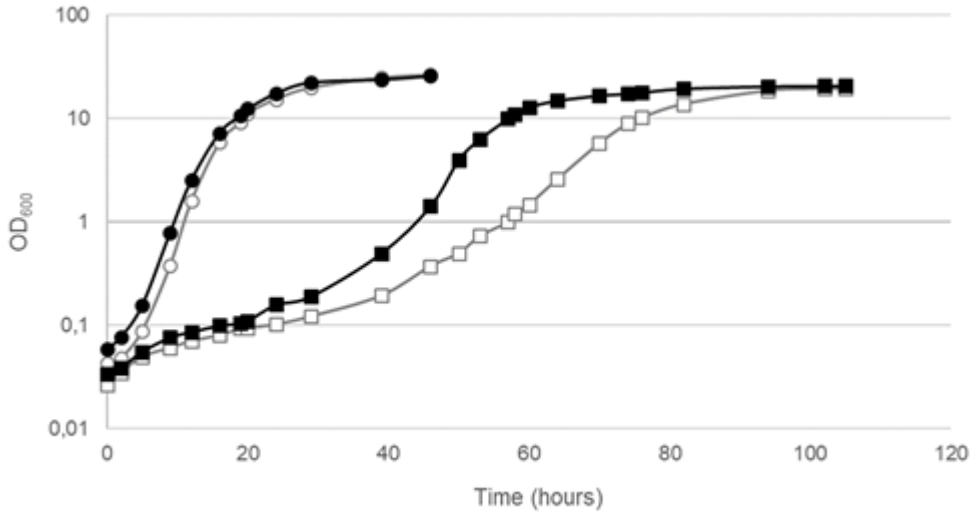


Figure 10 – Growth profiles of MWL9S1 and *Klmga2*Δ strain in light or dark exposure. Typical growth curves of the wild type MWL9S1 (circles) and the *Klmga2*Δ mutant strain (squares) in standard YPD medium are reported as optical density (OD_{600}) versus time (hours). Black and white symbols refer to darkness and light cultivation, respectively.

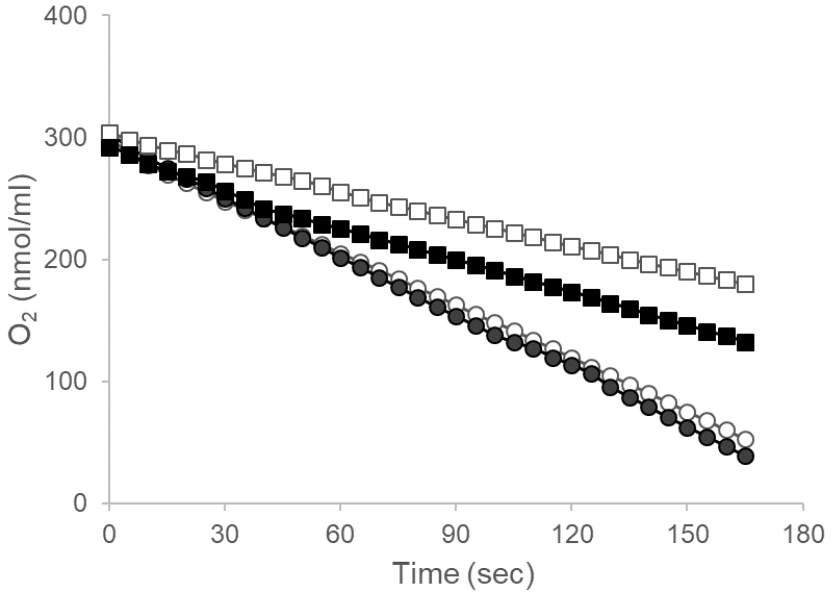
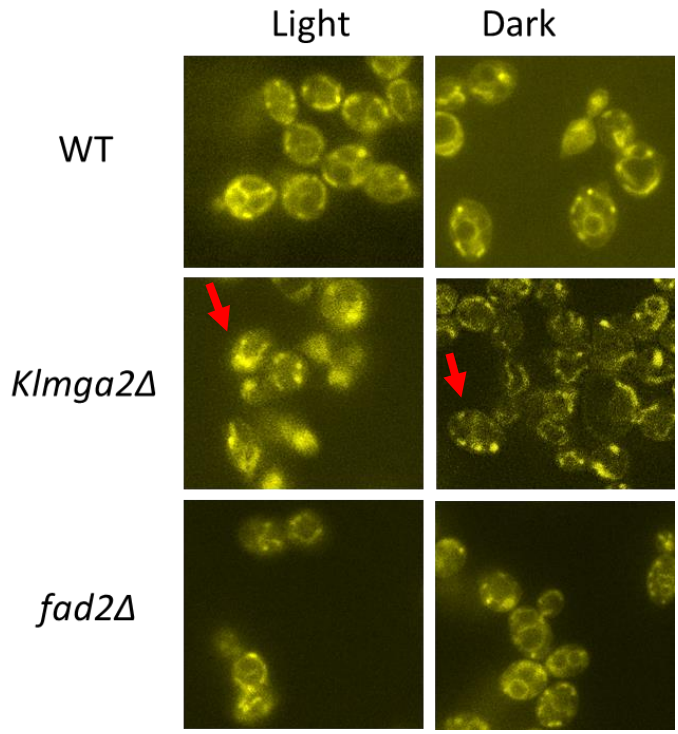


Figure 11 – Respiration rate of MWL9S1 and *Klmga2*Δ strain in light or dark exposure. Respiration was determined as oxygen consumption rate (nmol ml^{-1} sec^{-1}) of wild type MWL9S1 (circles) and of *Klmga2Δ* mutant cells (squares), grown exponentially in YPD medium. Black and white symbols refer to dark and light cultivation, respectively. Repeated experiments gave similar results: average values and standard deviations are reported in text.

A



B

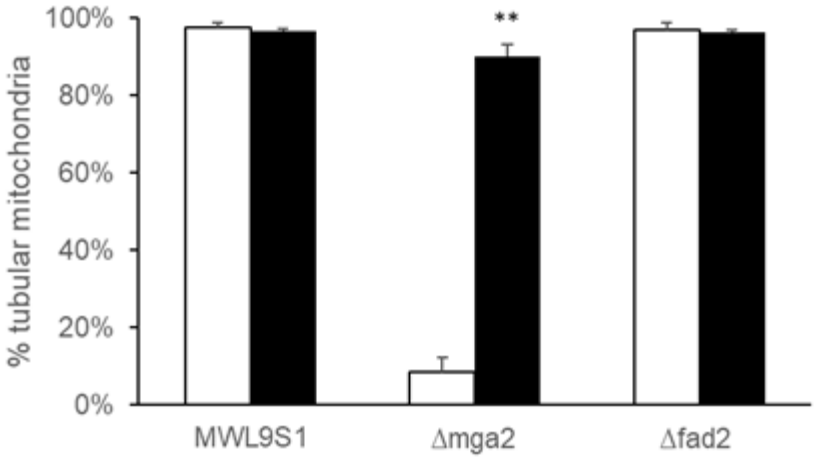


Figure 12 – Fluorescence microscopy analysis of wild-type and *Klmga2Δ* and *fad2Δ* mutant strains grown in overnight light or darkness. Panel A: DASPMI staining of the mitochondrial network of wild type MWL9S1, *Klmga2Δ* and *fad2Δ* strains. The red arrows show the non-tubular collapsed mitochondrial network. Panel B: histogram reporting the percentages of tubular mitochondria-containg cells. Black and white bars refer to darkness and light cultivation, respectively. Growth medium was YPD. Two or three independent cultures were performed for each strain/condition and 100 to 700 cells were analyzed. Standard deviations (SD) are reported. ** indicates p<0,01 for light versus dark condition.

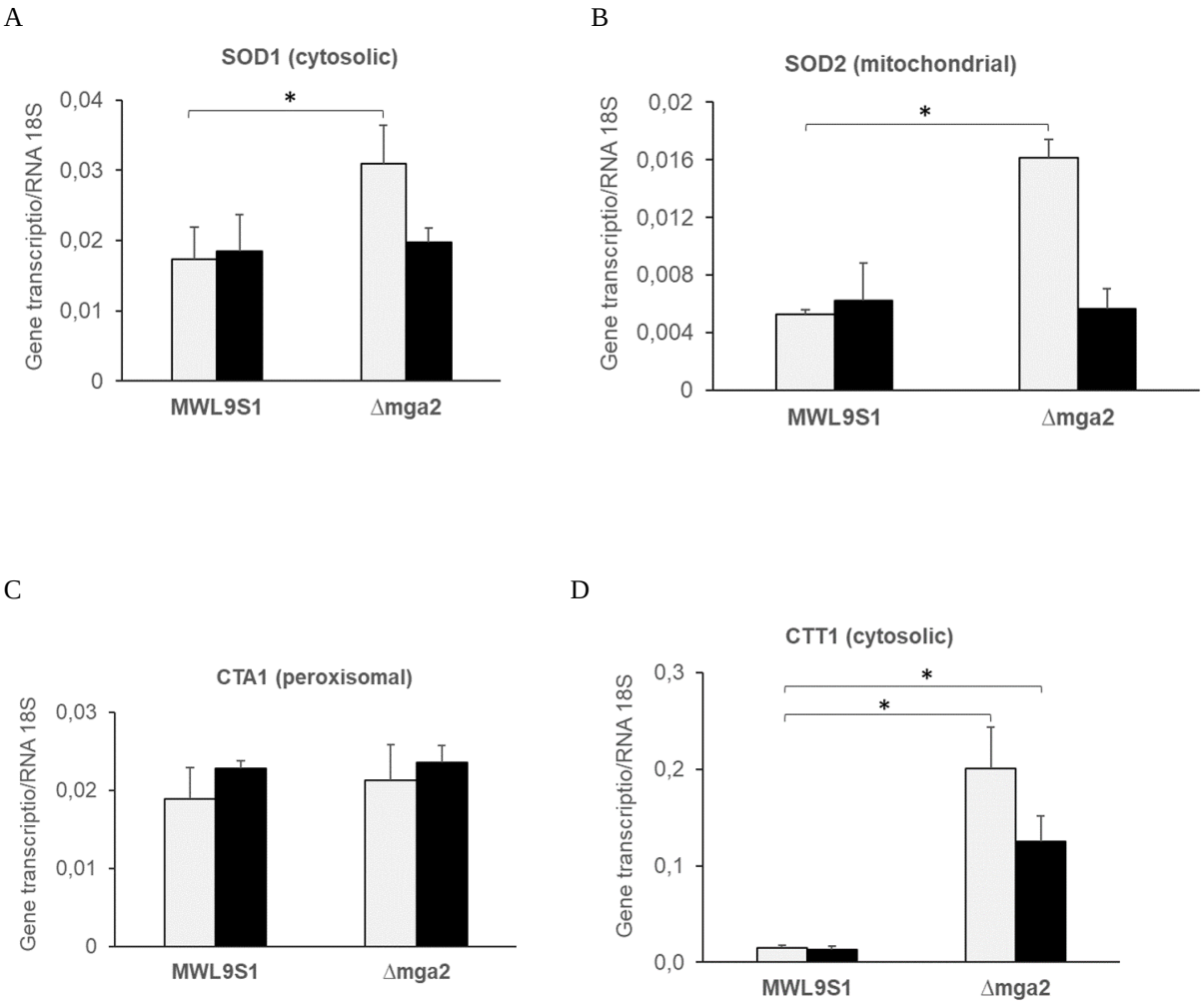
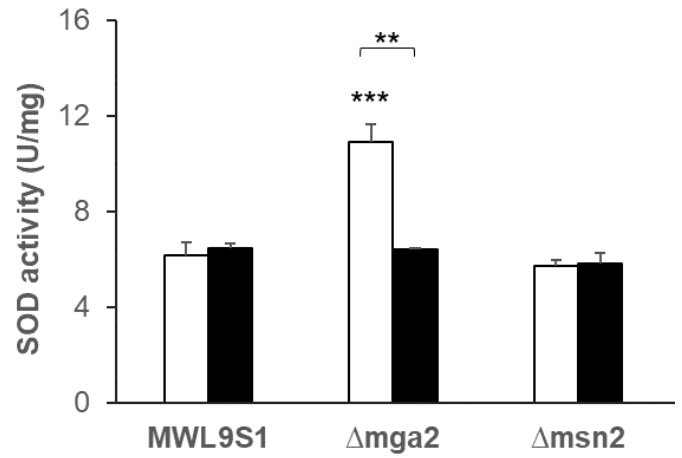


Figure 13 – Transcription of *KlSOD1, KlSOD2, KlCAT1 and KlCTT1* genes in wild type and *Klmga2Δ* mutant strain grown in light or darkness. Results of RT-qPCR analysis of Catalase (Cat) and SOD genes in wild type MWL9S1 and in *Klmga2*Δ mutant strain grown in YPD flask cultures in overnight light (white bars) or darkness (black bars) are reported. Panels A, B, C and D report *KlSOD1, KlSOD2, KlCAT1 and KlCTT1* transcription analysis, respectively. Ribosomal 18S gene transcription has been used as reference. Values are averages of three independent determinations, each measured by two technical repetitions, with SD reported.
* indicates $p<0,05$.

A



B

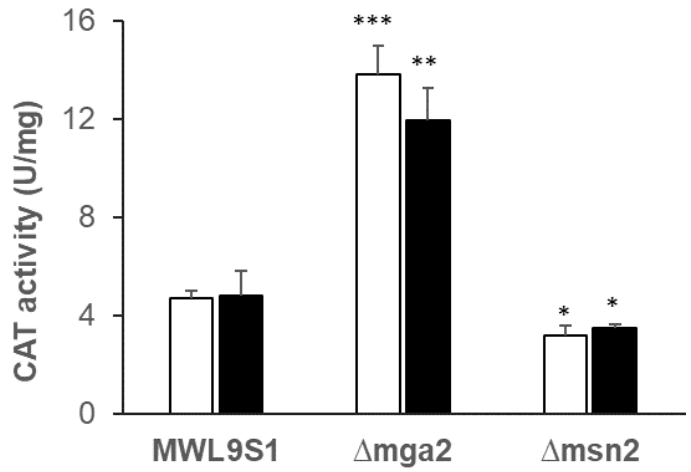


Figure 14 – Activity of SOD and catalases (Cat) enzymes in wild-type and *Klmga2Δ* and *msn2Δ* mutant strains grown in light or darkness. Total extracts from wild type MWL9S1, *Klmga2*Δ and *msn2Δ* strains, grown in overnight light (white bars) or dark (black bars) conditions up to exponential phase, were assayed for catalase and superoxide dismutase activities. Panel A shows SOD activities (U mg^{-1}): values are averages of three independent determinations, each measured by three technical repetitions, with standard deviations reported. Panel B shows Cat activities (U mg^{-1}): values are averages of two to four independent determinations, each measured by three technical repetitions, with SD reported. Significances: *=p<0,05; **= p<0,01 and ***=p<0,001 for mutants versus wild type light condition cells, except when otherwise specified.

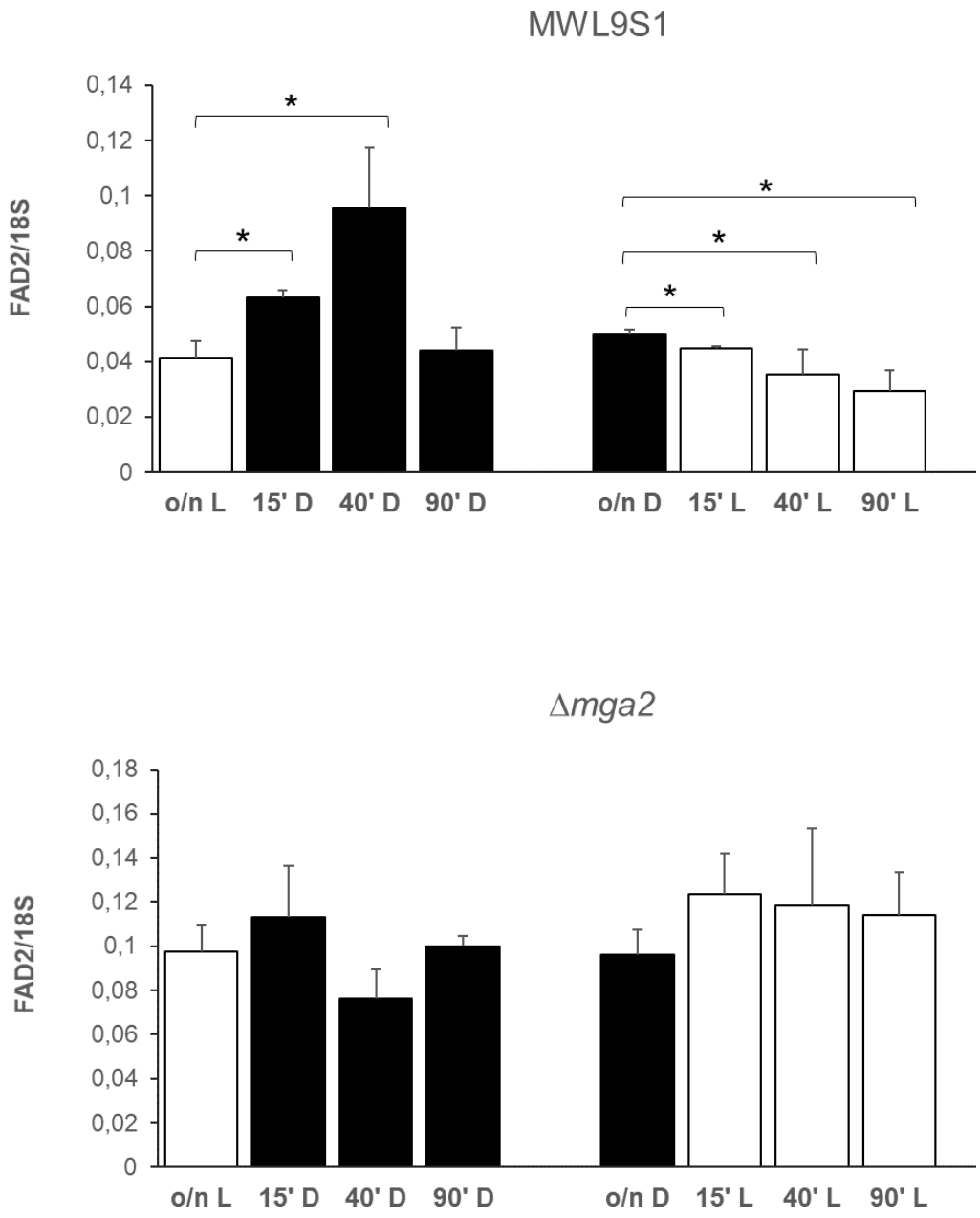


Figure 15 –*FAD2* transcription after light/dark shifts in wild-type strain MWL9S1 and *Klmga2Δ* strain. Results of RT-qPCR analysis of *FAD2* in wild type MWL9S1 and in *Klmga2*Δ mutant strain. Cells were grown overnight in light (o/n L) or darkness (o/n D) and then shifted to darkness (D) or light (L), respectively, for 15 min, 40 min and 90 min before nucleic acids extraction. Values are averages of three independent determinations with SD. Transcription of ribosomal 18S gene was used as reference. * indicates $p<0.05$.

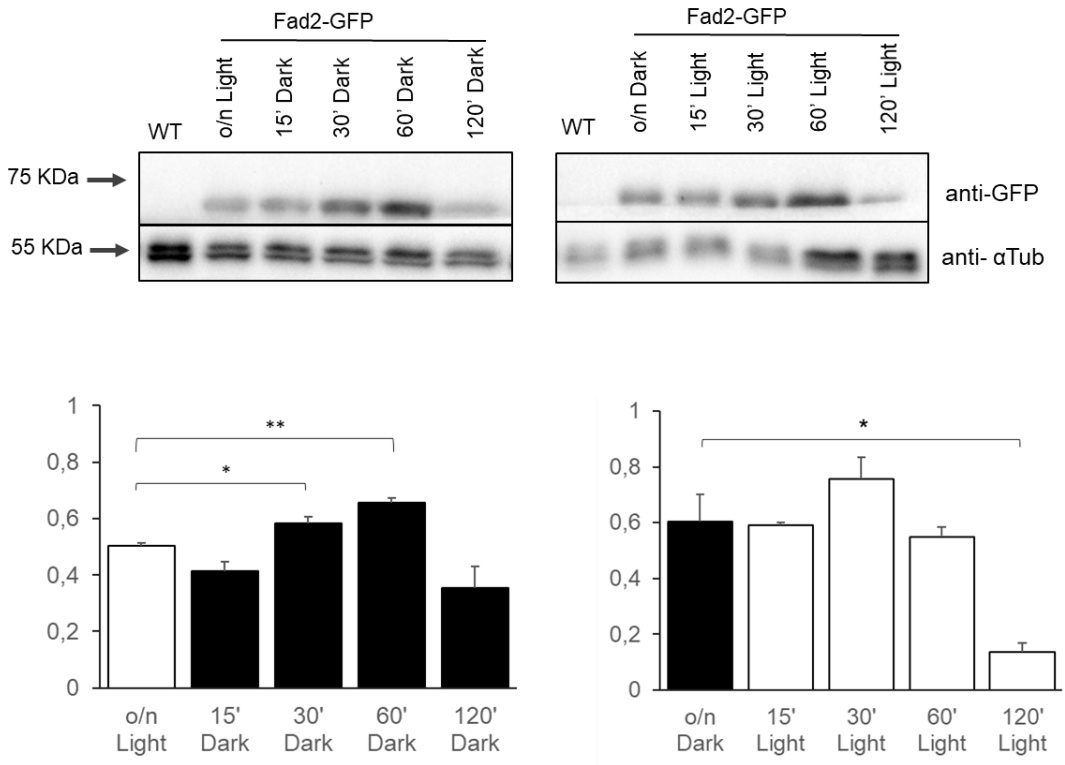


Figure 16 –*Fad2* expression in wild-type strain with *FAD2* fused to *EGFP* after light/dark shifts. Western blot against GFP-fused Fad2 protein, using strain LD2G (De Angelis *et al.* 2016). Cells were grown overnight in light or darkness and then shifted to darkness or light, respectively, for 15, 30, 60 and 120 min. α-Tubulin detection was used as loading control. Histogram reports Fad2-GFP signal quantification with respect to α-Tubulin signal: values are averages of three independent determinations with SD. Significances: *=$p<0.05$; **=$p<0.01$.

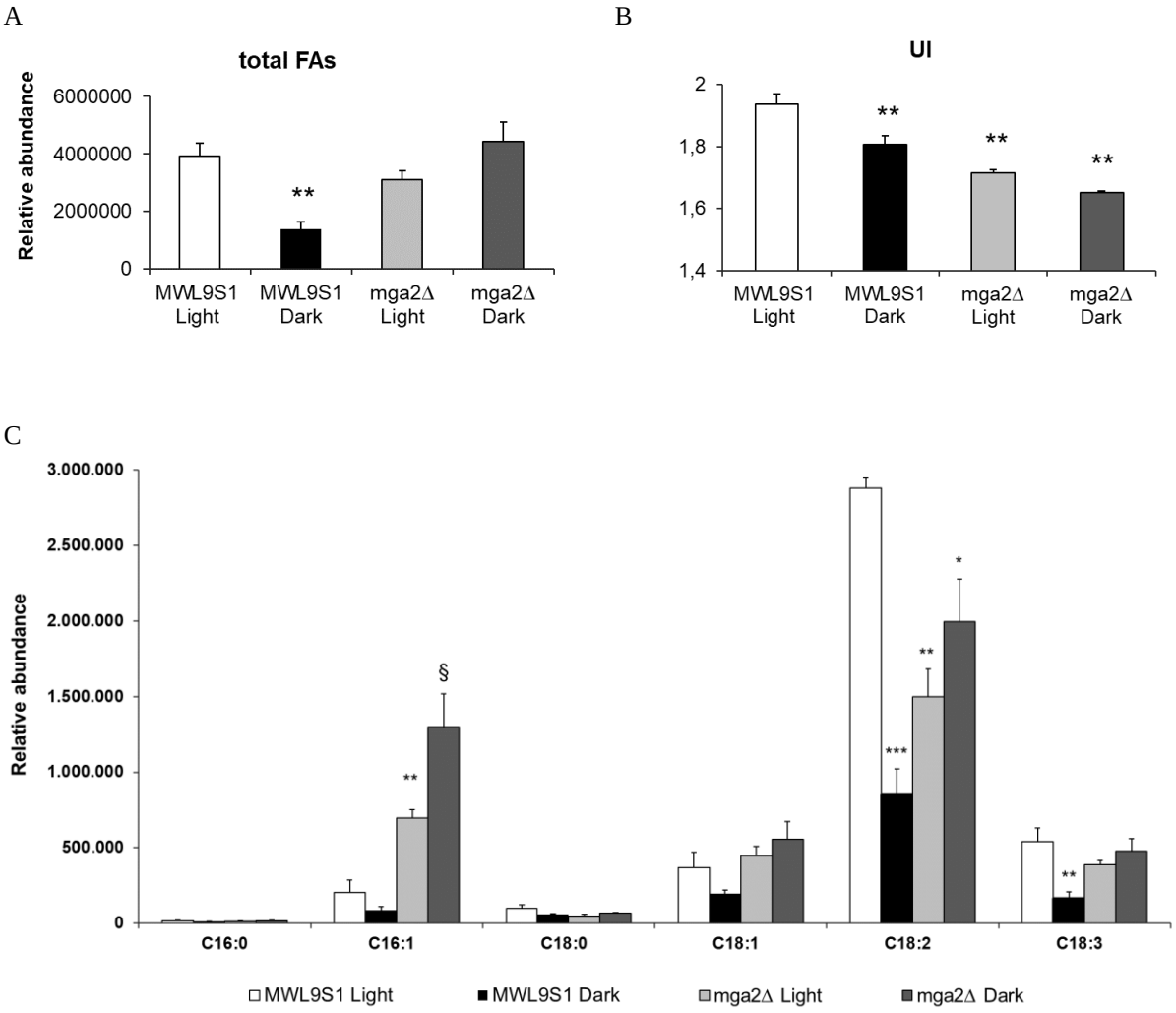


Figure 17 – Fatty acids analysis of the wild-type MWL9S1 and *Klmga2*Δ mutant strain grown in light or darkness. Cells were grown in overnight light or darkness in YPD medium. Panels A and B report total fatty acids (FAs) content (relative abundance respect to 9 hydroxy octadecadienoic d_4 acid, 9-HODE d_4) and unsaturation indexes (UI) of the wild-type and deleted mutant strain, respectively, with SD reported. Panel C reports amounts of palmitic (C16:0), palmitoleic (C16:1*cis*), stearic (C18:0), oleic (C18:1Δ9*cis*), linoleic (C18:2Δ9,12*cis*,*cis*) and linolenic (C18:3Δ9,12,15*cis*,*cis*,*cis*) acids (relative abundance respect to HODE). Results with SD are means of three independent determinations. Statistical relevance: * =p<0,05; ** = p<0,01 and *** =p<0,001 for mutants versus wild type light condition cells and §= p<0,05 for *Klmga2*Δ mutant light versus *Klmga2*Δ mutant dark cells.

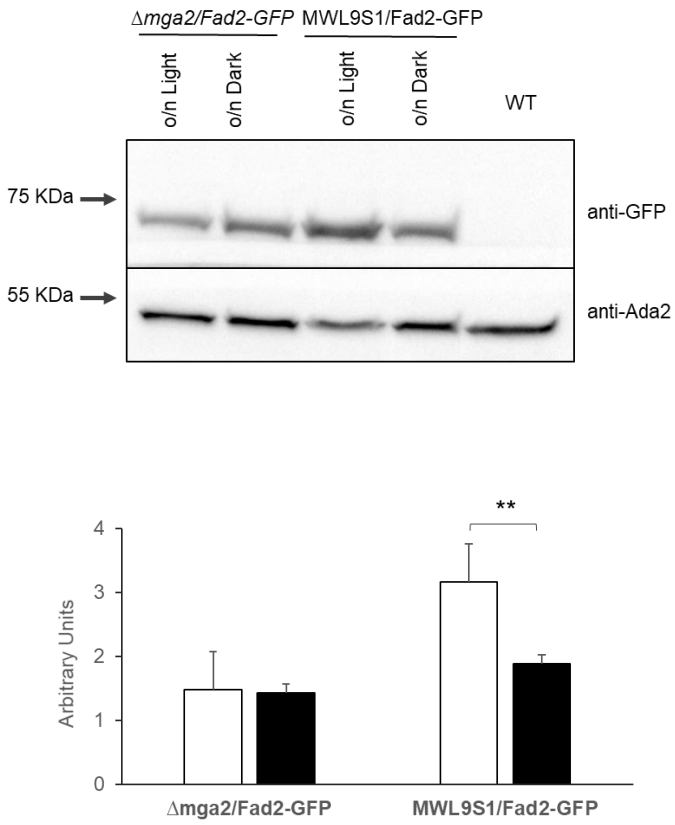


Figure 18 – Western blot showing *Fad2* expression in *Klmga2*Δ mutant strain and wild-type. Western blot against GFP-fused Fad2 protein, using wild type strain LD2G (MWL9S1/Fad2-GFP, De Angelis *et al.*, 2016) and mutant strain *Klmga2Δ/*Fad2-GFP (Tab. 3, this work). Cells were grown in overnight light (white bars) or darkness (black bars). Ada2 detection was used as loading control. Histogram reports Fad2-GFP signal quantification of repeated western blot experiments with respect to Ada2 signal. SD are indicated. Statistical significance: ** = p<.0.01.

4.7 Materials and methods

STAR★Methods

Key resources table

Reagent or resource	Source	Identifier
Yeast Extract	Becton Dickinson and Company	212750
Peptone	Becton Dickinson and Company	211677
Bacto-Agar	Becton Dickinson and Company	214010
Antimycin A	Sigma-Aldrich	A8674
Geneticin (G418)	Sigma-Aldrich	A1720
Vent DNA Polymerase	New England Biolabs	M0254L
Taq Pol	Jena Bioscience	PCR-202L
Electroporation cuvettes	Gene Pulser Cuvette, BioRad	1652082
Phenol-Chloroform-Isoamilic Acid, pH 4.7	Sigma-Aldrich	77619
Phenol-Chloroform-Isoamilic Acid, pH 5.2	Amresco	0966
Formaldehyde	Carlo Erba	415661
KAPA SYBR FAST Bio-Rad iCycler	Sigma-Aldrich	KK4608
Western Bright Quantum kit	Advansta	K-12042
TEMED	Sigma-Aldrich	T9281
SDS	Sigma-Aldrich	L4390
Blotting-Grade Blocker	BioRad	1706404
APS	Sigma-Aldrich	A3678
Acrylamide 30% solution	Sigma-Aldrich	A9926
PageRuler Prestained Protein Ladder	Thermo Scientific	26616
Immobilon-P PVDF Membrane	Merck Millipore	IPVH00010
Mouse anti-GFP (B-2)	Santa Cruz Biotechnology, Inc.	sc-9996
Mouse anti-HA(F-7)	Santa Cruz Biotechnology, Inc.	sc-7392
Mouse anti-flag (M5)	Sigma-Aldrich	F4042
Anti-Tub	Santa Cruz Biotechnology, Inc	sc-53030
Anti Ada2	AbCam	ab215524
Mouse-IgGκBP-HRP	Santa Cruz Biotechnology, Inc.	sc-516102
Hydrogen peroxide	Sigma-Aldrich	216763
Sod enzyme	Sigma-Aldrich	S2515
Menadione	Sigma-Aldrich	M5625
DASPMI	Sigma-Aldrich	280135
Critical Commercial Assay		
QuantiTect Reverse Transcription Kit	Qiagen	205313
SOD Assay kit-WST	Sigma-Aldrich	19160-1KT-F
Software and Algorithms		
Image Lab 5.2 Software	BioRad	N/A
Rotor Gene Q Software	Qiagen	N/A
GraphPad Software	Prism	N/A
Mass Hunter Quantitative software	Agilent	N/A
AxioVision 4.8	Zeiss	N/A

N/A = not available

Method Details

Media and growth condition – The yeast strains used are listed in Table 3. The YPD medium was composed of 1% Yeast Extract (Becton Dickinson and Company), 1% Peptone (Becton, Dickinson and Company) and 2% glucose (YPD medium). In some cases, instead of glucose, YP medium was supplemented with 2% of glycerol or ethanol. Solid media contained 2% Bacto-Agar (Becton, Dickinson and Company). For selection of transformed cells, we used YPD solid medium supplemented with Geneticin 100µ/mL (G418; Sigma-Aldrich) or SD solid medium (0.67% Yeast Nitrogen Base (Becton and Dickinson), 2% glucose and auxotrophic requirements as needed, without uracil). GAA medium, used to select rag^+ phenotype cells, was composed of YP medium with 5% of glucose and 5 µM Antimycin A (Sigma-Aldrich, St Louis, MO, USA). To assess mutant strains phenotypes, we used YP solid medium supplemented with 2% glycerol, 5% glucose, 20 µM and 60 µM menadione, a compound extensively used in studies of cellular oxidative stress (Kim, Sohn and Jin 2011). Except when otherwise specified, cultures growth was performed overnight at $28\pm1°C$ in complete darkness or in light (134 $\mu M/s/m^2$) under white LED lamps (4500K, 400 to 700 nm range). Cell density was determined by optical density at 600 nm or by cell counting in Burker chamber.

Construction of strains – Strains MWL9S1/*crz1*Δ, MWL9S1/*msn2*Δ and MWL9S1/*Klmga2*Δ were obtained by disruption of *CRZ1* (ORF KLLA0E08713g), *MSN2* (ORF KLLA0F26961g) or *KlMGA2* (ORF KLLA0E17953g) with the *KanMX4* cassette or *URA3* deletion cassettes, which have been constructed by the short flanking sequences-PCR method (Janke *et al.* 2004) (VentDNA Polymerase; New England Biolabs). DNAs were amplified using the primers 1 - 6 (Table 4) and plasmid pFA6-KanMX4 (Addgene, Table 3) as DNA template containing *KanMX4* or the plasmid pRS416 (Stratagene, Table 3) as DNA template containing *URA3* marker. The purified PCR products were then used to transform MWL9S1 strain by electroporation. Transformed colonies were selected on YPD solid medium supplemented with Geneticin (G418) on SD solid medium without uracil and then analyzed by PCR (Taq Pol, Jena Bioscience). Strain MWL9S1/*mga2Δ*TM was obtain in the same way by disruption of C-terminal membrane-anchoring domain in *KlMga2* (from codon 833 of protein KLLA0E17953p) using plasmid pYM14 (Euroscarf, Table 3) as template bearing the *3HA-KanMX4* cassette (primers 7 and 8, Table 4). *FAD2* gene was fused in frame with GFP sequence in *Klmga2Δ* strain, by a PCR-based strategy (Janke et al. 2004) using plasmid pYM27 (Euroscarf, Table 3) as template bearing the *EGFP-KanMX4* cassette to obtained *Klmga2Δ*/Fad2-GFP strain. Yeast strains were obtained by transformation with the electroporation procedure as previously described (Salani and Bianchi 2006).

RNA extraction ad analysis – Total RNAs were prepared by the hot phenol procedure as described by Köhrer and Domdey (1991) from cultures grown to $OD_{600}= 0.7\pm0.1$ in white light or dark conditions, and then collected or subjected to the opportune light/dark shift. Integrity of total RNAs extracts was controlled loading 1 µl of each sample on 1% agarose 6% formaldehyde gel. Concentration was determined by measuring the absorption at 260 nm. Transcript analysis was performed by RT-qPCR. 1 µg of total RNA extract was retro-transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen), following the manual instructions. One negative control sample was not treated with retro-transcriptase (RT) enzyme. Amplification was performed using KAPA Sybr Fast 2X (Sigma), containing the fluorophore, the buffer and the Taq polymerase, 0.4 µL of the appropriate couple of 10 µM primers (Table 4) and 2 µL of the cDNA (100 ng). All the samples, except for the negative controls (without cDNA, without RT) were loaded in double for a technical replicate. The experiments

were then conducted using the Rotor Gene Q (Qiagen) and data were analyzed using the provided software. The cycling was set with 3' at 95°C for enzyme activation, then 40 cycles at 95°C for 3'' and 30'' at 60°C, followed by a final step for generation of melting curve, ranging from 60° to 95°C. Quantification was performed by the construction of a standard curve with genomic DNA from MWL9S1 with 5 points of serial dilutions (the R^2 obtained for the curve was always superior to 0.99) and by the relative quantification of the samples of interest, using amplification of 18S rDNA as reference.

Protein extraction and Western blotting - Cultures were grown to OD_{600} = 0.7±0.1 and then collected or shifted to light or dark conditions for different time points, depending on the experiment. Then cells were harvested and suspended in 200µl of sterile water. An equal volume of 0.2 N NaOH was added and cells were incubated at room temperature for 5 min, then centrifuged for 2 min at 10000g. After elimination of the supernatant, the pellets were suspended in Laemmli buffer (TrisHCl 60 mM pH 6.8; glycerol 50%; SDS 2%; β-mercaptoethanol 5%; bromophenol blue 2%). Total protein extracts amount was quantified by measuring the absorption at 280 nm (NanoDrop™ 2000 spectrophotometer, ThermoFisher). The samples were boiled for 5 min or incubated at 65°C for 10 min, kept on ice for 2 min and centrifuged at 10 000 g for 1 min, then loaded on 8-10% acrylamide (Sigma-Aldrich) gel for SDS-PAGE. After electrophoretic separation, the proteins were transferred to a PVDF transfer membrane (Merck Millipore). Western blotting was performed with different primary antibodies (mouse monoclonal Anti-HA, Santa Cruz Biotechnology; mouse monoclonal anti-GFP, Santa Cruz Biotechnology; mouse monoclonal Anti-flag, Sigma-Aldrich) and horseradish peroxidase-conjugated secondary antibody (anti-mouse Santa Cruz Biotechnology). Detection was performed with ECL Western blotting detection reagents (LiteAblot EXTEND, EuroClone or Western Bright Quantum, Advansta) and visualized by ChemiDoc™ MP Imaging System (Biorad). α-tubulin (Santa Cruz Biotechnology) or Ada2 (AbCam) detections were used as loading controls.

Catalase and Superoxide dismutase activity – Cells (3 to 4 OD_{600} units) were collected from cultures and extracts were prepared by glass beads crushing in lysis buffer (50 mM Tris-HCl pH 6.8; 100 mM NaCl). Protein content in samples was determined at 280 nm (Nanodrop 1000). To determine catalase activity, 1–1.5 µl aliquots of samples were added to 0.5 ml of 11 mM H_2O_2 (Sigma-Aldrich) in 50 mM phosphate buffer pH 7.0, 1 µM EDTA. H_2O_2 decomposition was monitored at 25°C at 240 nm (ε_{240}=39.4 M^{-1} cm^{-1}). One Unit of catalase activity was the amount of enzyme that catalyzed the degradation of 1 µmol of H_2O_2/min. Superoxide dismutase activity was determined by measuring the rate of WST1-Formazan formation, using the SOD Assay kit-WST (Sigma-Aldrich), as suggested by the supplier. Calibration curve was determined using a commercial Sod enzyme (Sigma-Aldrich). 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.

Fluorescence microscopy. Exponentially growing cells on YPD medium were observed with a Zeiss Axio Imager Z1 fluorescence microscope with an AxioVision 4.8 digital image processing system, and objective lens ×63 oil. 100 µl of culture was centrifugated, washed with sterile water and then resuspended in 100 µl of 0.1 mM2-(p-dimethylaminostryryl)-l-methylpyridiniumiodine (DASPMI). Fluorescence could be detected 5-10 min after addition of the dye to the cells. The fluorescence was observed using DASPMI filter sets (550/25 nm excitation and 605/670 nm emission). DASPMI

maximal absorption wavelength is 429 nm and maximal emission wavelengths (excitation at 467 nm) is 557.5 nm in water (Bereiter-Hahn, 1976, Ramadass and Bereiter-Hahn, 2008).

Respiration. Respiration was analyzed by measuring the oxygen consumption rate using a Clark oxygen electrode (Hansatech Instruments) as described in De Luca et al. (2009). Cells ($1x10^6$) from exponential cultures ($1\div4\times10^6$ cells ml^{-1}) were collected, washed with 1 ml sterile water, suspended in 1 ml sodium phosphate buffer (10mM pH 7.4 containing 4 g l^{-1} glucose) and loaded in the reaction vessel of the previously calibrated oxygen electrode chamber.

Fatty acid extraction and HPLC-MS/MS analysis. FAs were extracted from cells grown to OD_{600} = 1 in light or dark exposure. Lyophilized cells of *K. lactis* were extracted following the method described in Ludovici et al. (Ludovici *et al.* 2014). Internal reference standard was the 9(S)-HODE-d4 (Cayman) at the final concentration of 1 µM. The samples were extracted with 2 mL of isopropyl alcohol : water : ethyl acetate (1:1:3 v/v) mixture with 0.0025% w/v of butylated hydroxytoluene. The extracts were dried by nitrogen flux and resuspended with 100 µL of Methanol. The samples have been analyzed with LC (HPLC 1200 series rapid resolution) coupled to a triple quadrupole MS (G6420 series triple quadrupole, QQQ) equipped with an electrospray ionization source (ESI). The equipment, the chromatographic column and the analysis software were from Agilent Technologies. The chromatographic separation has been performed with a Zorbax ECLIPSE XDB-C18 rapid resolution HT 4.6 × 50 mm1.8 µm p.s. column. Fatty acids were analyzed by Single Ion Monitoring (SIM) method in negative. The elution program requires the following mobile phase: phase A water/acetonitrile 97:3 v/v containing 0.1% formic acid and 3% acetonitrile, and phase B: acetonitrile/isopropyl alcohol 90:10 v/v. The injection volume was 10 µL. Setting instrument and elution program is reported in Ludovici et al., 2014. The SIM parameters have been obtained by flow injection of authentic standard and agreed with the literature (Yang *et al.* 2009). Fatty acid mass and relative parameters of analysis are reported in the Table 5. SIM data have been processed using Mass Hunter Quantitative software. Membrane fluidity, expressed as the fatty acid unsaturation index (UI), was calculated as follows: [(%C16:1+%C18:1)+(%C18:2×2)+(%C18:3×3)]/100.

Compound Name	Ion mass$[M-H]^-$	Fragmentor (V)	Polarity
16:0	255.2	140	Negative
16:1	253.2	140	Negative
18:0	283.2	140	Negative
18:1	281.2	140	Negative
18:2	279.2	140	Negative
18:3	277.2	140	Negative

Table 5 Fatty Acids SIM method.

5. Conclusions

Light is an ubiquitous and free source of energy. Light availability allowed the evolution of organisms endowed of biochemical systems able to capture and use this energy for biosynthesis. Also organisms lacking such molecules or proteins are subjected to light and are possibly set to respond to its exposure. Microorganisms like the unicellular yeasts *K. lactis* and *S. cerevisiae* do not have recognized light-responsive protein or photo-receptive molecules, but are transparent to light and their metabolism and physiology might be influenced by light.

Very few data, related to the light response in yeast, are available. In *S. cerevisiae*, the two regulatory factors Msn2 and Crz1 are environmental-stress regulators and a similar function has been recorded also for Msn2 in *K. lactis* (Barsoum *et al.* 2011). Msn2 and Crz1 are also light-dependent proteins in *S. cerevisiae*, translocated to nucleus under light exposure (Cai, Dalal and Elowitz, 2008; Bodvard *et al.*, 2011), suggesting a possible light-stress response dependent on the expression of specific target genes. Light signaling can be mediated in *S. cerevisiae* by the synthesis of hydrogen peroxide and the activity of PKA through the action of Pox1/Tsa1/Trx1 enzymes (Bodvard *et al.*, 2017). All these elements are also present in *K. lactis* (*TPK1/2*: *KLLA0B07205g*, *TPK2*: *KLLA0D03190g*, *BCY1*: *KLLA0E04181g*, *POX1*: *KLLA0F09933g*, *TSA1*: *KLLA0B01628g*, *TRX1*: *KLLA0E16347g*), suggesting that a similar mechanism might be active also in this yeast. In wild-type *K. lactis* cells, light had no effects on growth rate. Differently, in the absence of *KlMGA2* gene, growth in darkness proceeded faster than in light, suggesting that that light could act as stressing element respect to growth and that *Kl*Mga2 could counteract light effect, protecting cells from adverse events. The recovery of normal mitochondrial morphology and the increase of respiration rate of the *Klmga2*Δ strain in the darkness confirm that *K. lactis* can sense light and that *Kl*Mga2 could play a role to mediate or attenuate phenotypes specifically correlated to respiration and/or mitochondrial morphology in response to light.

Results regarding catalases expression and activity suggested a general overactive ROS response in the absence of *KlMGA2* when the cells were exposed to light, especially as far as SOD expression was concerned. Our results also suggested a possible role of *Kl*Mga2 as down-regulator of Msn2, because we observed high Cat activity in the absence of *KlMGA2*, which was instead reduced in the absence of *KlMSN2*. In the latter case, the mechanism of action seemed to be independent on light/dark exposure.

Finally, our study on the expression of *FAD2* desaturase gene in *K. lactis* indicated that *Kl*Mga2 mediates the transient light-dependent regulation of *FAD2* observed in wild type cells, suggesting a light-stress dependent modulation of membrane composition. Our results showed opposite light regulation of transcription of *FAD2* and *SOD1/2* or *CTT1* genes in the wild-type and in the *Klmga2*Δ mutant, suggesting that light stress influenced differently ROS metabolism and PUFAs biosynthesis in *K. lactis* and indicating the involvement of other specific elements in the *Kl*Mga2-dependent response mechanisms. Major differences between *K. lactis* cells cultivated in light and in darkness, were in the total amount of FAs and in the unsaturation index (UI). Light cultivation caused a consistent increase of cellular FAs and an increase of UI. Light also caused specific changes in the amount of individual fatty acid species. As far as FAs biosynthesis and accumulation was concerned, the absence of *Kl*Mga2 simulated a light stress-like status with abundant FAs content. On the other hand, light stress could be associated with increased membrane fluidity, but this response was not dependent on *Kl*Mga2. It remains to be investigated the specific connection between light stress and high FAs content and high UI.

We conclude that results reported in this work indicate that the yeast *K. lactis* is responsive to light and that the regulatory factor *Kl*Mga2 has a role in this response. It has been shown that light induces oxidative stress in *S. cerevisiae* (Bodvard *et al.*, 2017) and a similar activity could be envisaged also in

K. lactis with the involvement of *Kl*Mga2, because of the effect of light on the detoxifying enzymes catalase and SOD. The deletion of *KlMGA2* gene has pleiotropic effects suggesting that *Kl*Mga2, possibly because of its biochemical characteristics (Camponeschi *et al.*, 2020), participates to various pathways: uncovering the detail of these pathways requires further investigation.

One of the most relevant pathway regulated by *Kl*Mga2 is the biosynthesis of fatty acids, and we showed here that also this pathway is influenced by light stress. The connection among light stress, membranes composition or functionality and *Kl*Mga2 is highlighted by the fact that phenotypes of the *Klmga2*Δ strains, like growth rate, respiration rate and mitochondrial morphology, are partially or completely suppressed either by darkness or by unsaturated fatty acids (Micolonghi *et al.*, 2012; Ottaviano *et al.*, 2015; Santomartino *et al.*, 2019). Finally, our findings open new perspectives on the role of light in the biology of organisms (apparently) deprived of light sensing proteins and on the role of lipids and membranes in the response to light stress.

6. 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.

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.

Barsoum E, Rajaei N, Åström SU. RAS/cyclic AMP and transcription factor Msn2 regulate mating and mating-type switching in the yeast kluyveromyces lactis. *Eukaryot Cell* 2011;**10**:1545–52.

Bereiter-Hahn J. Dimethylaminostyrylmethylpyridiniumiodine (daspmi) as a fluorescent probe for mitochondria in situ. *Biochim Biophys Acta* 1976;**423**:1–14.

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.

Bodvard K, Jörhov A, Blomberg A *et al.* The Yeast Transcription Factor Crz1 Is Activated by Light in a Ca2+/Calcineurin-Dependent and PKA-Independent Manner. *PLoS One* 2013;**8**, DOI: 10.1371/journal.pone.0053404.

Bodvard K, Peeters K, Roger F *et al.* Light-sensing via hydrogen peroxide and a peroxiredoxin. 2017, DOI: 10.1038/ncomms14791.

Bodvard K, Wrangborg D, Tapani S *et al.* Continuous light exposure causes cumulative stress that affects the localization oscillation dynamics of the transcription factor Msn2p. *Biochim Biophys Acta - Mol Cell Res* 2011;**1813**:358–66.

Breunig KD, Bolotin-Fukuhara M, Bianchi MM *et al.* Regulation of primary carbon metabolism in Kluyveromyces lactis. *Enzyme Microb Technol* 2000;**26**:771–80.

Bussereau F, Casaregola S, Lafay JF *et al.* The Kluyveromyces lactis repertoire of transcriptional regulators. *FEMS Yeast Res* 2006;**6**:325–35.

Byfield GE, Upchurch RG. Effect of temperature on delta-9 stearoyl-ACP and microsomal omega-6 desaturase gene expression and fatty acid content in developing soybean seeds. *Crop Sci* 2007;**47**:1698–704.

Cai L, Dalal CK, Elowitz MB. Frequency-modulated nuclear localization bursts coordinate gene regulation. *Nature* 2008;**455**:485–90.

Camponeschi I, Damasco A, Uversky VN *et al.* Phenotypic suppression caused by resonance with light-dark cycles indicates the presence of a 24-hours oscillator in yeast and suggests a new role of intrinsically disordered protein regions as internal mediators. *J Biomol Struct Dyn* 2020;**0**:1–12.

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

Chang Y, Dickson RC. Primary Structure of the Lactose Permease Gene from the Yeast Kluyveromyces lactis. *J Biol Chem* 1988;**263**:16696–703.

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 act. *J Biol Chem* 2001;**276**:43548–56.

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.

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.

Cyert MS. Calcineurin signaling in Saccharomyces cerevisiae: How yeast go crazy in response to stress. *Biochem Biophys Res Commun* 2003;**311**:1143–50.

Dar AA, Choudhury AR, Kancharla PK *et al.* The FAD2 Gene in Plants: Occurrence, Regulation, and Role. *Front Plant Sci* 2017;**8**:1789.

De Deken RH. The Crabtree Effect: A Regulatory System in Yeast. *J Gen Microbiol* 1966;**44**:149–56.

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

Estruch F. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol Rev* 2000;**24**:469–86.

Fabrizio P, Longo VD. The chronological life span of Saccharomyces cerevisiae. *Methods Mol Biol* 2007;**371**:89–95.

Fillinger S, Chaveroche M-K, van Dijck P *et al.* Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus Aspergillus nidulans. *Microbiology* 2001;**147**:1851–62.

Goder V, Alanis-Dominguez E, Bustamante-Sequeiros M. Lipids and their (un)known effects on ER-associated protein degradation (ERAD). *Biochim Biophys Acta - Mol Cell Biol Lipids* 2020;**1865**:158488.

González-Siso MI, Freire-Picos MA, Ramil E *et al.* Respirofermentative metabolism in Kluyveromyces lactis: Insights and perspectives. *Enzyme Microb Technol* 2000;**26**:699–705.

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.

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.

Görner W, Durchschlag E, Martinez-Pastor MT *et al.* Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev* 1998;**12**:586–97.

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

Hao N, O'Shea EK. Signal-dependent dynamics of transcription factor translocation controls gene expression. *Nat Struct Mol Biol* 2011;**19**:31–9.

Hasan R, Leroy C, Isnard AD *et al.* The control of the yeast H2O2 response by the Msn2/4 transcription factors. *Mol Microbiol* 2002;**45**:233–41.

Hernández ML, Padilla MN, Sicardo MD *et al.* Effect of different environmental stresses on the expression of oleate desaturase genes and fatty acid composition in olive fruit. *Phytochemistry* 2011;**72**:178–87.

Hockberger PE, Skimina TA, Centonze VE *et al.* Activation of flavin-containing oxidases underlies light-induced production of H2O2 in mammalian cells. *Proc Natl Acad Sci U S A* 1999;**96**:6255–60.

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**:605–18.

Idnurm A, Verma S, Corrochano LM. A glimpse into the basis of vision in the kingdom Mycota. *Fungal Genet Biol* 2010;**47**:881–92.

Janke C, Magiera MM, Rathfelder N *et al.* A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* 2004;**21**:947–62.

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.

Kainou K, Kamisaka Y, Kimura K *et al.* Isolation of Δ12 and ω3-fatty acid desaturase genes from the yeastKluyveromyces lactis and their heterologous expression to produce linoleic and α-linolenic acids inSaccharomyces 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.

Kargiotidou A, Deli D, Galanopoulou D *et al.* Low temperature and light regulate delta 12 fatty acid desaturases (FAD2) at a transcriptional level in cotton (Gossypium hirsutum). *J Exp Bot* 2008;**59**:2043–56.

Kegel A, Martinez P, Carter SD *et al.* Genome wide distribution of illegitimate recombination events in Kluyveromyces lactis. *Nucleic Acids Res* 2006;**34**:1633–45.

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

Kim IS, Sohn HY, Jin I. Adaptive stress response to menadione-induced oxidative stress in Saccharomyces cerevisiae KNU5377. *J Microbiol* 2011;**49**:816–23.

Kis M, Zsiros O, Farkas T *et al.* Light-induced expression of fatty acid desaturase genes. *Proc Natl Acad Sci U S A* 1998;**95**:4209–14.

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

Larosa V, Remacle C. Insights into the respiratory chain and oxidative stress. *Biosci Rep* 2018:BSR20171492.

Lazarova G, Ootaki T, Isono K *et al.* Phototropism in Yeast: A New Phenomenon to Explore Blue Light-Induced Responses. *Zeitschrift fur Naturforsch - Sect C J Biosci* 1994;**49**:751–6.

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

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

Loor G, Kondapalli J, Schriewer JM *et al.* Menadione triggers cell death through ROS-dependent mechanisms involving PARP activation without requiring apoptosis. *Free Radic Biol Med* 2010;**49**:1925–36.

Los DA, Murata N. Structure and expression of fatty acid desaturases. *Biochim Biophys Acta*

1998;**1394**:3–15.

Ludovici M, Ialongo C, Reverberi M *et al.* Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis of Fusarium verticillioides and maize kernels. *Food Addit Contam - Part A Chem Anal Control Expo Risk Assess* 2014;**31**:2026–33.

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 H2O2. *Free Radic Biol Med* 2007;**43**:1458–65.

Medvedik O, Lamming DW, Kim KD *et al.* MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae. *PLoS Biol* 2007;**5**:2330–41.

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.

Natter K, Kohlwein SD. Yeast and cancer cells - Common principles in lipid metabolism. *Biochim Biophys Acta - Mol Cell Biol Lipids* 2013;**1831**:314–26.

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, Angelis L De *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.

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.

Ramadass R, Bereiter-Hahn J. How DASPMI reveals mitochondrial membrane potential: fluorescence decay kinetics and steady-state anisotropy in living cells. *Biophys J* 2008;**95**:4068–76.

Rangel DEN. Stress induced cross-protection against environmental challenges on prokaryotic and eukaryotic microbes. *World J Microbiol Biotechnol* 2011;**27**:1281–96.

Rensing L, Monnerjahn C, Meyer U. Differential stress gene expression during the development of Neurospora crassa and other fungi. *FEMS Microbiol Lett* 1998;**168**:159–66.

Rossi M, Buzzini P, Cordisco L *et al.* Growth, lipid accumulation, and fatty acid composition in obligate psychrophilic, facultative psychrophilic, and mesophilic yeasts. *FEMS Microbiol Ecol* 2009;**69**:363–72.

Saini P, Beniwal A, Kokkiligadda A *et al.* Response and tolerance of yeast to changing environmental stress during ethanol fermentation. *Process Biochem* 2018;**72**:1–12.

Salani F, Bianchi MM. Production of glucoamylase in pyruvate decarboxylase deletion mutants of the yeast Kluyveromyces lactis. *Appl Microbiol Biotechnol* 2006;**69**:564–72.

Santhanam A, Hartley A, Düvel K *et al.* PP2A phosphatase activity is required for stress and Tor kinase regulation of yeast stress response factor Msn2p. *Eukaryot Cell* 2004;**3**:1261–71.

Santomartino R, Camponeschi I, Polo G *et al.* The hypoxic transcription factor KlMga2 mediates the response to oxidative stress and influences longevity in the yeast Kluyveromyces lactis. *FEMS Yeast Res* 2019;**19**, DOI: 10.1093/femsyr/foz020.

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.

Spohner SC, Schaum V, Quitmann H *et al.* Kluyveromyces lactis: an emerging tool in biotechnology. *J Biotechnol* 2016;**222**:104–16.

Surma MA, Klose C, Peng D *et al.* A lipid E-MAP identifies Ubx2 as a critical regulator of lipid saturation and lipid bilayer stress. *Mol Cell* 2013;**51**:519–30.

Taymaz-Nikerel H, Cankorur-Cetinkaya A, Kirdar B. Genome-wide transcriptional response of Saccharomyces cerevisiae to stress-induced perturbations. *Front Bioeng Biotechnol* 2016;**4**, DOI: 10.3389/fbioe.2016.00017.

Thewes S. Calcineurin-Crz1 signaling in lower eukaryotes. *Eukaryot Cell* 2014;**13**:694–705.

Vasconcelles MJJ, 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.

Wésolowski-Louvel M. An efficient method to optimize Kluyveromyces lactis gene targeting. *FEMS Yeast Res* 2011;**11**:509–13.

Wèsolowski-Louvel M, Prior C, Bornecque D *et al.* Rag- mutations involved in glucose metabolism in yeast: isolation and genetic characterization. *Yeast* 1992;**8**:711–9.

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

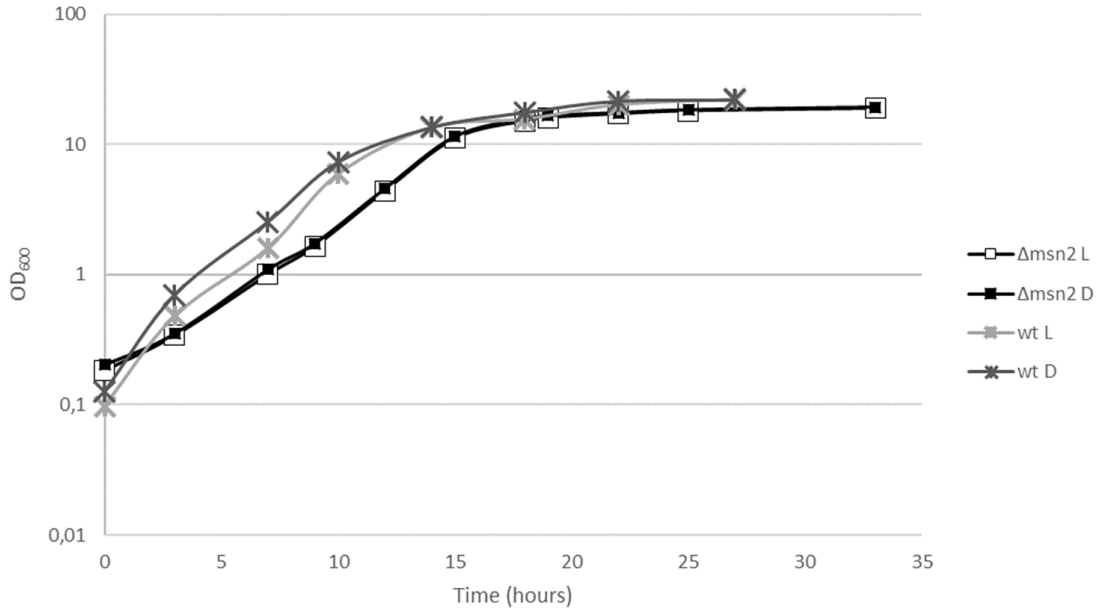
Yang J, Schmelzer K, Georgi K *et al.* Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem* 2009;**81**:8085–93.

Yuan S wei, Wu X long, Liu Z hong *et al.* Abiotic Stresses and Phytohormones Regulate Expression of FAD2 Gene in Arabidopsis thaliana. *J Integr Agric* 2012;**11**:62–72.

Zhang S, Skalsky Y, Garfinkel DJ. MGA2 or SPT23 is required for transcription of the delta9 fatty acid desaturase gene, OLE1, and nuclear membrane integrity in Saccharomyces cerevisiae. *Genetics* 1999;**151**:473–83.

7. Supplemetary material

A



B

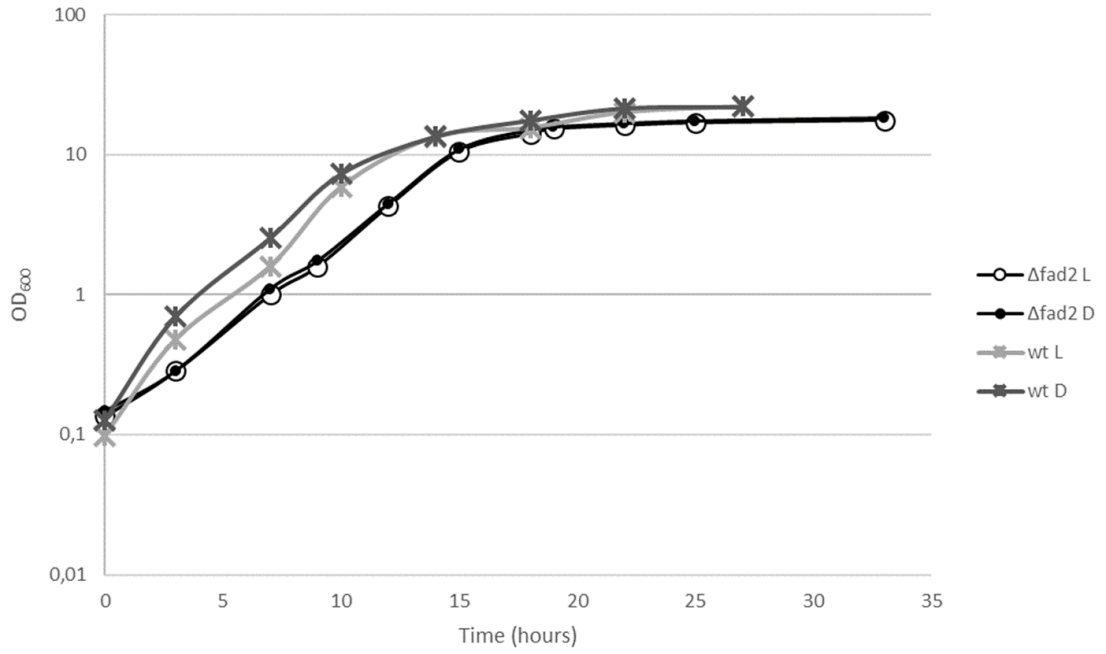
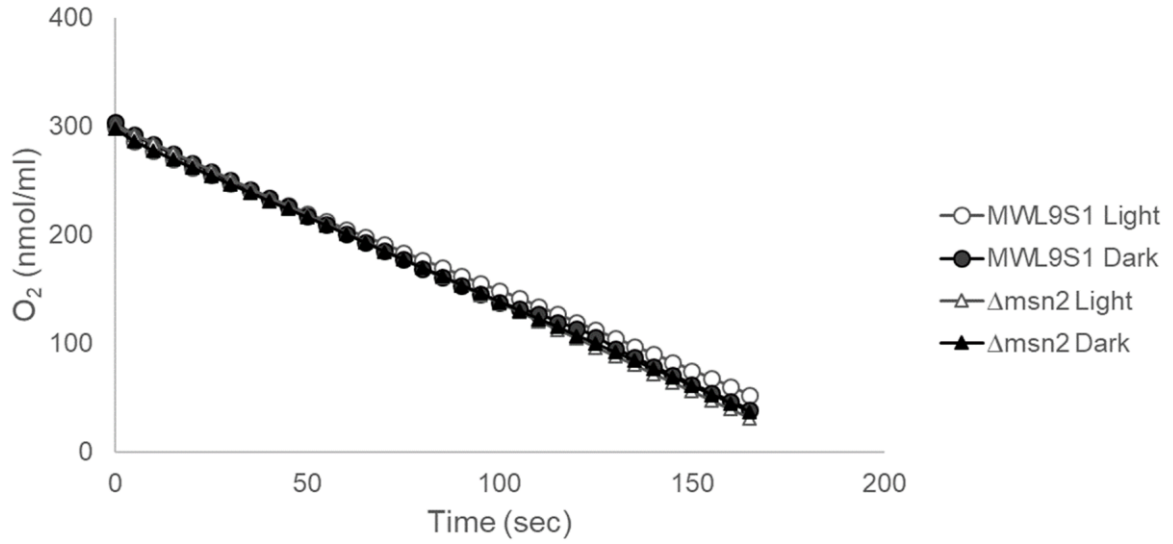


Figure S1 – Growth curve of *msn2*Δ and *fad2*Δ strains in white light or dark exposure. The growth in batch of the wild-type (asterisk) and the mutant strains, *msn2*Δ (squares, Fig. S1A) and *fad2*Δ (circles, Fig. S1B), is reported as optical density (OD_{600}) over time (hours). Black/dark gray and white/light gray symbols refer to darkness and light cultivation, respectively. Growth medium was YPD.

A



B

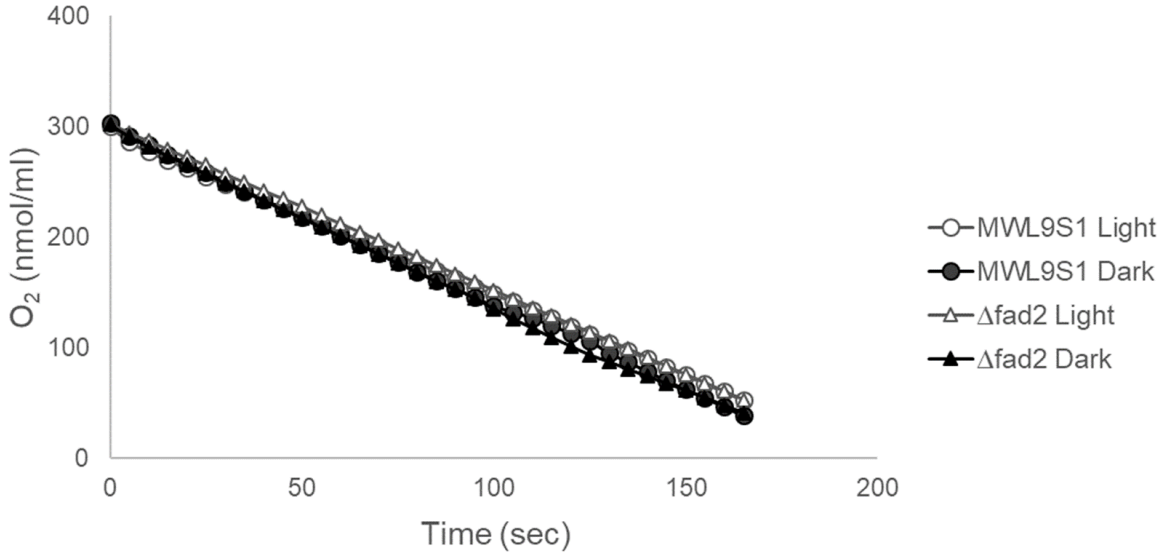


Figure S2 – Respiration of wild type strain MWL9S1 and mutant strains *msn2Δ* and *fad2Δ*. Respiration was determined as oxygen consumption rate (nmol/ml) of WT (circles) and mutant strains (triangles; *msn2*Δ, Fig. S2A and *fad2*Δ, Fig. S2B), grown in YP 2% glucose containing medium. Black and white symbols refer to darkness and light cultivation, respectively.

8. Synopsis of the Thesis

Kluyveromyces lactis is a non-conventional yeast widely used in both industrial applications and basic research. A main regulatory trait that differentiates it from the conventional yeast *Saccharomyces cerevisiae* is its preference for respiration. Indeed, *K. lactis* is a Crabtree-negative organism, having weak or absent glucose repression and the balance between respiration and fermentation dependent on oxygen availability and not glucose concentration. In our laboratory we are studying hypoxic role of *KlMGA2*. In *S. cerevisiae* Mga2 is a transcription factor constitutively expressed as inactive form and bound into endoplasmic reticulum (ER). It is activated in hypoxic conditions by proteasome cleavage. Following the cleavage, a soluble N-terminal fragment moves into the nucleus and induces the expression of low-oxygen responsive genes, like the desaturase gene *OLE1*. We have shown that also *Kl*Mga2 is a transcription factor involved in the regulation of lipid synthesis, but also in respiratory functions and in the general cellular fitness. Indeed, we previously demonstrated that deletion of the *KlMGA2*, coding for a hypoxic mediator in *K. lactis,* generated a viable strain, although suffering of several deficiencies, all restored by addition of UFAs (Unsaturated Fatty Acids) to the medium. We also showed that glucose signaling and glucose catabolism were involved in *Kl*Mga2 regulation. In this work, we showed that, in addition to these defects, the deletion of *KlMGA2* also caused increased resistance to oxidative stress and extremely extended lifespan. These phenotypes are associated with increased expression levels of catalase and superoxide dismutase genes. We propose that *Kl*Mga2 might act as a direct mediator not only of hypoxic response, but also of oxidative stress response/adaptation, thus revealing connections between hypoxia, glucose signaling, fatty acid biosynthesis and ROS metabolism.

In the second part of this work we wanted to investigate the possible *Kl*Mga2 role in presence of light, as a source of environmental stress. In unicellular organisms like yeasts, that cannot utilize specialized tissue for protection against environmental challenges, the presence of cellular mechanisms to respond and adapt to stress conditions is fundamental. *S. cerevisiae* has been reported to respond to light by increasing hydrogen peroxide (H_2O_2) levels. This molecule is a second messenger that triggers Msn2 nuclear accumulation, a typical stress- transcription factor in yeast, that rhythmically shuttles into and out of the nucleus in response to illumination. The homologue of this stress-sensitive transcription factor was identified also in *K. lactis*. For this reason, in the second part of the Thesis, we studied the response to light and darkness in absence of *Kl*Mga2 and its role in Msn2 oxidative stress response activation.

We have demonstrated that the *Klmga2*Δ mutant strain in a non-stressful condition (darkness growth) presents phenotypes identical or similar to wild type: improved growth rate, regular mitochondrial morphology, higher respiration rate and regular superoxide dismutase expression and activity. We proposed that these phenotypes depend on mutant's inability to adapt to the light stress thus suggesting that *K. lactis* is actually able to perceive and adapt to this environmental stimulus. Finally, we confirmed that *Kl*Mga2 is a transcription regulator of fatty acid (FA) biosynthesis and its absence affects the proportion of UFAs and the unsaturation index. Therefore, yeast is an organism lacking specialized light sensing proteins such as phytochromes, opsins and cryptochromes, but responds to environmental stimuli as prerequisite for survival. We conclude that *Kl*Mga2 in *K. lactis* has an important role in the regulation of the general metabolism and in various response and adaptation to environmental conditions including light stress.

9. Awards and grants

Funding for Start-up Research Project from "Sapienza" University of Rome, n°AR11916B70521233. Title: Effects of environmental stresses on phenotypic reversion in yeast.

10. Congress communications

Poster: **I. Camponeschi**, T. Rinaldi, C. Mazzoni, M.M. Bianchi. Lipid metabolism connects the response to oxidative stress and longevity in yeast. **The Biennial Congress of the Italian Association of Cell Biology and Differentiation (ABCD), 19-21 September 2019, Bologna, Italy. Abstract p.135.**

Poster: **I. Camponeschi**, R. Santomartino, A. Immesi, G. Polo, T. Rinaldi, C. Mazzoni, L. Brambilla, M.M. Bianchi. Connections among lipid biosynthesis, ROS metabolism and longevity revealed by the deletion of the hypoxic regulator *KlMGA2* in *Kluyveromyces lactis.* **Scientific Congress of Biology and Biotechnology Department "C. Darwin", "Sapienza" University of Rome, 2-3 July 2019.**

Poster: **I. Camponeschi**, R. Santomartino, A. Immesi, G. Polo, T. Rinaldi, C. Mazzoni, L. Brambilla, M.M. Bianchi. Connections among lipid biosynthesis, ROS metabolism and longevity revealed by the deletion of the hypoxic regulator *KlMGA2* in *Kluyveromyces lactis.* **VII Conference on Physiology of Yeasts & Filamentous Fungi (PYFF), 24-27 June 2019, Milan, Italy. Abstract p.205.**

Poster: **I. Camponeschi**, R. Santomartino, A. Immesi, G. Polo, T. Rinaldi, C. Mazzoni, L. Brambilla, M.M. Bianchi. Connections among lipid biosynthesis, ROS metabolism and longevity revealed by the deletion of the hypoxic regulator *KlMGA2* in *Kluyveromyces lactis.* **XV Congress of the Italian Federation of Life Sciences (FISV), 18-21 September 2018, Rome, Italy.**

Poster: M.M. Bianchi, R. Santomartino, **I. Camponeschi**, L. Falato, T. A. Landicho Alcarpio, A. Soulard, M. Lemaire. *Connection between hypoxia and glucose regulation in the expression of the glucose transporter gene RAG1 in Kluyveromyces lactis*. **XV Congress of the Italian Federation of Life Sciences (FISV), 18-21 September 2018, Rome, Italy.**

11. Publications

Camponeschi I, Damasco A, Uversky V N, Giuliani A, and Bianchi MM. 2020. Phenotypic suppression caused by resonance with light-dark cycles indicates the presence of a 24-hours oscillator in yeast and suggests a new role of intrinsically disordered protein regions as internal mediators. *J Biomol Struct Dyn.* 2020; 30:1-16. doi: 10.1080/07391102.2020.1749133.

Santomartino R, Ottaviano D, **Camponeschi I**, Landicho Alcarpio TA, Falato L, Visca A, Soulard A, Lemaire M, and Bianchi MM. 2019. The hypoxic expression of the glucose transporter RAG1 reveals the role of the bHLH transcription factor Sck1 as a novel hypoxic modulator in *Kluyveromyces lactis*. *FEMS Yeast Research* 2019; 19(4). https://doi.org/10.1093/femsyr/foz041.

Santomartino R, **Camponeschi I**, Polo G, Immesi A, Rinaldi T, Mazzoni C, Brambilla L and Bianchi MM. The hypoxic transcription factor *Kl*Mga2 mediates the response to oxidative stress and influences longevity in the yeast *Kluyveromyces lactis*. *FEMS Yeast Research* 2019; 19(3). doi: 10.1093/femsyr/foz020.

Nicita F, Tasca G, Nardella M, Bellacchio E, **Camponeschi I**, Vasco G, Schirinzi T, Bertini E, Zanni G. Novel homozygous KCNJ10 mutation in a patient with non-syndromic early-onset cerebellar ataxia. *Cerebellum* 2018; 17(4):499-503. doi: 10.1007/s12311-018-0924-7.

Paper submitted

Light-stress response mediated by the transcription factor *Kl*Mga2 in the yeast *Kluyveromyces lactis*. Camponeschi I., Montanari A., Beccaccioli M, Reverberi M, Mazzoni C and Bianchi MM.

12. Acknowledgements

I thank Professor Michele M. Bianchi for his guidance, patience and support throughout the course of this research. I would also love to thank Dr. Sergio Giannattasio and Professor Maurizio Ruzzi for accepting to review my PhD thesis and Professor Paola Goffrini for accepting to become as a member of my PhD dissertation committee.
A special mention goes to Dr. Arianna Montanari, Dr. Teresa Rinaldi, Professor Cristina Mazzoni, Professor Massimo Reverberi and Dr. Marzia Beccaccioli for their collaboration and advice and my friends and colleagues of lab 18 for making my PhD's time a wonderful experience.
In addition, I thank all my students, my family and my friends: Giorgia, Anisa, Sara, Francesca, Chiara, Doriana e Alessia.