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ORIGINAL ARTICLE

The *UPC2* gene in *Kluyveromyces lactis* stress adaptation

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Abstract

*Kl*Upc2p, a transcription factor belonging to the fungal binuclear cluster family, is an important regulator of ergosterol biosynthesis and azole drug resistance in *Kluyveromyces lactis*. In this work, we show that the absence of *Kl*Upc2p generates Rag$^-$ phenotype and modulates the *K. lactis* susceptibility to oxidants and calcofuor white. The *KlUPC2* deletion leads to increased expression of *KlMGA2* gene, encoding an important regulator of hypoxic and lipid biosynthetic genes in *K. lactis* and also *KlHOG1* gene. The absence of *Kl*Upc2p does not lead to statistically significant changes in glycerol, corroborating the expression of *KlGPD1* gene, encoding NAD$^+$-dependent glycerol-3-phosphate dehydrogenase, that is similar in both the deletion mutant and the parental wild-type strain. Increased sensitivity of *Klupc2* mutant cells to brefeldin A accompanied with significant increase in *KlARF2* gene expression point to the involvement of *Kl*Upc2p in intracellular signaling. Our observations highlight the connections between ergosterol and fatty acid metabolism to modulate membrane properties and point to the possible involvement of *Kl*Upc2p in *K. lactis* oxidative stress response.

Keywords *Kluyveromyces lactis* · *UPC2* · Ergosterol · Susceptibility · Oxidative stress · Lipid trafficking

Introduction

Fungi belonging to *Saccharomycotina* clade contain a specific family of transcriptional regulators (Zn_2Cys_6), many of which are essential for adaptation to various types of stresses (MacPherson et al. 2006). The main transcription factors involved in *Saccharomyces cerevisiae* ergosterol biosynthesis regulation, Upc2p, Ecm22p, and Sut1p, belong to this zinc cluster family. In the absence of ergosterol Upc2p and Ecm22p bind to the sterol response element (SRE) in the promoters of ergosterol (*ERG*) genes to activate their transcription (Vik and Rine 2001). Under anaerobic conditions, Upc2p also plays a role in the uptake of sterols by regulating the expression of genes encoding cell wall mannoproteins (*DAN1/TIR*) and ABC transporters (*AUS1*, *PDR11*) (Wilcox et al. 2002). Along with the SRE binding proteins, Upc2p and Ecm22p, the heme-binding protein Hap1p and the repressors Rox1p and Mot3p coordinate ergosterol gene expression in *S. cerevisiae* (Davies and Rine 2006; reviewed in Jorda and Puig 2020).

Kluyveromyces lactis, an ascomycetous yeast species closely related to *S. cerevisiae*, received considerable attention due to its distinct metabolic and physiological properties (Breunig et al. 2000; Rodicio and Heinisch 2013). In aerobiosis, *S. cerevisiae* primarily uses fermentative metabolism, whereas in *K. lactis*, respiratory metabolism is predominant. In *S. cerevisiae*, where respiration and mitochondrial functions are dispensable, glucose is consumed via glycolysis. On the contrary, in the respiratory *K. lactis* yeast, glucose is metabolized through the pentose phosphate pathway and the preferences between respiration and fermentation depend on oxygen availability (Kiers et al. 1998). *K. lactis* belongs to petite-negative yeast species that are not able to survive without mitochondrial DNA and proteosynthesis. Petite mutants can be obtained in specific *K. lactis* nuclear background only (Chen and Clark-Walker 1999). Wild-type *K. lactis* strains are able to grow on glucose in the presence of antimycin A, an inhibitor of mitochondrial respiration, although many *K. lactis* mutants (natural or induced) fail to grow in such conditions. Impaired growth on glucose medium supplemented with antimycin A is known as Rag$^-$ phenotype (resistance to antimycin A on glucose) and is usually ascribed to defective glycolysis and/or its

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regulation (reviewed in Breunig et al. 2000). However, Rag$^-$ phenotype in *K. lactis* was also observed as a result of *KlHSL1* (the *K. lactis* homologue of a gene encoding a serine-threonine protein kinase regulating the morphogenesis checkpoint in *S. cerevisiae*) and *KlMGA2* (transcriptional activator involved in various kinds of stresses) gene deletions (Cialfi et al. 2011; Micolonghi et al. 2012). The fact, that low oxygen induction of *KlMGA2* requires the plasma membrane glucose sensor Rag4 in *K. lactis*, suggests that low oxygen response is dependent on glucose availability in this yeast (Rolland et al. 2006; Micolonghi et al. 2012). Thus, the coordination of the oxygen-response pathways at the transcription factor level maintains metabolic homeostasis in the cell, primarily the requirement for acetyl-CoA in lipid biosynthesis (Burr and Espenshade 2018).

K. lactis has a lower redundancy of genes compared to *S. cerevisiae* because it did not undergo a whole genome duplication (WGD) (Bussereau et al. 2006; González-Siso et al. 2009). In *S. cerevisiae*, three ARF (ADP-ribosylation factors) GTPases have been identified, and two of them, the paralogs *ScARF1* and *ScARF2*, regulate the formation of coated vesicles in intracellular trafficking within the Golgi. *K. lactis* contains only one ortholog of *ScARF1* and *ScARF2*, the *KlARF2*. The activity of ARFs is regulated by small G-proteins that are essential for a variety of cellular processes (Labbaoui et al. 2017).

The absence of an uptake system for ergosterol precludes the growth of *K. lactis* in strictly anoxic conditions (Snoek and Steensma 2006). *K. lactis* contains only one orthologue of *S. cerevisiae* Upc2p and Ecm22p (Bussereau et al. 2006). In the previous work, we showed that *K. lactis* cells lacking *KlUPC2* exhibit hypersusceptibility to azole drugs and are not able to activate expression of *ERG* genes in the presence of azoles. *Kl*Upc2p was shown to be important for the appropriate regulation of plasma membrane ion transporters *Kl*Ena1p (Na^+-ATPase) and *Kl*Pma1p (H^+-ATPase) (Toth Hervay et al. 2020). In this work, we further extend these observations and show that *Kl*Upc2p is required not only for optimal growth on glucose and modulation of membrane properties but also for oxidative stress response.

Materials and methods

Strains and culture conditions

The following yeast strains were used: *K. lactis CBS2359ku80* (*MATα ku80::loxP*; Kooistra et al. 2004) and its isogenic *upc2Δ* deletion mutant (Toth Hervay et al. 2020). For cultivation standard YPD medium (1% yeast extract and 2% peptone supplemented with carbon sources as needed, typically 2% glucose) was used. The solid media contained 2% Bacto agar.

Drug susceptibility assays

Overnight yeast cultures grown in YPD were diluted to a density of 1.0×10^8 cells/mL. Cell suspensions from ten-fold dilutions were spotted onto agar plates containing the indicated concentrations of drugs in 5 µl aliquots. Growth at 28 °C was scored after 2 days. The drug concentrations used were as follows: CW, 14, 18, and 20 µg/mL; menadione, 10, 20, and 30 µg/mL; brefeldin A, 20 and 30 µg/mL; and H_2O_2, 5 and 6 mmol/L.

Quantitative real-time PCR

The levels of gene transcripts were assessed by quantitative real-time PCR as described previously (Konecna et al. 2016). For statistical analyses, the Student's *t*-test was used and the level of statistical significance was set to $P < 0.05$. The primers used are listed in Table 1.

Glycerol assay

Cells grown in liquid YPD with or without 5 µg/mL of calcofluor white were processed as described previously (Konecna et al. 2018). Glycerol concentration in supernatants gained from 3×10^8 cells was estimated using the Free Glycerol Reagent Kit (Sigma- Aldrich). For statistical analyses, the Student's *t*-test was used and the level of statistical significance was set to $P < 0.05$.

Results

KlUpc2p absence generates Rag$^-$ phenotype

The *Klupc2Δ* mutant showed slightly reduced growth in almost all conditions tested compared to its parental strain. However, growth of the *Klupc2Δ* mutant was further reduced

Table 1 List of oligonucleotides used in this study

Primers for qRT-PCR analysis	Nucleotide sequence (5′→3′)
KlACT1-F	AAT GCA AAC TGC TTC TCA AT
KlACT1-R	AAC AGA TGG ATG GAA CAA AG
KlARF2-F	TTG CCA GAA GCC ATG CC
KlARF2-R	TTC GTA CAA ACC TTC ACC AG
KlHOG1-F	CGC AGG TGT GAT CCA TAG AG
KlHOG1-R	CAC GTA ACC AGT CAT TTG AGG
KlMGA2-F	ATTGGACGATGAAGTACAGGA
KlMGA2-R	TTCTTCCGATGGCTTGACTG
KlGPD1-F	GAT GCT GCT AAG GAC GCT GA
KlGPD1-R	TAA CAC CAT CCT TAC CGA CG

when cultures were grown on YPD plates containing the respiratory inhibitor antimycin A (Fig. 1A). Inspired by the previous findings of Cialfi et al. (2011) and Micolonghi et al. (2012), we tested whether the addition of unsaturated fatty acids or ergosterol to the growth medium could restore the growth of *Klupc2Δ* mutant on glucose in the presence of antimycin A. As Fig. 1A shows, the presence of Tween 80, but not ergosterol, restored the growth of *Klupc2Δ* cells in glucose media containing antimycin A. This observation indicates a functional correlation between the defective growth and unsaturated fatty acid biosynthesis mediated by *Kl*Upc2p. The *KlUPC2* deletion leads to a distinct resistance of mutant cells to menadione (Fig. 1B). This observation corroborates the finding of Becerra et al. (2004), who observed a mild increase in mRNAs transcribed from *K. lactis* genes participating in the *K. lactis rag2/Klpgi1* mutant (lacking phosphoglucose isomerase) oxidative stress defence.

KlUPC2 gene deletion results in increased *KlHOG1* expression

Figure 1B shows increased tolerance of mutant cells to calcofluor white as the result of *KlUPC2* gene deletion. Foregoing research showed that the antifungal effect of calcofluor white is either the result of its binding to the cell wall chitin or depends on a functional HOG pathway (García-Rodriguez 2000; Roncero 2002). However, difference in the chitin content between the *Klupc2Δ* deletion mutant and the parental wild-type strain was not detected (data not shown). To find out whether the *KlUPC2* gene deletion could influence the *KlHOG1* transcription, the *KlHOG1* expression was assayed in the mutant strain. Figure 2A shows an increase of mRNA transcribed from the *KlHOG1* in the *Klupc2Δ* mutant cells compared with that in the parental strain. Following incubation of cells in the medium containing calcofluor white before the RNA isolation, the transcript levels of *KlHOG1* gene further increased in both the mutant and wild-type strains (Fig. 2A). The Hog1p activation could lead to increased expression of *GPD1* (glycerol-3-phosphate dehydrogenase) resulting in stimulation of de novo glycerol synthesis. Therefore, we determined the glycerol content in the parental strain and the *Klupc2Δ* mutant. As shown in Fig. 2B, the changes in glycerol content in the *Klupc2Δ* mutant were not statistically relevant as compared to that in the parental wild-type strain and this pattern was basically unaffected by the presence of calcofluor white. This finding was corroborated by estimating the levels of *GPD1* gene transcripts in the *Klupc2Δ* mutant and parental cells, that were not significantly different (Fig. S1)

KlUPC2 gene deletion results in increased *KlMGA2* expression

The cellular processes needed to combat redox stresses in yeast are different for different oxidants or reductants. The ability of *Klupc2Δ* mutant cells to grow in the presence of hydrogen peroxide and diamide was reduced compared to that of the parental wild-type strain (Fig. 3A). To analyze the possible relationship between *Kl*Upc2p and *Kl*Mga2p, we determined the transcript levels of *KlMGA2* gene in both the parental wild-type strain and the *Klupc2Δ* mutant. Figure 3B shows

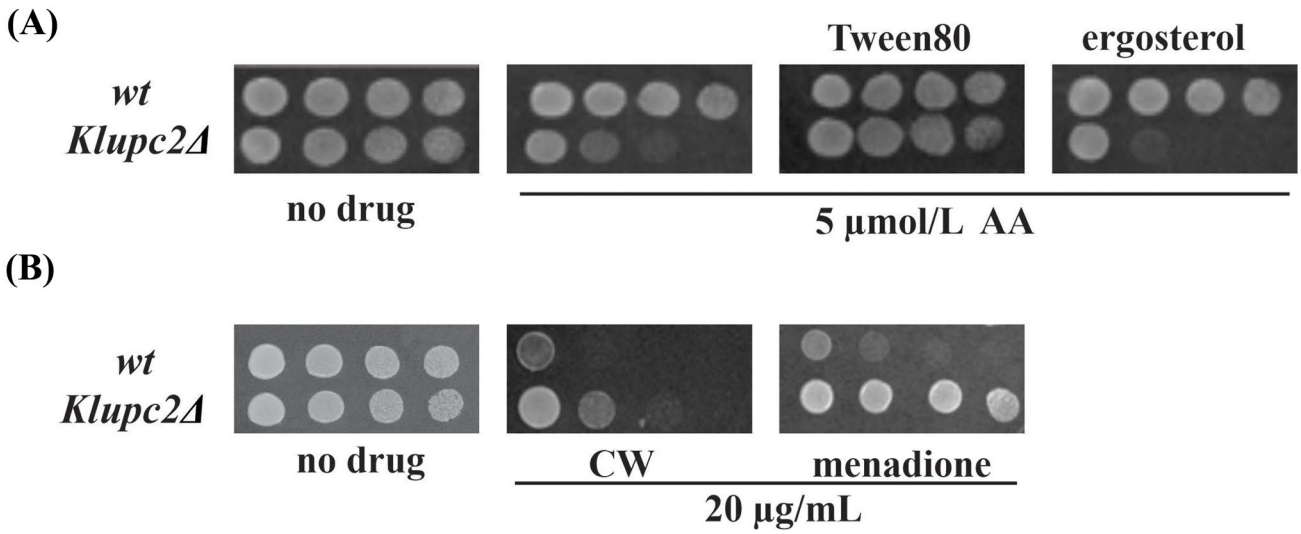

Fig. 1 Susceptibility of *Kluyveromyces lactis* cells to AA (supplemented with Tween 80 or ergosterol) (**A**); CW and menadione (**B**). Spotting assays were performed with a tenfold dilution of overnight culture grown on YPD medium containing the indicated compounds. The plates were incubated for 2 days at 28 °C. AA antimycin A, CW calcofluor white

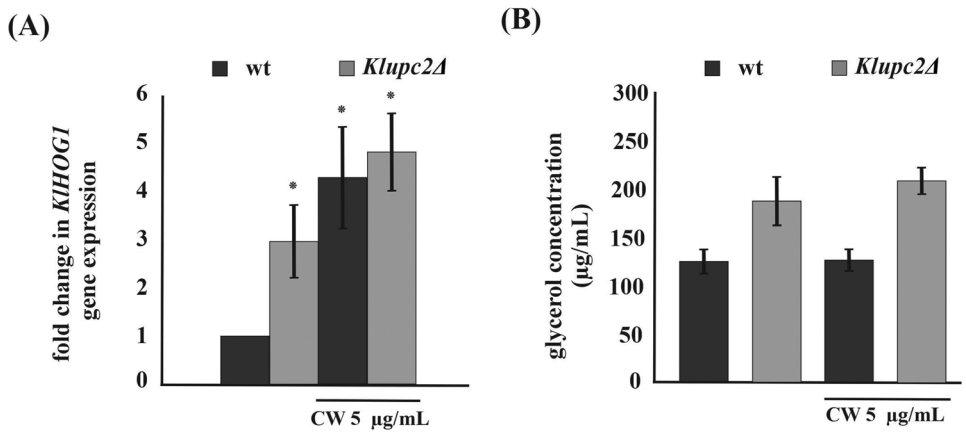

Fig. 2 *KlHOG1* gene expression (**A**) and glycerol content (**B**) in *Klupc2Δ* mutant and the corresponding wild-type strain. The gene transcript level in parental wild-type strain was set as 1. The values were normalized to the β-actin mRNA level. Glycerol concentration was determined as described in "Materials and methods." Data represent the mean of three independent experiments (performed in triplicate). Error bars indicate the SDs. For statistical analyses, the Student's *t*-test was used and the level of statistical significance was set to $P < 0.05$

an increased expression of *KlMGA2* gene in the *Klupc2Δ* mutant as compared to the parental wild-type strain. The transcript level of *KlMGA2* gene in *Klupc2Δ* mutant cells, following the incubation of cells in the presence of hydrogen peroxide (1 mmol/L, for 1 h), was even higher (Fig. 3B). This result demonstrates the possibility that *Kl*Upc2p prevents the induction of *KlMGA2* expression in the presence of hydrogen peroxide.

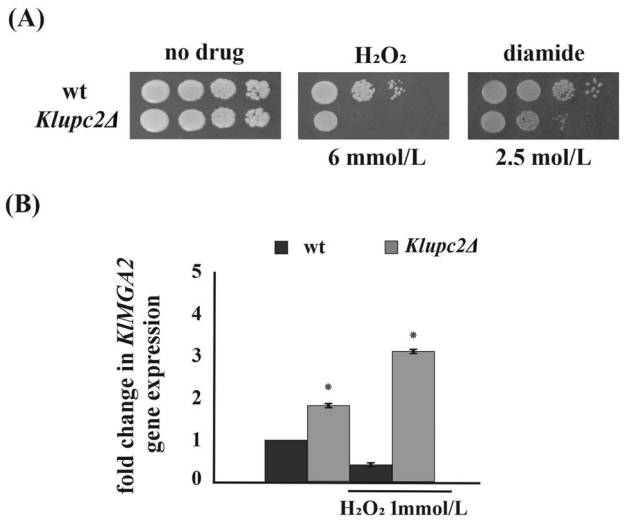

Fig. 3 *KlUPC2* gene deletion results in increased susceptibility of *Klupc2Δ* mutant cells to hydrogen peroxide and diamide (**A**) and increased *KlMGA2* gene expression (**B**) that is even higher in the presence of hydrogen peroxide, compared with that in the corresponding wild-type strain. The gene transcript level in parental wild-type strain was set as 1. The values were normalized to the β-actin mRNA level. For statistical analyses, the Student's *t*-test was used and the level of statistical significance was set to $P < 0.05$

*Kl*Upc2p controls vesicle trafficking within the Golgi

A possible explanation of Klupc2Δ drug sensitivity could be the defect in the ergosterol biosynthesis or troubles in transporting this lipid to the cell membrane. Figure 4A demonstrates increased susceptibility of *Klupc2Δ* cells to brefeldin A, an inhibitor of the guanine nucleotide exchange protein, necessary for Arf2p activation. Therefore, we decided to examine the *KlARF2* expression in the parental wild-type strain and *Klupc2Δ* mutant cells. As Fig. 4B shows, the *KlUPC2* gene deletion results in almost six fold increase in *KlARF2* expression relative to that in the parental wild-type strain. The upregulation of *KlARF2* expression in the absence of *Kl*Upc2p could be considered as a cellular strategy to compensate for its decrease in activity.

Discussion

In this study, we further analyzed the impact of the *KlUPC2* deletion in *K. lactis*. *UPC2* gene in *Saccharomycotina* yeast species encodes the Zn_2Cys_6 transciption factor, member of the fungus specific family, that functions as the major regulator of ergosterol biosynthesis. Other factors beyond the paralogues Upc2p/Ecm22p, the heme-binding protein Hap1p, Mga2p, and the repressors Rox1p and Mot3p, coordinate ergosterol genes expression in *S. cerevisiae* (Davies and Rine 2006; reviewed in Jorda and Puig 2020). Recent reports point to a link between Pdr1p, the main transcription factor involved in the yeast *S. cerevisiae* and *C. glabrata* multidrug resistance, and Upc2p (Vu et al. 2019; Moye-Rowley 2020). Recently, we have shown that the *KlUPC2* deletion increased the cell's susceptibility to azole treatment. Reduced ergosterol

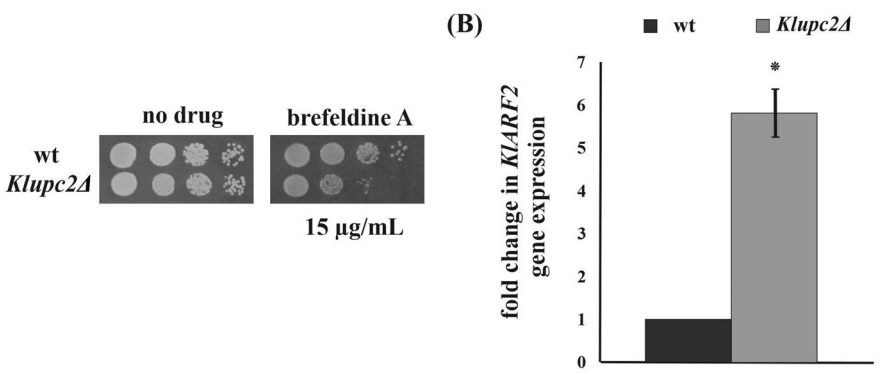

Fig. 4 *KlUPC2* gene deletion leads to increased susceptibility of mutant strain to brefeldin A (**A**) and marked increase in the *KlARF2* gene expression (**B**). The gene transcript level in parental wild-type strain was set as 1. The values were normalized to the β-actin mRNA level. For statistical analyses, the Student's *t*-test was used and the level of statistical significance was set to $P < 0.05$

content in the *Klupc2Δ* cells modulates their plasma membrane properties — the membrane is hyperpolarized and its fluidity decreased. Normal ergosterol homeostasis could be crucial for the proper regulation of plasma membrane ion transporters — the Ena1p Na^+-ATPase or the Pma1p H^+-ATPase in *K. lactis* (Toth Hervay et al. 2020). Here, we extend the published observations showing that deletion of *KlUPC2* leads to a specific *K. lactis* Rag^- phenotype, suggesting the link between ergosterol and carbohydrate metabolism in this yeast species. Coordination of carbohydrate response with lipid metabolism was also observed in *C. parapsilosis*, where sterol and carbohydrate biosynthesis are both regulated by Upc2p (Guida et al. 2011). We show that the presence of Tween 80 (a source of unsaturated fatty acids) in the growth medium suppresses the Rag^- phenotype of the *Klupc2Δ* cells indicating a link between Upc2p and unsaturated fatty acid metabolism. Rag^- phenotype in *K. lactis* was also generated as a result of *KlMGA2* gene deletion (Micolonghi et al. 2012). *KlMGA2* encodes the hypoxia responsive and lipid biosynthesis transcription factor involved also in glucose metabolism, cellular fitness, and light-stress response (Ottaviano et al. 2015; Santomartino et al. 2019; Camponeschi et al. 2021). The phenotypes generated by both the *KlUPC2* and *KlMGA2* gene deletions are restored by the addition of unsaturated fatty acids. In our previous paper, we showed decreased membrane fluidity of *Klupc2Δ* mutant. Lower fluidity of the membrane indicates lower Unsaturation Index (UI), due to a reduced content of unsaturated carbon–carbon bonds in fatty acid molecules. The decrease in the unsaturated fatty acid content can thus be compensated by the addition of Tween 80 to the growth media. The increased *KlMGA2* expression observed as a result of *KlUPC2* gene deletion could amend the *KlupcΔ* cells with the unsaturated fatty acids. Mga2p is constitutively expressed as inactive form bound to ER. Its activation requires appropriate stimuli (e.g., hypoxia), to induce ubiquitylation and the consequent proteolytic activation by the proteasome. Activation releases the transcriptional activator from the ER; the soluble Mga2p enters the nucleus where it activates gene expression through the stress responsive elements (STRE) in the promoters of regulated genes. Santomartino et al. (2019) showed stronger protection against ROS in *Klmga2Δ* cells. The increased expression of *KlMGA2* gene observed in our *Klupc2Δ* mutant in the presence of hydrogen peroxide could be the result of inability to produce ergosterol in the *Klupc2Δ* mutant cells that is converted to an oxidative stress signal.

Along with the common antioxidant systems, one of the major factors contributing to the detoxification of ROS and the tolerance to oxidative stress in *K. lactis* is considered to be the ability to redirect the flux of metabolites from glycolysis to the pentose phosphate pathway providing the NADPH by the glucose-6-phosphate dehydrogenase. Such production of the reducing metabolite for the cellular redox systems is conserved between yeast and animals, underlining its importance in the process of oxidative stress adaptation (Grant 2008). The activation of stress signaling pathways as a result of *KlUPC2* gene deletion is also in line with the increased *KlHOG1* expression. However, we did not observed statistically relevant changes in glycerol content between the wild-type and the *Klupc2Δ* mutant strain. This is in compliance with the detected transcription level of *KlGPD1* (glycerol-3-phosphate dehydrogenase). We propose that *KlUPC2* gene deletion leads to activation of Hog pathway that is not functional. The observed increased sensitivity of the *Klupc2Δ* mutant to brefeldin A together with increased expression of *KlARF2* gene points to the possible defect in membrane/protein trafficking in the *Klupc2Δ* mutant cells. Brefeldin A blocks Arf2p activation catalyzed by guanine nucleotide-exchange proteins. Arf2p, localized in the Golgi, controls intra-Golgi and Golgi-to-ER transport of cargo proteins by the formation of COPI carriers (Suda et al. 2018). Such trafficking could allow for changes in plasma membrane lipid composition, and strengthens the barrier function during stress condition (Babst 2020). *K. lactis* does not import exogenous sterols and relies mostly on the biosynthesis of endogenous ergosterol that has to be transported from ER to the plasma membrane (Snoek and Steensma 2006).

Promoter sequence analysis of the *KlMGA2*, *KlHOG1*, *KlGPD1*, and *KlARF2* genes led to the identification of putative Stress response element (STRE), Yap1 recognition element (YRE) and Stb5p binding sites in all studied genes (Table S1). As both *Kl*Yap1p and *Kl*Stb5p are involved in oxidative stress response, it further points to the *Kl*Upc2p involvement in oxidative stress response. Regulation of gene expression in response to the levels of oxygen involves several transcription factors. Similar to *S. cerevisiae*, the transcription factor *Kl*Hap1p mediates the induction of genes involved in respiration, lipid metabolism, and oxidative stress response. However, it seems to have lower number of target genes, some of which respond only under hypoxic conditions (Bao et al. 2008). Genes encoding enzymes of lipid metabolism are also expressed under hypoxic conditions in a *KlMGA2*-dependent manner (Micolonghi et al. 2012). Unlike in *S. cerevisiae*, *Kl*Rox1p is not involved in the hypoxic response in *K. lactis*, but rather contributes to metal ion resistance (Torres et al. 2012). Due to the crosstalk of different environmental signals, many aspects of regulation by these transcription factors are still unknown, but the coordination of the oxygen- responsive pathways at the transcriptional level efficiently maintains the requirement for acetyl-CoA in lipid biosynthesis (Burr and Espenshade 2018).

In our *Klupc2Δ* mutant, varying concentrations and types of sterols and a lack of ergosterol contributed to changes in the plasma membrane structure resulting in a decrease in plasma membrane fluidity. The disturbance in sterol content could be compensated by changes in the proportions of unsaturated fatty acids. Such plasticity in make-up of plasma membrane might increase the ability of cells to withstand of stress. We cannot exclude that the altered cellular sterol composition affects intracellular signaling and trafficking pathways, including the localization and activity of membrane proteins (Suchodolski and Krasowska 2019). As shown in Figs. 2A and S1, the *KlUPC2* gene deletion leads to changes in the *KlHOG1* but not in the *KlGPD1* gene expression. This is in line with the work of Tanigawa et al. (2012), who observed the activation of HOG pathway as a result of the inhibition of sterol biosynthesis. Mojardín et al. (2018) proposed that *Kl*Gpd1p is part of a signaling pathway that governs transcriptional regulation to maintain the redox balance under changing environmental conditions. However, the exact molecular mechanism how the signal generated by the redox state is converted into the proper transcriptional response in the nucleus remains to be elucidated.

Altogether, Upc2p is a transcription regulator taking part in *K. lactis* ergosterol biosynthesis, carbon metabolism, intracellular signaling/trafficking pathways, and oxidative stress response.

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Declarations

Conflict of interest The authors declare no competing interests.

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