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*Review Article*

# *Kluyveromyces lactis*: A Suitable Yeast Model to Study Cellular Defense Mechanisms against Hypoxia-Induced Oxidative Stress

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Studies about hypoxia-induced oxidative stress in human health disorders take advantage from the use of unicellular eukaryote models. A widely extended model is the fermentative yeast *Saccharomyces cerevisiae*. In this paper, we describe an overview of the molecular mechanisms induced by a decrease in oxygen availability and their interrelationship with the oxidative stress response in yeast. We focus on the differential characteristics between *S. cerevisiae* and the respiratory yeast *Kluyveromyces lactis*, a complementary emerging model, in reference to multicellular eukaryotes.

## 1. Introduction

Interest in hypoxic and oxidative stress studies is increasing in recent years, mostly in relation to aging or diseases such as neurodegenerative disorders or cancer. These processes in human cell lines show a very complex regulation, and therefore the availability of simple models is extremely useful. Yeasts have demonstrated to be suitable unicellular eukaryotic models since, in addition to generalized culture facilities, global "omic" analysis is fully developed and molecular mechanisms are generally well conserved. For instance, although obviously without nervous system, most of the molecular signaling pathways and the proteins involved in human neurological diseases are functionally conserved in yeasts [1]. Besides, functional characterization of human genes is sometimes achieved by means of their heterologous expression in mutant yeasts.

Most studies about the hypoxic and oxidative stress responses and their connections have been carried out hitherto on *Saccharomyces cerevisiae*, a yeast model with a predominantly fermentative metabolism [2]. In the same way, *S. cerevisiae* mutants have been frequently used as research models in aging [3] and in human pathologies [4]. Frequently, the mechanisms discovered with this yeast proved to be conserved in multicellular eukaryotes. However, human tissues such as the neuronal network have oxidative metabolism, and therefore the use of alternative yeast models has been suggested [5]. We analyze *Kluyveromyces lactis* from the perspective of an alternative eukaryote model in these studies or similar studies, since this yeast has a predominantly respiratory metabolism.

Molecular mechanisms that support the metabolic differences between *S. cerevisiae* and *K. lactis* and the specific responses to hypoxia or oxidative stress have been studied. Redox metabolism is a key differential point between *S. cerevisiae* and *K. lactis*, both thiol-redox and NAD(P)H-redox reactions. *K. lactis* is characterized, opposite to *S. cerevisiae*, by a higher glucose flow through the pentose phosphate pathway (PPP) than through glycolysis [6] and as a consequence by a higher production of NADPH in the cytosol. In fact, one of the molecular keys supporting the difference in the Crabtree phenotype (inhibition of respiration by fermentation) of the two yeast species lies in the mechanisms involved in the re-oxidation of the NADPH [7, 8]. A significant part of this reoxidation is carried out in *K. lactis* by mitochondrial external alternative dehydrogenases (NDEs), which use NADPH, the enzymes of *S. cerevisiae* being NADH-specific. Unlike *S. cerevisiae*, transcription of NDEs genes in *K. lactis* is not regulated by the carbon source. Since NDEs may compete with alcohol dehydrogenases for the cytosolic NADH, their repression at high glucose concentrations, as it occurs in *S. cerevisiae*,

correlates with an increase of reoxidation of glycolytic NADH by the alcohol dehydrogenases and therefore with the prevalence of aerobic fermentation and the Crabtree-positive phenotype [9]. Interestingly, NDEs have been reported to influence ROS production and life span in *S. cerevisiae* [10, 11]. Moreover, the NADPH-dependent pathways of response to oxidative stress also contribute to NADPH reoxidation. In *S. cerevisiae*, they play a main role together with glutamate dehydrogenase and also operate, although to a lesser extent, in *K. lactis*, [8, 12, 13].

In this paper, we focus on the complex interdependence of multiple mechanisms, which arise as a consequence of the decrease of oxygen availability and on the responses elicited to compensate this stress. A general overview of all the subjects analyzed is shown in Figure 1. Along the text, special reference is made on the differences found between *S. cerevisiae* and *K. lactis*, looking for the potential advantages and disadvantages of these models in reference to each other and in comparison to multicellular eukaryotes.

The first intracellular signal sensing low levels of oxygen is the heme content. The biosynthesis of heme includes enzymes that directly use oxygen as electron acceptor during the catalysis, and, besides, several steps are regulated by oxygen availability. Other important pathway that directly uses oxygen is the biosynthesis of ergosterol, which is also regulated by oxygen availability. The intake of ergosterol from the media through the membrane is also regulated by oxygen levels. Downstream in these sensing strategies is heme and ergosterol dependent transcriptional factors, which act in the nucleus to regulate the transcription of more than 100 genes, those conditioning the "hypoxic response" and improving the use of the low levels of oxygen. Hypoxia signals the activation of mechanisms that regulate the transcription of genes involved in the oxidative stress response. Simultaneously, the decrease in oxygen levels causes a complete rerouting of nutrients through different metabolic pathways. This principally affects glucose and other sugars, which can follow fermentative or respiratory transformations and, in turn, condition the systems of redox exchange between cytoplasm and mitochondria and the mechanisms that produce ROS. Reoxidation of reduced NAD(P)H also has regulatory effects on the diverse metabolic routes that need the oxidized coenzyme forms to function. ROS also elicit other mechanisms of cell defense, including reoxidation of NAD(P)H and life span adjustment, programmed cell death, and mitophagy (Figure 1).

## 2. The Hypoxic Response in *K. lactis*

During hypoxia, it is advantageous for cells to adapt the pattern of gene expression in order to improve oxygen utilization. The hypoxic response is well documented in the model yeast *Saccharomyces cerevisiae,* whose cells sense oxygen via the levels of heme and sterols [14–17]. The response of *S. cerevisiae* to hypoxia produces increased expression of genes related to ergosterol synthesis, cell wall composition, and glycolytic genes and reduced expression of components of the respiratory chain, ATP synthesis, and the citric acid cycle [14, 18, 19]. However, this knowledge is not directly applicable to other yeasts, even to those close-related in phylogeny, which became evident in the last years with the advances in the study of the hypoxic response in *K. lactis* and other yeasts. It has been proposed that a whole-genome duplication (WGD) contributed to yeast evolution from strict aerobes to facultatives/fermentatives [20–22]. Functional specialization between duplicated genes explains the existence in *S. cerevisiae* of homologous genes (COX5a/COX5b; CYC1/CYC7; HYP2/ANB1; AAC1/AAC2/AAC3) differentially expressed in aerobic and hypoxic conditions [14, 23–25]. *Kluyveromyces lactis* does not present duplication of genes with specialized aerobic, hypoxic transcription, but the unique copy is regulated by oxygen availability [26]. Probably, *K. lactis* and *S. cerevisiae* are diverged from one common ancestor yeast previously to the WGD event and this could explain the multiple differences observed when comparing the response to hypoxia in both yeasts, as explained below.

Although *K. lactis* is unable to grow under strictly anoxic conditions [27], probably due to the absence of expression of genes related to the import of sterols in this condition [28], this yeast ferments sugars and grows in hypoxic conditions defined as oxygen availability below 1% of fully aerobic levels [7, 29]. Several reports of genes upregulated during hypoxia in *K. lactis* have been published. A *K. lactis* heme-deficient strain, obtained by *KlHEM1* disruption, was used to assess the functional significance of heme-directed regulation in *K. lactis*; *KlHEM13*, encoding the coproporphyrinogen oxidase (EC 1.3.3.3), an oxygen-requiring enzyme that catalyzes the sixth step of heme biosynthesis, was the first hypoxic gene functionally characterized in this yeast [30, 31]. Other gene from the heme biosynthetic pathway, *KlHEM1*, is upregulated during hypoxia in *K. lactis* [32]. The *KlPDC1* gene, encoding for the enzyme pyruvate decarboxylase, is also induced by hypoxia [33]. After the completion of the Génolevures sequencing project [34], the availability of the complete sequence of the *K. lactis* genome allowed the design of specific DNA arrays containing selected DNA probes putatively related to the aerobic-hypoxic response by their similarity to the orthologs in *S. cerevisiae* [26]. The nature of the hypoxic transcriptional response in *K. lactis*, as revealed by using these arrays, differed notably from *S. cerevisiae*, but confirmed the existence of hypoxic upregulated genes in *K. lactis* such as *KlOYE2 (KLLA0A09075g)*, *KlGSH1* (*KLLA0F14058g*) and *KlOLE1* [26]. Besides *KlOLE1,* the transcription of other lipid biosynthetic genes like *KlERG1*, *KlFAS1*, *and KlATF1* is also induced by hypoxia [35].

In *S. cerevisiae*, adaptation to hypoxia requires the transcriptional induction or derepression of multiple genes organized in regulons controlled by specific transcriptional regulators. Considering that in *K. lactis* the hypoxic response exists but the target genes are not coincident and are not equally regulated, the question arose about the functionality of sensors and transcriptional regulators. The principal sensors in the yeast response to hypoxia are heme and ergosterol. The heme biosynthetic pathway is well conserved in different organisms throughout evolution [36], and this is also true between *S. cerevisiae* and *K. lactis.* Both yeasts have eight highly homologous genes necessary for the biosynthesis of

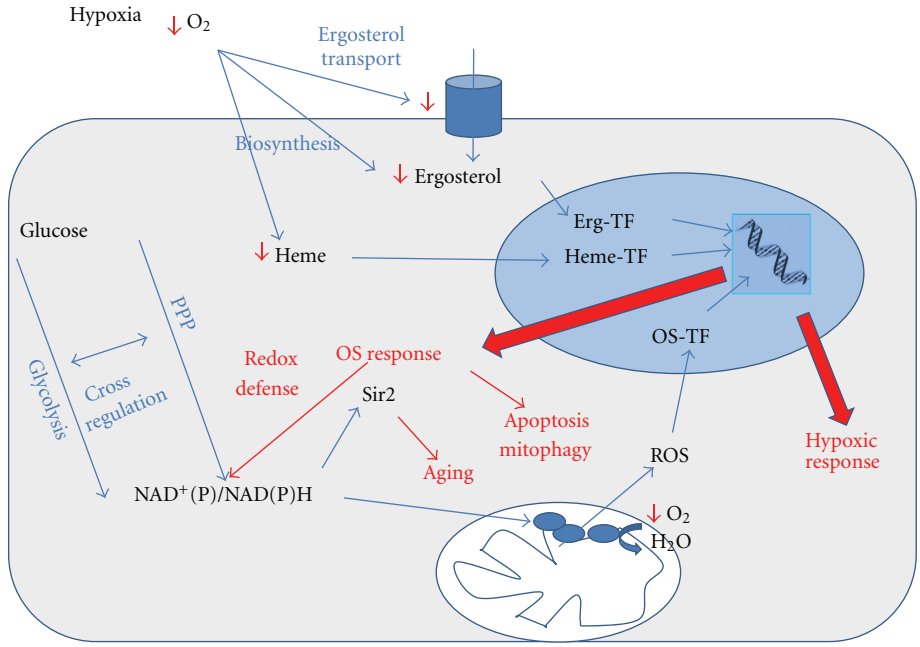


Figure 1: A panorama of the multiple connections between hypoxia, metabolic rerouting, oxidative stress response, and cell defense mechanisms. Erg: ergosterol; PPP: pentoses phosphate pathway; TF: transcriptional factors.

heme. For the three genes of the heme biosynthetic pathway characterized in *K. lactis* to date (*KlHEM1*, *KlHEM12*,and *KlHEM13)*, functional equivalence with their *S. cerevisiae* homologs has been confirmed experimentally by cross-complementation [31, 32, 37]. As it happens in *S. cerevisiae*, the transcriptional regulation of *KlHEM12* is not a key point for regulation of heme synthesis in *K. lactis* [38] and its transcriptional regulation in different carbon sources [38] is also similar to that reported for its homolog in *S. cerevisiae* [39, 40]. However, notable differences exist in the regulation of the other two characterized genes. In *S. cerevisiae*, the expression of *HEM1* is constitutive [41], but in *K. lactis* the first step of the heme biosynthesis is under double-feedback regulation by heme, at the level of gene transcription [37] and mitochondrial import of the protein [42]. Although in *S. cerevisiae* the aerobic repression of *HEM13* is mediated by the transcriptional regulator Rox1p, diverse data indicate that the mechanism is different in *K. lactis* and a similar repressor does not operate [43, 44].

In *S. cerevisiae*, intracellular levels of heme regulate the activity of the transcriptional regulator Hap1 [14]. The CCAAT-binding complex Hap2/3/4/5, an evolutionarily conserved multimeric transcriptional activator in eukaryotes [45], is also necessary for the activation of many genes involved in respiratory metabolism [45], although its direct activation by heme has not been demonstrated. Targets of Hap1 include genes required for respiration and for controlling oxidative damage [46–48] and also the aerobic repressor Rox1. When the oxygen levels drop, heme does not bind to Hap1. Then, the interaction with Ssa1, Ydj1, and Sro9 maintains to Hap1 inactive [49, 50]. As a result, *ROX1* is not expressed and no longer represses aerobic expression of genes involved in the hypoxic response. Moreover, Mot3 collaborates in the repression exerted by Rox1 in target promoters [51], and Ixr1 has been related to the hypoxic response of *S. cerevisiae* in cross-regulation with *ROX1* [52–54]. Although several homologs to the components of the Hap2/3/4/5 complex have been cloned in *K. lactis* [55–57], the respiratory system of *K. lactis,* escapes from *HAP2* control [56]. Contrary to data previously described for the homologous gene of *S. cerevisiae*, the function of the *KlHAP1* gene does not affect growth in media with carbon sources used by fermentative or respiratory pathways in *K. lactis* and *Kl*Hap1 is not a transcriptional activator of the expression of genes related to respiration or sterol biosynthesis [58] but represses the expression of the major glucose transporter [59]. In a similar way, *KlROX1* does not regulate the hypoxic response in *K. lactis* [60] and the *KlROX1* promoter is not regulated by *Kl*Hap1 or *Kl*Rox1 in response to changes aerobiosis/hypoxia [44].

Parallel, Hap1-Rox1-independent, oxygen response pathways exist in yeast. For instance, in *S. cerevisiae*, the transcription of the hypoxic gene *OLE1* depends on cytochrome *c* oxidase [61] and requires the transcription factor Mga2 that is functional in hypoxia [62]. In *K. lactis,* this regulatory circuit is also different and, although *KlMGA2* shows homology to the *MGA2* gene from *S. cerevisiae, Kl*Mga2does not regulate *KlOLE1* hypoxic expression [35]. Sut1 and Sut2 are also involved in the transcriptional induction of hypoxic genes and in sterol uptake and synthesis in *S. cerevisiae* [63, 64].

Another sensing pathway includes the regulators of sterol biosynthesis Upc2 and Ecm22 [65]. Sterol depletion leads to activation of the paralogous genes *UPC2* and *ECM22* [16], which control expression of a subset of hypoxic genes. Both bind to a sequence motif known as the sterol

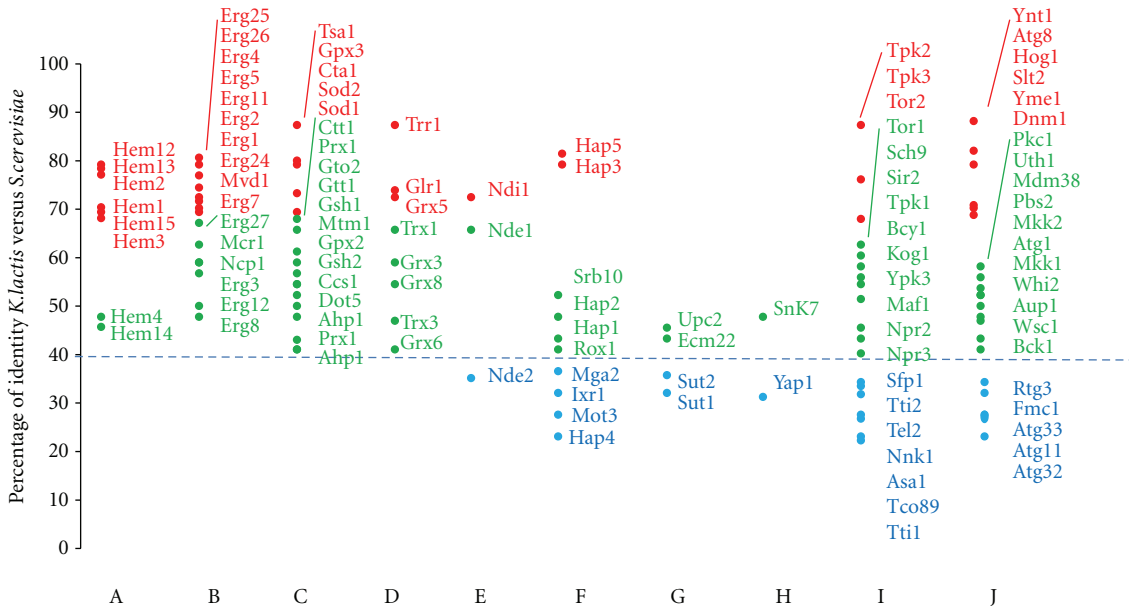


Figure 2: Homologies (percentage of identity calculated according to BLASTp in Génolevures) between *K. lactis* and *S. cerevisiae* proteins related to the pathways summarized in Figure 1. Red, 100–70% identity; green 69–40% identity; blue, <40% identity. (A) heme biosynthesis; (B) ergosterol biosynthesis and supply; (C) NAD(P)H consuming oxidative defense reactions; (D) other oxidative defense reactions; E, NAD(P)-dehydrogenases from the inner membrane of mitochondria; (F) heme/respiration-related transcriptional factors; (G) sterol-related transcriptional factors; (H) peroxide-related transcriptional factors; (I) life span-related proteins; (J) mitophagy-related proteins.

regulatory element (SRE) in the promoters of their target genes, but Ecm22 is an aerobic repressor and Upc2 an anaerobic activator, which is upexpressed during hypoxia. They regulate expression of ergosterol biosynthesis genes and the DAN/TIR family of cell wall proteins [65–67]. This regulatory circuit remains unstudied in *K. lactis,* although analysis of the genome sequence shows that the hypoxic genes from the sterol biosynthetic pathway are conserved in the two yeasts [26].

Quantitation of the homology between proteins translated from verified or putative orthologous genes of *K. lactis* and *S. cerevisiae* reveals that, with few exceptions, the proteins related to biosynthetic routes producing intracellular changes in heme and ergosterol are more conserved than the transcriptional factors, which are regulated by their levels and produce the hypoxic response (Figure 2).

## 3. Oxidative Stress Response in *K. lactis*

The oxidative stress response in *K. lactis* is a mostly unexplored field. The number of published works is less than 1.5% compared to *S. cerevisiae*. Several studies based on comparative genomics have been performed, combining *in silico* and experimental approaches [26, 68, 69]. The search in the *K. lactis* genome of putative *S. cerevisiae* orthologs related to the oxidative stress response (genes coding for superoxide dismutases and their chaperones, catalases and peroxidases, proteins of the glutathione, and thioredoxin systems) has suggested that pathways and proteins are generally conserved, with a few exceptions mainly affecting gene redundancy or predicted subcellular location of the proteins [69]. These exceptions comprise the groups of genes encoding thioredoxin peroxidases, thioredoxin reductases, thioredoxins, glutaredoxins, and glutathione transferases (Figure 2). However, several functional differences affecting mainly connections between redox pathways related to carbohydrate metabolism, respiratory functions and oxidative stress response are observed [69]. In support of these results, a recent study about glutathione transferases, and synthetases in yeast species representatives of fermentative, respiratory, and oxidative metabolism [70] found significant differences in homology and predicted intracellular sorting.

Among the reported differences, it is remarkable to outline the role of the thiol-redox pathways, specifically glutathione reductase (GLR), the enzyme that catalyzes the interconversion of oxidized (GSSG) and reduced glutathione (GSH) using NADPH as reducing power. Whereas in *S. cerevisiae* the expression of GLR increases in response to oxidative stress produced after addition of peroxides by a Yap1-mediated mechanism, this effect is absent in *K. lactis* [26, 71, 72]. Surprisingly, both *S. cerevisiae* and *K. lactis* GLR depletion mutants are more sensitive to oxidative stress [12, 13]. In such *K. lactis* GLR mutants, increase in ROS production, catalase, and thioredoxin reductase (TRR) activities are observed and the expression of a pool of other antioxidant enzymes and oxidoreductases is also upregulated [73]. It is likely that TRR and other NADPH-dependent oxidoreductases might replace GLR in maintaining the GSH/GSSG ratio. In fact, purified *K. lactis* TRR shows GLR activity *in vitro* (our unpublished results). In support of this explanation, it has been reported that the thioredoxin-TRR system can reduce GSSG in *S. cerevisiae* [74]. Other reported functional differences affecting the OS defense enzymes of *K. lactis* and *S. cerevisiae* are regarding the mechanism of

cation handling of the superoxide dismutase Sod1 [75] and the transcriptional regulation of the *SOD1* gene after a shift to hypoxia [26, 76]. Also the transcriptional regulation of the genes encoding catalases [12, 26, 77, 78] and glutathione synthetases [26, 79] under aerobic/hypoxic conditions and under peroxide-treatment is different in the two yeasts. About comparative analyses between the transcriptional factors related to the oxidative stress response in *S. cerevisiae* and *K. lactis*, Yap1 and Snk7 share, respectively, 33% and 50% identities (Figure 2), and *Kl*Yap1 has been functionally characterized in relation to the oxidative stress response induced by metals and peroxides [80].

Several evidences support that in *K. lactis* the OS response has a regulatory role upon fermentation/respiration balance. Thus, there is a positive correlation between the increase of GLR activity and the glucose-6-phosphate-dehydrogenase activity (from PPP) when oxygen levels increase [12]. Besides, the glucose respiration rate, in *K. lactis* cells that metabolize all the monosaccharide through the PPP, increases upon GLR depletion and decreases upon GLR overexpression [13]. Proteome analysis reveals that there is a different response to $H_2O_2$-treatment, which is dependent on GLR in such a way that the expression of several enzymes of the glycolysis and the Krebs' cycle decreases in the wild-type strain, while enzymes of these pathways and the PPP increase in the GLR depleted mutant [73]. Other indirect evidence is that *K. lactis* GLR activity decreases in a Gcr1-mutant [13] being Gcr1 at the same time a positive transcriptional regulator of *KlNDI1* [9]; *KlNDI1* is the gene encoding the internal mitochondrial alternative dehydrogenase (NDI), the enzyme that replaces the respiratory chain complex I found in other eukaryotes [81]. GLR depletion mutants grow better in glucose than the wild type, overall when all the glucose is metabolized through the PPP, which might be explained by rerouting the oxidation of the NADPH produced in the PPP from GLR to NDEs, thus increasing ATP production in the respiratory chain [12, 13].

Yeasts alternative dehydrogenases of the mitochondrial inner membrane are also related to OS and are other point of connection with the response to metabolic changes produced by oxygen availability. The transcription of the two *K. lactis* genes encoding NDEs decreases when cells are under oxidative stress and the NADPH-related defense mechanisms are activated [9, 82]. Regarding *K. lactis* NDI, we have proved that the transcription of the *KlNDI1* gene is induced in nonfermentable carbon sources through a process mediated by the factor Adr1 and that the expression of the gene did not decrease after an hypoxic shift [9]. The homologous *S. cerevisiae* enzyme has been more widely studied and its role in aging and ROS production has been reported [83]. Differences found in yeasts NDI are of clinical interest since they have been used in gene therapy of diseases [84] such as Parkinson's disease [85] or hereditary optic neuropathy [86, 87]. Heterologous expression of the *S. cerevisiae NDI1* gene reduces the overproduction of ROS caused by mitochondrial complex I defects in multicellular eukaryotes [88].

These connections between thiol-redox OS reactions and carbohydrate metabolism described above in *K. lactis* are also supported in other organisms. Ralser et al. [89] discovered that *S. cerevisiae* cells with reduced activity of the key glycolytic enzyme triose-phosphate isomerase exhibit an increased resistance to the thiol-oxidizing reagent diamide. This phenotype is conserved in *Caenorhabditis elegans* and the underlying mechanism is based on a redirection of the metabolic flux from glycolysis to the PPP, altering the redox equilibrium of the cytoplasmic NADP(H) pool. Another key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is known to be inactivated in response to various oxidant treatments, and this causes a similar redirection of the metabolic flux [89].

## 4. The Hypoxic-Induced Oxidative Stress Response in *K. lactis*

A connection between the hypoxic and oxidative stress responses has been reported in the fermentative yeast *S. cerevisiae*. Several *S. cerevisiae* genes that are induced during hypoxia are related to the oxidative stress response. *CUP1* and *CUP2*, which are necessary for the removal of superoxide radicals, are upregulated 11.6-fold during hypoxia in a Rox1 and Srb10-dependent mechanism [18]. Other genes related to oxidative stress (*HSP12*, *FMP46*, and *GRE1*) DNA repair (*ALK1*) or mitochondrial genome maintenance (*MGM1*)also increase their expression during hypoxia [18]. The level of mitochondrial and cytosolic protein carbonylation, the level of mitochondrial and nuclear DNA damage measured by 8-OH-dG modification, and the expression of *SOD1*, encoding superoxide dismutase, increases transiently during a shift to anoxia [76]. Besides, the specific proteins, which become carbonylated during the shift to anoxia, are the same proteins that become carbonylated during peroxidative stress. These results demonstrate that yeast cells exposed to anoxia experience transient oxidative stress and suggest that ROS generated could also signal the variation in oxygen levels and trigger the nuclear response to hypoxia affecting transcription [76]. However, the specific connection between ROS production, protein, or DNA modifications and transcriptional regulation has not yet been elucidated in yeasts. The question about whether mitochondrial or cytosolic proteins, which are specifically oxidized in cells exposed to anoxia, play a role in signaling pathways from the mitochondrion to the nucleus that function to induce hypoxic genes remains unanswered.

In *K. lactis,* after analyzing 30 genes related to oxidative stress, only two (*KlGSH1* and *KlOYE2*) increased their expression after the hypoxic shift [26]. However, a whole-genome approach has not been carried out in this yeast and, therefore, a direct comparison of these data with those reported from *S. cerevisiae* is not accurate. An interesting observation, which suggests that also in *K. lactis* the hypoxic response might be triggered by ROS production, is that the hypoxic response is highly dependent on the relative flux of glucose through glycolysis or the pentose phosphate pathway (PPP). The predominant use of PPP *versus* glycolysis is accompanied by a higher expression of mitochondrial cytochrome *c* [7], which might be associated with the mitochondrial chain activation and changes in ROS production.

Indeed, in a *rag2* mutant, lacking phosphoglucose isomerase and committed to reroute the glucose-6-phosphate through PPP, in order to bypass the blocked glycolytic step, a more intense hypoxic response than the wild-type strain, and that affects the genes of heme metabolism and the oxidative stress response, is observed [26].

A new link between oxidative stress and hypoxia comes from the analysis of multiple functions attributed to transcriptional regulators initially characterized in the aerobic/hypoxic response. In *S. cerevisiae,* Hap1 not only acts as an aerobic activator but has a regulatory function during hypoxia [17, 90]. Hap1 also controls the expression of genes related to sterol biosynthesis [14, 91–94] and *SOD2,* involved in the oxidative stress response [95].

Although putative homologues of two of the principal regulators of the aerobic/hypoxic response in *S. cerevisiae* (Hap1 and Rox1) have been characterized in *K. lactis*, their sequence and function diverge notably from those described in *S. cerevisiae* [44, 58]. Remarkably, their functions in *K. lactis* are somehow related to the metal-induced oxidative-stress response. Deletion of *KlHAP1* increases the resistance to oxidative stress or cadmium [58]. Moreover, the induction by 0.5 mM $H_2O_2$ of two genes related to the oxidative stress response, *KlYAP1* and *KlTSA1,* is repressed by *Kl*Hap1p [58]. *KlROX1* mediates the response to arsenate and cadmium [44]. KlRox1p binds to the *KlYCF1* promoter, a gene related to cadmium detoxification, and causes its activation [44].

## 5. Adjustment of Cell Survival and the Oxidative Stress Response

Besides the transcriptional, proteomic, and metabolomic reorganization caused by the oxidative stress, ROS also elicit other mechanisms of cell defense, including life span adjustment, programmed cell dead, autophagy, and mitophagy. Mitochondria, being the major intracellular source of ROS, are involved in aging and life span regulation [96] Yeasts have been proved to be good models for studying these processes. In *S. cerevisiae,* a cross-regulation between glycolysis and PPP has been proposed in order to prevent oxidative stress when cells switch from anaerobic to oxidative metabolism. Low activity of the glycolytic enzyme pyruvate kinase causes accumulation of PEP and blocks the pathway diverting the glucose flux into the PPP [97]. This mechanism helps to balance the increased ROS production during oxidative metabolism [97]. Also in mammals, during oxidative damage in cancer cells, a similar redirection of metabolic fluxes contributes to ROS clearance [98]. Therefore, it is possible that the hypoxic and the oxidative stress responses, influenced by the reorganization of the utilization of different metabolic pathways, also contribute to modulate these cell defense mechanisms in yeasts and other cells.

Studies pioneered in *S. cerevisiae* by measuring life span have revealed several molecular mechanisms underlying cellular aging and which are well conserved in eukaryotes. Two basic experimental approaches have been applied in unicellular organisms. Replicative life span (RLS) is defined as the number of daughters a single cell produces during its life [99]. Chronological life span (CLS) is defined as the time a population of cells survive in stationary phase [100]. In *S. cerevisiae*, a Crabtree-positive yeast, calorie restriction by glucose limitation increases both RLS and CLS, a feature that coincides with increased cytochrome content, and NADH-cytochrome *c* reductase activity [101]. In. *K. lactis*, a Crabtree-negative yeast for which glucose limitation does not promote an enhancement of the respiratory capacity [2], the increase in CLS by glucose limitation is not produced [102]. These results suggest that calorie restriction-dependent increase in longevity may be due to mitochondrial control and more particularly the regulation of oxidative phosphorylation activity.

An additional nexus between aging and the redox cell balance came from the discovery of sirtuins. They are $NAD^+$-dependent enzymes and they belong to a highly conserved family of proteins that in yeasts, invertebrates, and mammals act in diverse functions related to longevity [103]. In *S. cerevisiae,* Sir2 is induced in cells treated with 4 mM $H_2O_2$ or 10 mM menadione [104] and these data suggest a connection between oxidative stress, Sir2 activation and longevity. The existence of complexes of Sir2 with other metabolic enzymes $NAD^+$-dependent, like those formed with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase Tdh3 or the alcohol dehydrogenase Adh1 [105], might indicate that the ratio $NAD^+$/NADPH in the microenvironment surrounding Sir2 could act as its modulator. Recently, a third complex of Sir2 with Mdh1, the mitochondrial malate dehydrogenase, has been proposed based on multiple common network interactions involving also the proteins Rad53, Aat1, Fob1, and Hst1 [106], although not yet proved by physical interactions. Since Mdh1 is overexpressed under conditions of calorie restriction [107] and it causes extension of Sir2-dependent RLS [108], further investigation is promising. In *K. lactis* sirtuins or its regulators have not been studied but there is an ORF (KLLA0F14663g) with 62% identities to *S. cerevisiae* Sir2 (Figure 2). Considering the importance of sirtuins and their modulators in the etiology and treatment of human pathologies such as metabolic, cardiovascular, and neurodegenerative diseases or cancer [104, 109] and the similarities found between the high respiratory metabolism of *K. lactis* and certain human cell types like neurons [5], sirtuins and related proteins from *K. lactis* are good targets for study.

About the signaling pathways that affect life span in yeast (reviewed in [103]), the serine threonine kinases Tor1, Sch9, and PKA that control nutrient signaling pathways also regulate aging in yeasts, and their homologs in animals share conserved functions in aging. Depletion of Tor1 kinase increases both RLS and CLS in budding yeast, flies, and *C. elegans*. Likewise, PKA kinase activation lengthens life span in budding yeast and longevity in mice. The kinase S6K1, which is known to be related to the control of aging in mice, Drosophila, *C. elegans,* and the yeast Sch9 kinase that controls RLS and CLS in yeast represent other group or orthologous genes. Purification of TOR from yeast and human cells revealed that TOR can exist in at least two multiprotein complexes, termed TORC1 and TORC2 [110]. Interestingly, it has been found that superoxide anions

Table 1: Putative main actors of aging and its signaling pathways in *K. lactis* and *S. cerevisiae*. Degree of identity (%) between homologs is indicated in Figure 2.

| Protein | ORF *K. lactis* | ORF *S. cerevisiae* | Function |
|---|---|---|---|
| Sir2 | YDL042C | KLLA0F14663g | $NAD^+$ -dependent histone deacetylase |
| Tpk1 | YJL164C | KLLA0B12716g | PKA catalytic subunit |
| Tpk2 | YPL203W | KLLA0D03190g | PKA catalytic subunit |
| Tpk3 | YKL166C | KLLA0B07205g | PKA catalytic subunit |
| Bcy1 | YIL033C | KLLA0E04181g | PKA regulatory subunit |
| Ypk3 | YBR028C | KLLA0F24618g | An AGC kinase phosphorylated by cAMP-dependent protein kinase (PKA) in a TORC1-dependent manner |
| Asa1 | YPR085C | KLLA0D09086g | Subunit of the ASTRA complex involved in the stability or biogenesis of PIKK*s such as TORC1 |
| Tor1 | YJR066W | KLLA0B13948g | PIK-related protein kinase and rapamycin target, subunit of TORC1, a complex that controls growth in response to nutrients by regulating translation, transcription, ribosome biogenesis, nutrient transport, and autophagy, involved in meiosis |
| Tor2 | YKL203C | KLLA0B13948g | PIK-related protein kinase and rapamycin target, subunit of TORC1 and TORC2, a complex that regulates cell-cycle dependent polarization of the actin cytoskeleton, involved in meiosis |
| Nnk1 | YKL171W | KLLA0A06776g | Protein kinase, implicated in proteasome function, interacts with TORC1, Ure2, and Gdh2 |
| Tco89 | YPL180W | KLLA0E18855g | Subunit of TORC1 (Tor1 or Tor2-Kog1-Lst8-Tco89) |
| Kog1 | YHR186C | KLLA0A09471g | Subunit of TORC1, it may act as a scaffold protein to couple TOR and its effectors |
| Tti1 | YKL033W | KLLA0F25762g | Subunit of the ASTRA complex, involved in chromatin remodeling, telomere length regulator involved in the stability or biogenesis of PIKK*s such as TORC1 |
| Tti2 | YJR136C | KLLA0B04026g | Subunit of the ASTRA complex, involved in chromatin remodeling, telomere length regulator involved in the stability or biogenesis of PIKK*s such as TORC1 |
| Sch9 | YHR205W | KLLA0B03586g | AGC family protein kinase and functional ortholog of mammalian S6 kinase, phosphorylated by Tor1p and required for TORC1-mediated regulation of ribosome biogenesis, translation initiation, and entry into G0 phase, integrates nutrient signals and stress signals from sphingolipids to regulate life span |
| Maf1 | YDR005C | KLLA0E17535g | Negative regulator of RNA polymerase III, binds to the N-terminal domain of the Rpc160 subunit of Pol III to prevent closed-complex formation, localization and activity are regulated by phosphorylation, mediated by TORC1, protein kinase A, and Sch9 |
| Tel2 | YGR099W | KLLA0D15158g | Subunit of the ASTRA complex, involved in the stability or biogenesis of PIKK*s such as TORC1 |
| Sfp1 | YLR403W | KLLA0B03047g | Regulates transcription of ribosomal protein, response to nutrients and stress, G2/M transitions during mitotic cell cycle, and DNA-damage response and modulates cell size, regulated by TORC1 and Mrs6 prion |
| Npr2 | YEL062W | KLLA0D01067g | Npr2/3 complex mediates downregulation of TORC1 activity upon amino acid limitation |
| Npr3 | YHL023C | KLLA0F18238g | Npr2/3 complex mediates downregulation of TORC1 activity upon amino acid limitation |

*PIKK phosphoinositide 3-kinase related kinase.

regulate the TORC1 complex and its ability to bind the Fpr1-rapamycin complex [111], thus establishing another link between OS and aging. In *S. cerevisiae,* 15 genes are functionally related to TORC1 function and Tor, Sch9, and PKA signaling are interconnected (Table 1). In *S. cerevisiae,* the genes *TPK1* (alias *PKA1*, *SRA3*; ORF, YJL164C), *TPK2* (alias *PKA2*, *YKR1*, *PKA3*; ORF, YPL203W), and *TPK3* (YKL166C) encode for three forms of the cAMP-dependent protein kinase catalytic subunit of the cyclic AMP-dependent protein kinase (PKA) and *BCY1* (YIL033C) for the regulatory subunit. In *K. lactis,* homologous genes of main participants in these signaling pathways are present (Table 1) and homology (recorded using BLASTp in Génolevures at http://www.genolevures.org/) is summarized in Figure 2 The most remarkable observation is that in *K. lactis,* there is only one ORF (KLLA0B13948g), which encodes for a protein with 71% identity to *S. cerevisiae* Tor2 and 68% identity to *S. cerevisiae* Tor1. This opens a question about the existence and composition of two TORC complexes in *K. lactis* as previously reported in *S. cerevisiae* [110] and outlines this issue as a differential point to study in relation to divergences in life span signaling. Besides, the *K. lactis* proteins in this group with less than 40% identity to their *S. cerevisiae* counterparts are also good targets for further studies.

Apoptosis is one type of programmed cell death (PCD) with great importance for the development and homeostasis of multicellular organisms. Basal apoptosis machinery exists also in yeast, unicellular fungus, and in some filamentous

fungi [112]. Regarding the respiratory yeast *K. lactis*, once more the number of studies performed hitherto is very scarce [113]. A mutant in the essential gene *KlLSM4*, an ortholog to *LSM4* of *S. cerevisiae,* which encodes an essential protein involved in both pre-mRNA splicing and mRNA decapping, shows phenotypic markers of apoptosis such as chromatin condensation, DNA fragmentation, accumulation of ROS, and increased sensitivity to different drugs. Mechanisms of Bax-induced [114, 115] and lactose-induced [116] cell death have also been described in *K. lactis*. We have recently investigated PCD in *K. lactis*, using the drugs staurosporine (STS) and phytosphingosine (PHS), which induce PCD in other organisms, and found that glutathione and GLR played an important role. While *K. lactis* seemed to be insensitive to STS, PHS induced PCD. The insensitivity of *K. lactis* to STS might be dependent upon the higher levels of GSH found in cells treated with STS. In human cells, PCD induced by STS causes GSH efflux, but GSH exporter proteins are absent in *K. lactis*. In addition, GLR appears to be involved in PHS-triggered PCD because cells lacking this enzyme are more resistant to the drug than the wild-type strain. Moreover, the addition of GSSG or GSH to the medium partially restores growth of the wild-type *K. lactis* strain on PHS [117].

The strictly regulated removal of oxidized structures is a universal stress response of eukaryotic cells that targets damaged or toxic components for vacuolar or lysosome degradation. Autophagy stands at the crossroad between cell survival and death. It promotes survival by degrading proteins and organelles damaged during oxidative stress, but it is also activated as a part of death programs, when the damage cannot be overcome. Evidence is accumulating that the cellular sites of ROS production and signaling (including mitochondria) may be primary targets of autophagy [118]. The surplus ROS damage the mitochondria themselves and the damaged mitochondria produce more ROS in a vicious circle, ultimately leading to mitochondrial DNA deletion, a form of the so-called petite-mutant phenotype [119]. Selective mitochondria autophagy is called mitophagy and contributes to the maintenance of mitochondrial quality by eliminating damaged or excess mitochondria [120]. Although little is known about the mechanism, glutathione influences mitophagy [121, 122]. The interplay between mitochondria and autophagy seems to be evolutionarily conserved from yeast to higher eukaryotes. Defects in one of these elements could simultaneously impair the other, resulting in risk increments for various human diseases [123]. Autophagy is associated with tumor genesis, neurodegenerative diseases, cardiomyopathy, Crohn's disease, fatty liver, type 2 diabetes, defense against intracellular pathogens, antigen presentation, and longevity [121, 122, 124].

Recent studies in yeast identified several mitophagy-related proteins, which have been characterized with regard to their function and regulation, allowing to compare the similarities and differences of this degradation process between yeast and mammalian cells [120]. Up to our knowledge, no studies at all about mitophagy or even autophagy have been published at present in *K. lactis*. We have performed a custom Blastp in Génolevures for the main actors of mitophagy [125] and its signaling pathways [126] in *S. cerevisiae versus K. lactis* and we found sequences with different degrees of similarity as shown in Table 2 and Figure 2. Further research is required to analyze if the sequence similarity corresponds to similarity of function or not. This is a new field of research in the respiratory yeast *K. lactis*. As occurs with other pathways, it is likely that functional differences exist according to fermentative or respiratory predominant metabolism in yeasts. *K. lactis* proteins in this group (Figure 2) are good targets for comparative studies in mitophagy.

## 6. Conclusions and Perspectives

*K. lactis* is proposed as a respiratory eukaryote model, complementary to the fermentative *S. cerevisiae*, for the study of the pathways of hypoxia-induced oxidative stress. The experimental studies carried so far reveal that there are many differences in all the steps analysed from a comparative perspective, even when high homology exists between the acting proteins from the two yeasts. Some of these differences are briefly summarized in Table 3, although they are probably much wider than here exposed and they will increase with future studies. Besides, *in silico* analysis reveals that transcriptional factors and several actors from the cell-defense response (life span and mitophagy) are among the poorly homologous proteins, and therefore those become good candidates for functional characterization.

Many yeast genes related to the hypoxic, oxidative, and cell-defense responses are related to human diseases [127]. Although most of the studies performed hitherto about *K. lactis* physiology are focused on the respiro-fermentative metabolism, and much less is known about other pathways, there are representative examples of differences between *K. lactis* and *S. cerevisiae* that might be of interest for their applications in therapy of human health disorders and in the potential use of *K. lactis* as a model for this research. Among the potential genes or proteins of interest, *SOD1* is homolog of the human gene involved in amyotrophic lateral sclerosis [128]. NDI is involved in gene therapy of complex I defects [88, 129] and is important in neurological diseases [84–86]. Several genes of ergosterol biosynthesis are targets to look for pharmacological drugs (anticholesterol, antifungal, anticancer, etc.) [125]. Diamine oxidases and catalases have been used as therapeutic approaches for the treatment of inflammatory bowel diseases, intestinal cancers, or pseudoallergic reactions [130]. Hereditary coproporphyria (HCP), an autosomal dominant acute hepatic porphyria, results from mutations in the gene that encodes coproporphyrinogen III oxidase [131]. Sirtuins have been associated to diabetes type 2 [132] and Huntington disease [104] as well as cardiopathies or cancer [109].

Neither *S. cerevisiae* nor other currently used models, even multicellular, manifest the complex set of alterations associated to each health disorder in humans. This makes necessary the combination of the information obtained from several models, as representative as possible of the diversity of human cell types (*S. cerevisiae*, *K. lactis,* and others), in order to advance in puzzling out the molecular basis of the diseases and in developing new preventive and therapeutic tools.

Table 2: Putative main actors of mitophagy and its signaling pathways in *K. lactis* and *S. cerevisiae*. Degree of identity (%) between homologs is indicated in Figure 2.

| Protein | ORF *K. lactis* | ORF *S. cerevisiae* | Function |
|---|---|---|---|
| Atg1 | KLLA0C17160g | YGL180W | Autophagy-dedicated protein serine/threonine kinase |
| Atg11 | KLLA0B12133g | YPR049C | Cytoplasm-to-vacuole targeting (Cvt) pathway and peroxisomal degradation (pexophagy) |
| Atg32 | KLLA0A00660g | YIL146C | Mitochondrial receptor specific to mitophagy |
| Atg33 | KLLA0A02695g | YLR356W | Detects or presents aged mitochondria for degradation at the stationary phase |
| Atg8 | KLLA0E20593g | YBL078C | Component of autophagosomes and Cvt vesicles |
| Aup1 | KLLA0F06985g | YCR079W | Mitochondrial protein phosphatase |
| Bck1 | KLLA0F14190g | YJL095W | MAP kinase kinase kinase acting in the protein kinase C signaling pathway |
| Dnm1 | KLLA0F12892g | YLL001W | Dynamin-related GTPase |
| Fmc1 | KLLA0F04081g | YIL098C | Assembly at high temperature of mitochondrial ATP synthase |
| Hog1 | KLLA0F20053g | YLR113W | MAP kinase involved in osmoregulation |
| Mdm38 | KLLA0B11748g | YOL027C | Mitochondrial distribution and morphology |
| Mkk1 | KLLA0D07304g | YOR231W | MAP kinase kinase acting in the protein kinase C signaling pathway |
| Mkk2 | KLLA0D07304g | YPL140C | MAP kinase kinase acting in the protein kinase C signaling pathway |
| Pbs2 | KLLA0E15313g | YJL128C | MAP kinase kinase in the osmosensing signal-transduction pathway |
| Pkc1 | KLLA0E06447g | YBL105C | Protein kinase C |
| Rtg3 | KLLA0E06513g | YBL103C | Transcription factor to activate the retrograde (RTG) and TOR pathways |
| Slt2 | KLLA0B11902g | YHR030C | MPK1 MAP kinase |
| Uth1 | KLLA0E14939g | YKR042W | Regulator outer membrane protein |
| Whi2 | KLLA0F15972g | YOR043W | Full activation of the general stress response |
| Wsc1 | KLLA0D14377g | YOR008C | Sensor transducer of the stress-activated PKC1-MPK1 kinase pathway |
| Yme1 | KLLA0E06711g | YPR024W | Protease catalytic subunit for degradation of unfolded or misfolded mitochondrial gene products |
| Ynt1 | KLLA0C06534g | YDR394W | Subunit of the 26S proteasome |

Table 3: Main differences reported hitherto between *K. lactis* and *S. cerevisiae*, two alternative unicellular eukaryote models for hypoxic and oxidative stress responses.

| | *K. lactis* | *S. cerevisiae* |
|---|---|---|
| Crabtree effect | Negative | Positive |
| Glucose catabolism in aerobic conditions | Mainly respiratory | Mainly fermentative |
| Ratio PPP/glycolysis for glucose catabolism | High | Low |
| Reoxidation of NADPH from PPP | Mainly by mitochondrial alternative external dehydrogenases | Mainly by cytosolic NADPH oxidoreductases |
| Catabolic repression of respiration | Low | High |
| Respiratory capacity | Unlimited | Limited |
| Petite phenotype | Positive in specific mutant genetic backgrounds | Positive |
| Caloric restriction increases longevity | No | Yes |
| Aerobic/hypoxic gene pairs | Absent | Present |
| Upregulated by hypoxia | Genes related to ergosterol synthesis, cell wall composition, and glycolytic genes. OS genes: *CUP1* and *CUP2*, *HSP12, FMP46* and *GRE1*, and *SOD1*. | Genes from the heme biosynthetic pathway, pyruvate decarboxylase, and lipid biosynthesis. OS genes: *KlOYE2, KlGSH1*. This response is highly dependent on the relative flux of glucose through glycolysis or PPP |
| Transcriptional regulators Hap1 and Rox1 | Not related to heme-mediated oxygen response | Related to heme-mediated oxygen response |

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