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RESEARCH ARTICLE

Yeast *YPK9* deficiency results in shortened replicative lifespan and sensitivity to hydrogen peroxide

Wei Zhao · Fang Guo · Lingyue Kong · Jiaxin Liu · Xiaoshan Hong · Zhiwen Jiang · Haochang Song · Xiaojing Cui · Jie Ruan · Xinguang Liu

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Abstract *YPK9/YOR291W* of *Saccharomyces cerevisiae* encodes a vacuolar membrane protein. Previous research has suggested that Ypk9p is similar to the yeast P5-type ATPase Spf1p and that it plays a role in the sequestration of heavy metals. In addition, bioinformatics analysis has suggested that Ypk9p is a homolog of human ATP13A2, which encodes a protein of the subfamily of P5 ATPases. However, no specific function of Ypk9p has been described to date. In this study, we found, for the first time, that *YPK9* is involved in the oxidative stress response and modulation of the replicative lifespan (RLS). We found that *YPK9* deficiency confers sensitivity to the oxidative stress inducer hydrogen peroxide accompanied by increased intracellular ROS levels, decreased mitochondrial membrane potential, abnormal mitochondrial function, and increased incidence of early apoptosis in budding yeast. More importantly, *YPK9* deficiency can lead to a shortened RLS. In addition, we found that overexpression of the catalase-encoding gene *CTA1* can reverse the phenotypic abnormalities of the *ypk9Δ* yeast strain. Collectively, these findings highlight the involvement of Ypk9p in the oxidative stress response and modulation of RLS.

Keywords *YPK9* · Deficiency · Oxidative stress · Replicative lifespan

Fang Guo contributed equally to this work and should be considered co-first author.

W. Zhao · F. Guo · L. Kong · J. Liu · Z. Jiang · H. Song · X. Cui · J. Ruan (✉) · X. Liu (✉)
Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics, Institute of Aging Research, Guangdong Medical University, Dongguan 523808, China
e-mail: ruanjie@gdmu.edu.cn

X. Liu
e-mail: xgliu@gdmu.edu.cn

W. Zhao · F. Guo · L. Kong · J. Liu · Z. Jiang · H. Song · X. Cui · J. Ruan · X. Liu
Institute of Biochemistry and Molecular Biology, Guangdong Medical University, Dongguan 523808, China

X. Hong
Institute of Gynecology, Women and Children's Hospital of Guangdong Province, Guangzhou 511442, China

Abbreviations

AbA	Aureobasidin A
ATP	Adenosine triphosphate
CHP	Cube hydroperoxide
CLS	Chronological life span
CR	Caloric restriction
DAPI	4′,6-Diamidino-2-phenilindole
DCFH-DA	2′,7′-Dichlorofluorescein diacetate
D. melanogaster	Drosophila melanogaster
ETC	Electron transport chain
FITC	Fluorescein isothiocyanate
H_2O_2	Hydrogen peroxide
LiAc	Lithium acetate

MMP	Mitochondrial membrane potential
mtDNA	Mitochondrial DNA
mt-nucleoids	Mitochondrial nucleoids
OD	Optical density
PCR	Polymerase chain reaction
Rh123	Rhodamine 123
RLS	Replicative lifespan
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative PCR
S. cerevisiae	*Saccharomyces cerevisiae*
TBHP	Tert-butyl hydroperoxide
TTC	2,3,5-Triphenyltetrazolium chloride
WT	Wild-type

Introduction

YPK9/YOR291W of *Saccharomyces cerevisiae* (*S. cerevisiae*) encodes a protein localized at the vacuolar membrane. Previous studies have reported that Ypk9p is similar to the yeast P5 ATPase Spf1p (Gitler et al. 2009; Sørensen et al. 2018). In addition, bioinformatics analysis has suggested that among proteins, Ypk9p has the greatest homology to human ATP13A2, which encodes a protein of the subfamily of P5 ATPases (Kühlbrandt 2004; Li et al. 2021; Palmgren and Nissen 2011).

P-type ATPases form a large superfamily of cation and lipid pumps that carry out many fundamental biological processes, including generation of membrane potential for muscle contraction and removal of toxic ions from cells (Kühlbrandt 2004; Tadini-Buoninsegni 2020). Based on sequence homology, the P-type ATPase family is divided into five distinct subfamilies, which are referred to as types P1–P5 (Kühlbrandt 2004; Palmgren and Axelsen 1998; Palmgren and Nissen 2011). Interestingly, P1–P4 ATPases have specific substrates; for example, P2C ATPases are Na^+/K^+ and H^+/K^+ pumps in animals, and P2D ATPases are Na^+ pumps in fungi (Palmgren and Nissen 2011). However, substrate specificities and biological roles have not been elucidated for any P5 pumps (Palmgren and Nissen 2011).

Previous studies in yeast have reported that deletion of *YPK9* leads to sensitivity to certain metals (Gitler et al. 2009; Heins-Marroquin et al. 2019; Schmidt et al. 2009), implicating *YPK9* in the transport or regulation of metal cations. Mutations in the ATP13A2 gene are involved in the pathogenesis of neurodegenerative diseases such as Parkinson's disease (Kong et al. 2014; Spataro et al. 2019), Kufor-Rakeb syndrome (Kett et al. 2015; Ramirez et al. 2006) and neuronal ceroid lipofuscinosis (Bras et al. 2012; Estrada-Cuzcano et al. 2017), implying a potential role of ATP13A2 in senescence; however, the underlying mechanism is largely unknown.

In this paper, we provide the first evidence that *YPK9* deficiency leads to sensitivity to oxidative stress, increased intracellular reactive oxygen species (ROS) levels, abnormal mitochondrial function, increased incidence of early apoptosis and shortened replicative lifespan (RLS) in budding yeast. These observations highlight the involvement of Ypk9p in the oxidative stress response and modulation of RLS.

Materials and methods

Yeast strains and plasmids

All *S. cerevisiae* strains used in this paper (listed in Table 1) were derivatives of the wild-type BY4742 strain.

To generate a *YPK9* deletion strain, we used the plasmid pRS306 with a *URA3* selection marker as a template and amplified the deletion cassette by polymerase chain reaction (PCR) (Baudin et al. 1993). The primers were 5′-AGCCCAGACTTACTGATAGATCTTGCATATACTCCGGTAAGATTGTACTG.AGAGTGCAC-3′ and 5′ - CATGGTACTTGTACACATACATAGATAAAAATCTTTGCTCTGTGCGGTATTTCACACCG-3′. The PCR product was transformed into BY4742 yeast cells with the standard lithium acetate (LiAc) method, and then the *YPK9* gene sequence fragment was replaced via homologous recombination (Jamshad and Darby 2012). The transformants were selected on selective plates (SD-URA). The positive clones were confirmed by PCR.

To generate the *CTA1* overexpression plasmid pAUR123CTA1, the yeast *CTA1* ORF was amplified from wild-type yeast genomic DNA using PCR. The forward primer (5′-ATAGTCGACATGTCGAAATTGGGACAAGA-3′) contained a *Sal* I site, and the reverse primer (5′-CGCTCTAGATCAAAATTTGGAGTTACTCG-3′) contained a *Xba* I site (Zhao et al. 2018). The PCR products were

Table 1 The *S. cerevisiase* strains used in this study

Strain name	Genotype	Comments	Source
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Wild-type	Gift from Matt Kaeberlein
ypk9Δ	BY4742 *ypk9:URA3*	Deletion of *YPK9* in BY4742	This study
*ypk9Δ*pAUR123*CTA1*	BY4742 *ypk9:URA3CTA1OX*	pAUR123*CTA1* was transformed into *ypk9Δ*	This study

cloned into the empty plasmid pAUR123. The recombinant plasmid pAUR123CTA1 was transformed into the *E. coli* strain DH5α. DNA sequencing of the recombinant plasmids was performed by Sangon (Shanghai, China).

The plasmid pAUR123CTA1 was transformed into *ypk9Δ* yeast cells with the standard LiAc method to generate *ypk9Δ CTA1* overexpression yeast strain (*ypk9Δ CTA1* OX). The transformants were screened on YPD medium plates containing 0.2 g/ml aureobasidin A (AbA) at 30 °C for 2 days. The positive clones were confirmed by PCR.

Culture conditions

For all experiments, the strains were removed from storage at − 80 °C, thawed, inoculated in YPD solid medium, and incubated in a thermostatically controlled incubator at 30 °C for 2 to 3 days. YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose glucose was used for yeast cell culture. The solid YPD plates contained 2% (w/v) agar. The selective SD-URA plates contained 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, 2% (w/v) agar and 0.77 g/l URA dropout amino acid mixture.

Growth curve determination

The growth rates were detected with a Bioscreen C instrument (Growth Curves, USA). First, single colonies were inoculated into YPD medium and grown overnight at 30 °C with shaking. Second, the overnight cell cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.1 by dilution with YPD medium or H_2O_2-containing YPD medium in culture plates. The strains were then cultured at 30 °C with shaking, and the OD_{600} was automatically measured every 2 h for 2–3 days until cell growth plateaued (Jasnos et al. 2005). The results represent the averages from three separate experiments. The Friedman test was used for analysis, and a p value less than 0.05 was considered to indicate statistical significance.

Spot assay

Single colonies of the tested yeast strains were inoculated into YPD medium and grown overnight at 30 °C with shaking. The overnight cell cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.1. The cells were then diluted with sterile PBS in a fivefold series, and 5 µl of each dilution was spotted onto solid agar plates with or without the stress agent. The plates were incubated at 30 °C, and images were taken 48 h after plating.

Real-time quantitative PCR (RT-qPCR)

Briefly, exponential-phase cells were pelleted and washed twice with PBS after they had been treated with or without 3 mM H_2O_2 for 1 h. Total RNA was then extracted by using a Yeast RNA Kit (Omega Biotek, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using a FastKing RT Kit (with gDNase) (Tiangen, China), and RT-qPCR was performed in a LightCycler 480 instrument with the SYBR Green method. The relative expression of the target genes was quantified relative to the expression of the housekeeping gene *PRP8*. The gene-specific primers for RT-qPCR are listed in Tables 2 and 3. The results represent the averages from three independent experiments. Student's t test was used for analysis, and a p-value less than 0.05 was considered to indicate statistical significance.

Table 2 The real-time PCR primers used for studying oxidative stress response

Gene	Primers	Sequence
PRP8	Forward	TCATGGCTGCGTCTGAAGTA
	Reverse	GGCACCGTTATTAGCAGCAT
SOD1	Forward	AATCCGAGCCAACCACTGTC
	Reverse	CGACGCTTCTGCCTACAACG
SOD2	Forward	GCATTACACCAAGCACCAT
	Reverse	CTCGTCCAGACTGCCAAAC
CTA1	Forward	CCAACAGGACAGACCCATTC
	Reverse	TTACCCAAAACGCGGTAGAG
CTT1	Forward	GATTCCGTTCTACAAGCCAGAC
	Reverse	GGAGTATGGACATCCCAAGTTTC
GPX1	Forward	ATCCATTCCCCTTCAACTCC
	Reverse	TCCAGACTTCCCGCTTAC
GPX2	Forward	AAAAGCCAAAAAGCAGGTTTACT
	Reverse	CCAAGGACGATGGTTTTGTT
GPX3	Forward	TAAAGGGAAAAGTGGTGC
	Reverse	TTCATAATGGGGAAAGTCA
TRX2	Forward	AAAGTTTGCAGAACAATATTCTGACG
	Reverse	TTGGCACCGACGACTCTGGTAACC
MXR1	Forward	ACAGATTTTGCGGAGGTTTTAC
	Reverse	CCATTTTGGTTGCCATTCTT
TSA1	Forward	TCTTTTCGCCTCCACTGACT
	Reverse	CGATGATGAACAAACCTCTCAA
GLR1	Forward	CGAACACCAAGCATTACGATTA
	Reverse	GTAGCGAGGTCAGAAGCATACC
GSH1	Forward	GACACCGATGTGGAAACTGA
	Reverse	CCCTTTTTGGCATAGGATTG
GSH2	Forward	CACAGAGCAGGAAATAGCG
	Reverse	TTGGAGCCAGATAATTGAGT
YAP1	Forward	ATGATGTCGTTCCATCTAAGGAAGG
	Reverse	CAACCCCTCTTTCTGAACATTTTGC
SKN7	Forward	CCCGAGGAAAGACAGAGATGTA
	Reverse	CAAAAGAGACCCAGAAGGATTG

Table 3 The real-time PCR primers used for mitochondrial function assay

Gene	Primers	Sequence
PRP8	Forward	TCATGGCTGCGTCTGAAGTA
	Reverse	GGCACCGTTATTAGCAGCAT
ABF2	Forward	GCCCACATCTGCTTATTTC
	Reverse	GGTCCTGCTGGTTTCTTTG
ACO1	Forward	GTTGATGTTATGGCAGGTC
	Reverse	AGTAGCGGAGAAGGTGTC
ATP1	Forward	AAGCCGCTCCTCTACAAT
	Reverse	TCACCACCTTGGGTTTCA
HSP60	Forward	TACAAGACCAGCCAAGCA
	Reverse	GGCAACACCAGAAGCATC
ILV5	Forward	TGGTGTCCGTAAAGATGG
	Reverse	GAGAAACCGTGGGAGAAG
KGD1	Forward	GGCTCCTCCCAGTATCAG
	Reverse	TAGATTTCCCGTCCCTTG
KGD2	Forward	TGGTGCCATTGAAGGTGA
	Reverse	ACGGCTGTTTGTGGTGAA
LPD1	Forward	ACAGGTGCTCTTTCGTTA
	Reverse	GTTCTTGTCGTCGTTTCT
MGM101	Forward	ACTCTAAACTCGGCGGGA
	Reverse	CGCCTCTTGCCACACTGA
MIP1	Forward	TGGTAGTTGCGGTGGTAA
	Reverse	CGTGATGAGCGTCTTCTT
RPO40	Forward	CCAGAATCGGTTCGGTAT
	Reverse	ATTGTGGTTGGACAGAGG

Yeast petite mutation assay

Petite mutants were identified by the 2,3,5-triphenyltetrazolium chloride (TTC) overlay technique. First, exponential-phase cells were harvested after they had been treated with or without 3 mM H_2O_2 for 1 h. An aliquot was diluted in PBS and spread onto YPD plates. The plates were incubated at 30 °C for 2 days until colonies formed and then overlain with TTC agar. In this assay, yeast colonies formed by respiration-deficient yeast mutants were white, whereas colonies formed by normal yeast were red (Cho et al. 1998; Laskowski 1954).

Detection of generated ROS

The 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Beyotime, China) method was used to measure intracellular ROS levels. When DCFH-DA is taken up by cells, it is de-esterified into the ionized free acid DCFH. DCFH is then further oxidized into fluorescent DCF by intracellular ROS (Eruslanov and Kusmartsev 2010). For the ROS generation assay, exponential-phase cells were harvested and washed twice with PBS after they had been treated with or without 3 mM H_2O_2 for 1 h. Then, the cells were

incubated with 5 M DCFH-DA at 30 °C in the dark for 1 h. The cells were again harvested and washed three times with precooled PBS to remove free reagent, and the mean green fluorescence intensity was evaluated by flow cytometry (BD FACSCanto II, USA). The results represent the averages from three separate experiments. Student's t test was used for analysis, and a p value less than 0.05 was considered to indicate statistical significance.

Detection of MMP

Rhodamine 123 (Rh123) (Beyotime, China) was used as a fluorescent probe to detect MMP (Ludovico et al. 2001). Briefly, yeast cells in the exponential phase (treated with or without 3 mM H_2O_2 for 1 h) were harvested, washed three times with PBS, and then incubated in liquid YPD medium containing 10 μM Rh123 for 60 min at 30 °C in the dark. Next, the cells were washed three times with PBS, and the fluorescence intensity was analyzed using a flow cytometer (BD FACSCanto II, USA). The results represent the averages from three separate experiments. Student's t test was used for analysis, and a p value less than 0.05 was considered to indicate statistical significance.

Apoptotic marker assay

Apoptosis was measured using fluorescein isothiocyanate (FITC)-annexin V and caspase-3 activity assays as previously described.

Exposure of phosphatidylserine on the outer cell surface is an early event in apoptosis. Phosphatidylserine normally resides in the inner leaflets of cells facing the cytoplasm but is rapidly translocated to the outer leaflets of the cells facing the surface at the early stage of apoptosis. Surface exposure to phosphatidylserine can be specifically detected by staining with fluorescent FITC-labeled annexin V (Herker et al. 2004).

Briefly, yeast cells in the exponential phase (treated with or without 3 mM H_2O_2 for 1 h) were harvested, and the cell walls were then digested with zymolyase in a buffer containing 0.5 mM $MgCl_2$, 1.2 M sorbitol, and 35 mM H_3PO_4 at pH 6.8 and 30 °C. After the cell walls were digested, the cells were washed with PBS. Next, the protoplasts were resuspended in 500 μl of annexin V binding solution, incubated with FITC-conjugated Annexin V for 15 min at room temperature, and then analyzed by flow cytometry (BD FACSCanto II, USA).

Caspase-3 is a key enzyme in apoptosis (Nicholson et al. 1995; Qi et al. 2020). The activity of yeast caspase-3 was determined as described previously (Rona et al. 2015) with a Caspase 3 Activity Assay Kit (Beyotime, China) Briefly, yeast cells in the exponential phase (treated with or without 3 mM H_2O_2 for 1 h) were harvested and then resuspended in cell lysis buffer (50 mM Tris–HCl, pH 8.0; 50 mM KCl; 2 M citrate; 10% glycerol; and 1 mM PMSF). The cells were then broken with glass beads by vigorous shaking, and the supernatants were used to determine caspase-3 activity according to the manufacturer's instructions. All experiments were performed at 4 °C.

The results represent the averages from three separate experiments, Student's t tests were used for analysis, and a p value less than 0.05 was considered to indicate statistical significance.

Catalase activity assay

To determine the intracellular catalase activity, a single yeast colony was inoculated into 5 ml of YPD and grown to the exponential phase at 30 °C. Next, the yeast cells were harvested and suspended in precooled RIPA lysis buffer containing protease inhibitors. The cells were then broken with glass beads by vigorous shaking, and the supernatant was collected by centrifugation at 4 °C. The protein concentration was determined using a Bradford Protein Assay Kit (Beyotime, China). Catalase activity was quantified using commercial assay kits (Beyotime, China).

Determination of RLS

The RLS assay was performed as previously described to count the total number of daughter cells generated by individual mother cells by using an optical microscope equipped with a micromanipulator (Postnikoff and Harkness 2014). All lifespan experiments were carried out on YPD plates. Statistical significance was calculated with the Wilcoxon rank-sum test, and $p < 0.05$ was considered to indicate statistical significance.

DAPI staining

Staining of exponential phase cells by 4′,6-diamidino-2-phenilindole (DAPI) was carried out as follows. Cells were fixed with 70% (v/v) ethanol for 30 min at room temperature. Then, the cells were washed once with NS buffer (20 mM Tris–HCl pH 7.6, 0.25 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 0.1 mM $CaCl_2$, 0.8 mM PMSF, 0.05% 2-mercaptoethanol) and resuspended in NS buffer containing 0.5 µg/ml DAPI for 5 min (Massardo et al. 2000). The cells were then dropped on a glass slide, squashed lightly, and observed under UV excitation (Leica DMi8, Germany).

Results and discussion

ypk9Δ yeast cells are sensitive to H_2O_2

The generation of yeast mutants knocked out for a certain gene and their employment to explore the response to distinct environmental stresses constitute an ideal methodology for discovering gene functions (Santos and Riezman 2012). H_2O_2 - is a widely used radical-generating reagent that can elicit harmful effects on cells, including enhanced DNA damage, oxidative stress and aging (Janero et al. 1991; Popa et al. 2010).

To explore the potential role of *YPK9* in the oxidative stress response, the *ypk9Δ* mutant was generated by PCR-mediated gene disruption. Specifically, the *YPK9* gene was replaced with the selectable marker *URA3* in the haploid BY4742 background. Then, the *ypk9Δ* mutant was subjected to oxidative stress induced by H_2O_2. We found that there was no significant difference between the wild-type strain and the *ypk9Δ* strain under unstressed conditions. When stressed with 3 mM H_2O_2, the *ypk9Δ* strain exhibited obvious growth defects, and the growth defects were more severe under the high (3.5 mM) H_2O_2 concentration (Fig. 1A). The hypersensitivity to H_2O_2 of the *ypk9Δ* cells was further confirmed by the results of the spot assay, the *ypk9Δ* cells exhibited defective growth compared with wild-type cells (Fig. 1B). In addition, the *ypk9Δ* cells also exhibited hypersensitivity to oxidative stressor tert-butyl hydroperoxide (TBHP) and cube hydroperoxide (CHP) (data not shown). These observations strongly suggest a role of *YPK9* in the oxidative stress response.

YPK9 deficiency induces changes in the mRNA expression levels of oxidative stress-related genes

The growth defects of the *ypk9Δ* strain under H_2O_2-stressed conditions implied that the intracellular oxidation and antioxidation systems were imbalanced. Therefore, we monitored the changes in the expression patterns of oxidative stress-related genes in the *ypk9Δ* strain under H_2O_2-stressed and unstressed conditions (Jamieson 1998). These genes included superoxide dismutase genes (*SOD1*, *SOD2*), catalase genes (*CTA1*, *CTT1*), glutathione peroxidase genes (*GPX1*, *GPX2*, *GPX3*), the thioredoxin-2 gene (*TRX2*), a methionine-s-sulfoxide reductase gene (*MXR1*), a thioredoxin peroxidase gene (*TSA1*), a cytoplasmic and mitochondrial glutathione oxidoreductase gene (*GLR1*), glutathione synthase genes (*GSH1*, *GSH2*), and transcription factor genes (*YAP1* and *SKN7*).

The mRNA expression levels of *TRX2, MXR1, YAP1, CTA1,* and *GPX1* were decreased, while those of *GSH1* and *GPX2* were increased in *ypk9Δ* cells compared to wild-type control cells under unstressed conditions. After stress with 3 mM H_2O_2, most of the investigated genes were upregulated in the *ypk9Δ* strain, such as *GPX3*, *TSA1*, *GSH1*, *SKN7*, *CTA1*, *GLR1*, *GSH1*, *SOD1*, *GPX2, CTT1*, and *SOD2* (Fig. 2). We hypothesize that the upregulation of antioxidative gene expression in *ypk9Δ* cells under stressed conditions may be a compensatory response to oxidative stress, suggesting that the cellular redox status is altered in *ypk9Δ* cells.

Notably, the catalase-encoding gene *CTA1*, which can catalyze the degradation of H_2O_2 in cells into H_2O and O_2 (Jamieson 1998), was downregulated in the *ypk9Δ* strain compared to the wild-type control strain under unstressed conditions, implying a relationship between *YPK9* and *CTA1* in the oxidative stress response.

In addition, we found that *MXR1*, a methionine-s-sulfoxide reductase involved in the oxidative stress response and RLS regulation (Kaya et al. 2010; Moskovitz et al. 1997), was also downregulated in the *ypk9Δ* strain compared to the wild-type control strain under unstressed conditions. Interestingly, a synthetic genetic array analysis showed that *MXR1* has

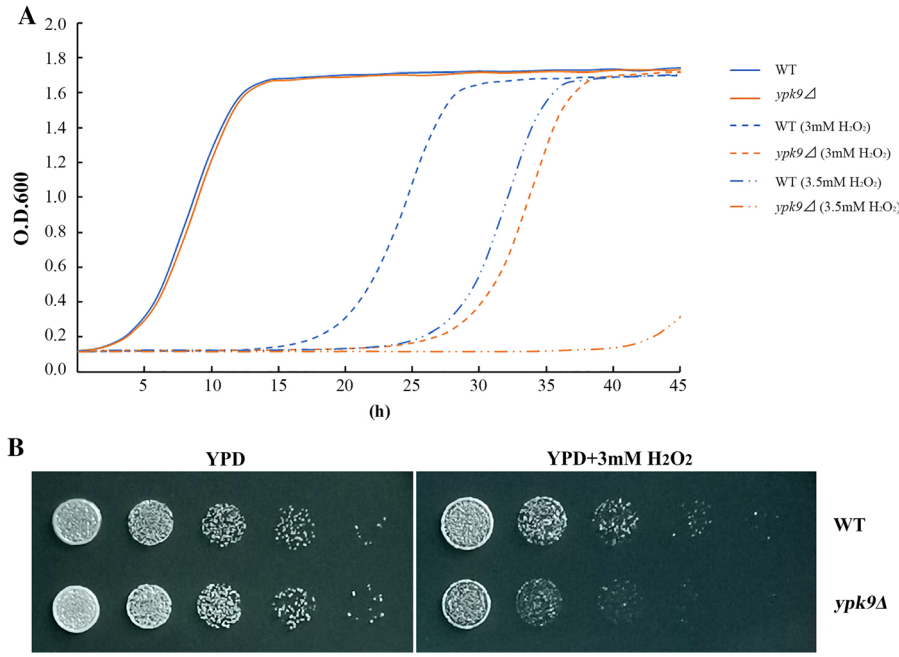

Fig. 1 *ypk9*-deleted yeast cells are sensitive to H_2O_2. The growth curves (**A**) of the wild-type strain and *ypk9Δ* strain were assessed with a Bioscreen C machine under 3 mM H_2O_2 and 3.5 mM H_2O_2 stressed and unstressed conditions. The results represent the averages from three separate experiments, and a *p*-value less than 0.05 was considered to indicate statistical significance. (**B**) Wild-type and *ypk9Δ* cells were fivefold serially diluted, spotted onto YPD plates (left) and YPD plates containing 3 mM H_2O_2 (right) and then incubated at 30 °C until colonies formed

a negative genetic interaction with *YPK9* (Costanzo et al. 2016), and our observation highlights the potential role of *YPK9* in RLS regulation.

YPK9 deficiency increases ROS levels, decreases MMP and results in a high rate of respiration-deficient mutants under H_2O_2 treatment

H_2O_2 can induce the production of intracellular ROS that subsequently attack cellular components, including lipids, proteins, and DNA (Janero et al. 1991; Popa et al. 2010). Therefore, we measured the intracellular ROS levels by the DCFH-DA method. As expected, we found that ROS levels were significantly higher in *ypk9Δ* cells than in wild-type cells under both unstressed and stressed conditions, indicating the occurrence of intracellular oxidative stress in *ypk9Δ* cells (Fig. 3A).

Intracellular ROS can cause damage to DNA, especially mitochondrial DNA (mtDNA) (Kurihara et al. 2012). Damage to mtDNA causes electron transport chain (ETC) dysfunction, generates oxidative stress, impairs MMP regulation and leads to abnormal MMP (Lenaz and Genova 2012).

Given the significant increases in intracellular ROS levels, we next determined whether *YPK9* deficiency also affected mitochondrial function. We observed that MMP was not significantly different between wild-type cells and *ypk9Δ* cells under unstressed conditions; however, after stress with H_2O_2, MMP of the *ypk9Δ* yeast strain was lower than that of the wild-type control strain (Fig. 3B).

Generally, under normal physiological conditions, the mitochondrial respiratory system maintains MMP at an adequate level to guarantee intracellular ATP synthesis (Larsen et al. 2012; Moon et al. 2016). The decrease in MMP suggested that mitochondrial respiratory dysfunction occurred in *ypk9Δ* yeast cells. Therefore, the proportions of respiration-deficient petite cells (result from the loss/mutation of nuclear-encoded proteins, which are essential for the mitochondrial respiration capacity, or from mtDNA mutation or loss) were determined by the TTC overlay technique. Respiring colonies could reduce TTC to a

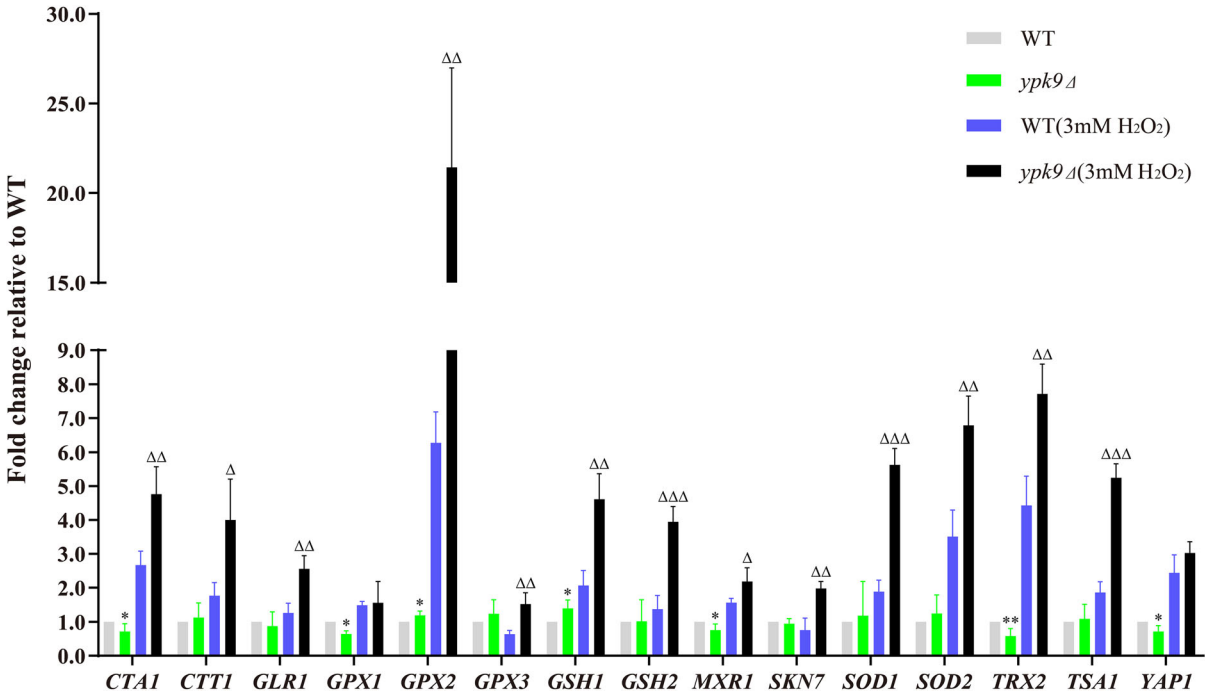

Fig. 2 Oxidative stress-related gene expression pattern of *ypk9Δ* yeast cells. The relative mRNA expression levels of *SOD1*, *SOD2*, *CTA1*, *CTT1*, *GPX1*, *GPX2*, *GPX3*, *TRX2*, *MXR1*, *TSA1*, *GLR1*, *GSH1*, *GSH2*, *YAP1* and *SKN7* in the wild-type strain and *ypk9Δ* strain were measured by RT-qPCR under 3 mM H_2O_2-stressed and unstressed conditions. All data are expressed as the fold-changes relative to the levels in unstressed wild-type cells, which were set to 1. Differences between groups were analyzed by Student's t test, and a *p*-value less than 0.05 was considered to indicate statistical significance. $*p < 0.05$, unstressed *ypk9Δ vs.* unstressed wild-type; $**p < 0.01$, unstressed *ypk9Δ vs.* unstressed wild-type; Δ indicates $p < 0.05$, stressed *ypk9Δ vs.* stressed wild-type; ΔΔ indicates $p < 0.01$, stressed *ypk9Δ vs.* stressed wild-type; ΔΔΔ indicates $p < 0.001$, stressed *ypk9Δ vs.* stressed wild-type

red color, while those with reduced respiratory function are unable to reduce TTC and remain white.

We found that under unstressed conditions, the proportion of petite yeast cells did not differ between the wild-type yeast strain and the *ypk9Δ* yeast strain. However, after stress with 3 mM H_2O_2, the ratio of petite yeast cells in the *ypk9Δ* yeast strain was significantly greater than that in the wild-type control strain (Fig. 3C), suggesting that mitochondrial respiratory dysfunction was serious under stressed conditions in *ypk9Δ* yeast cells.

There are two kinds of specific types of petite cells: rho^0 cells, where the mtDNA has been completely lost, and rho^- cells, where there are one or more mutations in the mtDNA that prevent respiratory metabolism, but some residual mtDNA remains in the cell (Ferguson and von Borstel 1992). Therefore, we further determined that the petite mutants of *ypk9Δ* are rho^0 or rho^- by the DAPI staining method. Four randomly selected petite colonies (white colonies on the TTC agar plate) of *ypk9Δ* cells were cultivated in YPD medium to the exponential phase, and the cells were harvested for DAPI staining as previously described (Massardo et al. 2000).

We found no obvious mitochondrial bodies in these *ypk9Δ* petite cells (only a few cells could be stained with one or two mitochondrial bodies, and these mitochondrial bodies had very weak fluorescence), while obvious mitochondrial bodies could be detected in the wild-type and *ypk9Δ* cells (red colonies on the TTC agar plate), and the mitochondrial DNA appeared as small beads on strings, as previously described (Massardo et al. 2000). We note that the petite cells of the wild-type were stained with more mitochondrial bodies than *ypk9* petite cells (Fig. 3D). In addition, we extracted the total genomic DNA of the 4 *ypk9* petite cells and used gene-specific PCR primers (5′-AGAAGTGTAGTAAGAGGT-3′ and 5′-TCAATAG-TAGAGATAACAGG-3′) to amplify the fragment (194 bp) of the *CYTB* gene, which is located in

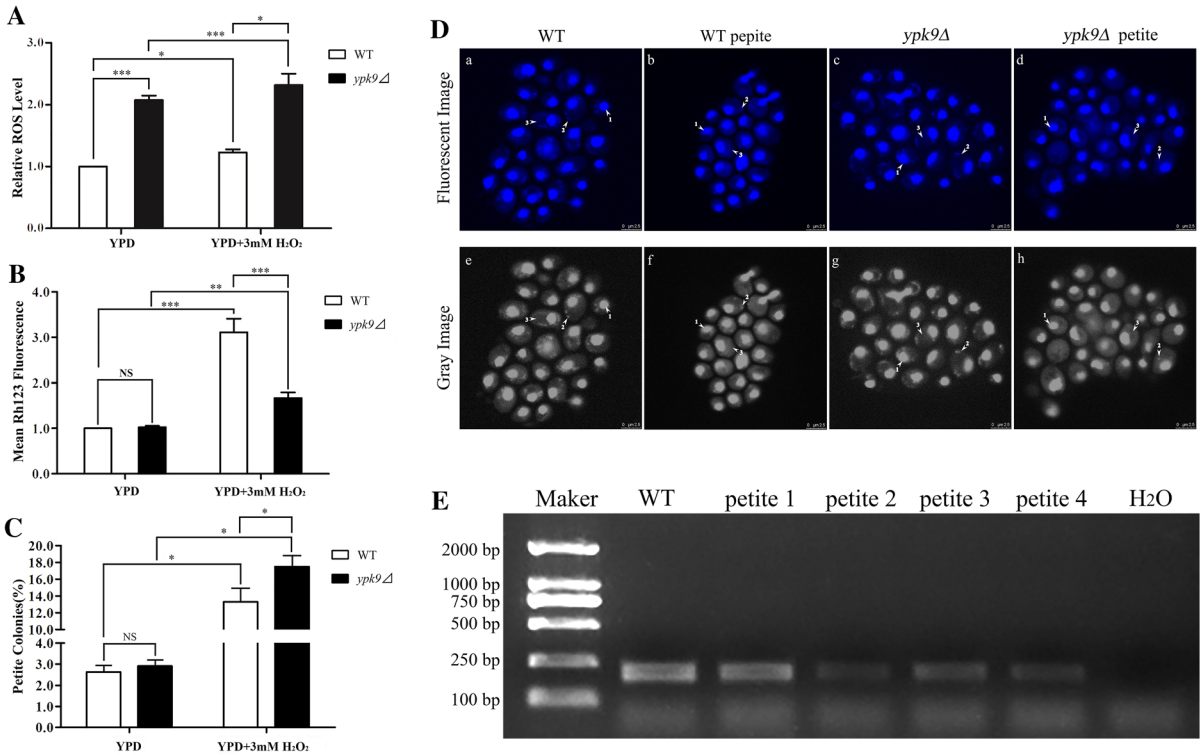

Fig. 3 Increased ROS production and (**A**) decreased MMP (**B**) in *ypk9Δ* yeast cells. Wild-type yeast cells and *ypk9Δ* yeast cells in the exponential phase were stressed or not stressed with 3 mM H_2O_2 for 1 h and then subjected to ROS or MMP assays. The results are shown as the mean relative fluorescence intensities from three separate experiments. *ypk9Δ* yeast cells formed more petite colonies than wild-type when treated with 3 mM H_2O_2 (**C**). Differences between groups were analyzed by Student's t test, and a p value greater than 0.05 was considered to indicate statistical significance. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. (**D**) Mitochondrial and nuclear DNA stained with DAPI. 1, indicates nuclear DNA, 2, indicates Mitochondrial DNA, 3, indicates cytoplasm. **a**–**d**: fluorescent image; **e**–**h**: gray image. A bar represents 2.5 μM. (**E**) The electrophoretogram of the PCR product of CYTB gene

the mitochondrial genome of yeast (Tzagoloff et al. 1976). The electrophoretogram of the PCR product showed that all 4 *ypk9Δ* petite cells had the specific expected product of wild-type (Fig. 3 E). Both the DAPI staining and PCR results indicated that the *ypk9Δ* petite cells were rho^- not rho^0. These findings raise the possibility that *YPK9* is involved in stabilizing mitochondria.

In budding yeast, mtDNA is packaged into protein–DNA complexes that are called mitochondrial nucleoids (mt-nucleoids) (Chen and Butow 2005). More than 20 proteins have been identified as potential components of mt-nucleoids in yeast. These proteins play crucial roles in mitochondrial organization and maintenance. For example, *ABF2* deficiency leads to an unstable mitochondrial genome and hypersensitivity to damage from endogenously produced free radicals (Diffley and Stillman 1991; O'Rourke et al. 2002). Therefore, we measured the mRNA expression levels of eleven canonical component proteins of mt-nucleoids (Chen and Butow 2005). The results revealed that most of the genes studied, including genes involved in mtDNA packaging and protection (*ABF2*), mtDNA assembly (*ILV5*), mtDNA stability (*ACO1* and *HSP60*), citric acid cycle (*KGD1*), ATP synthesis (*ATP1*), mtDNA replication (*MIP1*), and mtDNA transcription (*RPO41*), were downregulated in *YPK9*-deficient cells under unstressed conditions (Fig. 4). We speculate that the downregulated mt-nucleoid gene expression pattern may be another reason for the observed high rates of respiration-deficient petite cells in the *ypk9Δ* yeast strain under stressed conditions, although we do not know why these mt-nucleoid genes were differentially expressed in *YPK9*-deficient cells.

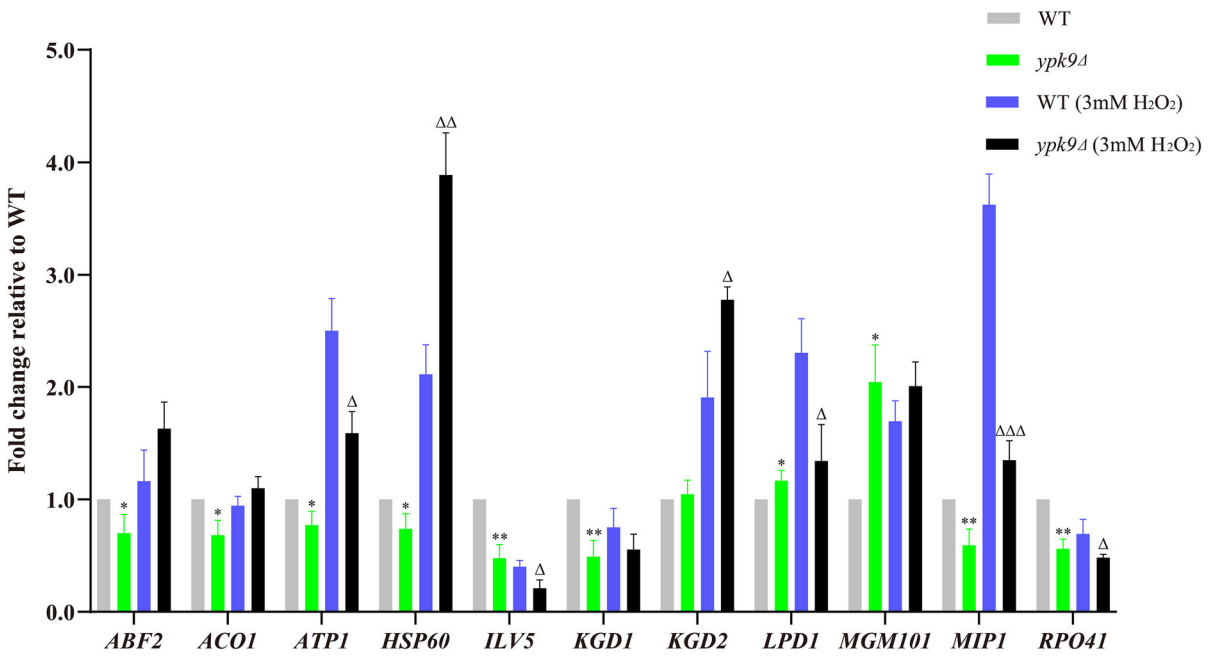

Fig. 4 Mt-nucleoid genes were differentially expressed in *ypk9*-deficient cells. The relative mRNA expression levels of *ABF2*, *ACO1*, *ATP1*, *HSP60*, *ILV5*, *KGD1*, *KGD2*, *LPD1*, *MGM101*, *MIP1* and *RPO41* in the wild-type strain and *ypk9Δ* strain were measured by RT-qPCR under 3 mM H_2O_2-stressed and unstressed conditions. All data are expressed as the fold-changes relative to the levels in unstressed wild-type cells, which were set to 1. Differences between groups were analyzed by Student's t test, and a *p*-value less than 0.05 was considered to indicate statistical significance. $^*p < 0.05$, unstressed *ypk9Δ vs.* unstressed wild-type; $^{**}p < 0.01$, unstressed *ypk9Δ vs.* unstressed wild-type; Δ indicates $p < 0.05$, stressed *ypk9Δ vs.* stressed wild-type; ΔΔ indicates $p < 0.01$, stressed *ypk9Δ vs.* stressed wild-type; ΔΔΔ indicates $p < 0.001$, stressed *ypk9Δ vs.* stressed wild-type

YPK9 deficiency induces early apoptosis in yeast

Cells undergoing apoptosis always exhibit a decrease in MMP, which precedes nuclear signs of apoptosis (Dai et al. 2009; Fehrmann et al. 2013). Given the observed decrease in MMP, apoptosis was evaluated by FITC-labeled annexin V staining and caspase-3 activity assays.

The exposure of phosphatidylserine at the outer cell surface is an early event in apoptosis that is remarkably conserved from yeast to mammals and can be specifically detected by staining with FITC-labeled annexin V (Herker et al. 2004). As expected, the mean FITC fluorescence intensity was stronger in *ypk9Δ* cells than in wild-type control cells under both unstressed and stressed conditions (Fig. 5A).

In addition, caspase-3, one of the most important caspases, plays a central role in mediating nuclear apoptosis, and its activation is a hallmark and early marker of apoptosis. *S. cerevisiae* contains a metacaspase (encoded by *YCA1* gene) that is similar to mammalian metacaspases (Guaragnella et al. 2006; Uren et al. 2000). As expected, caspase activity was also increased in *ypk9Δ* yeast cells under both unstressed and stressed conditions (Fig. 5B).

As mentioned above, the observed overproduction of intracellular ROS may have been a causal factor for mitochondrial dysregulation and apoptosis in *ypk9Δ* cells. Previous research has reported that inhibition of Na^+/K^+-ATPase (belonging to the P2-type ATPase subfamilies) with ouabain can result in increased ROS levels in cardiac cells through a mechanism that may be mediated by Ras and p42/44 mitogen-activated protein kinases (MAPKs) (Xie et al. 1999). Activation of Na^+/K^+-ATPase attenuates high glucose-induced H9c2 cell apoptosis by suppressing ROS accumulation and MAPKs (Yan et al. 2016). However, based on sequence homology and structural organization analysis, Ypk9p is similar to P5-type ATPases but not similar to Na^+/K^+-ATPase (Kühlbrandt 2004; Palmgren and Nissen 2011).

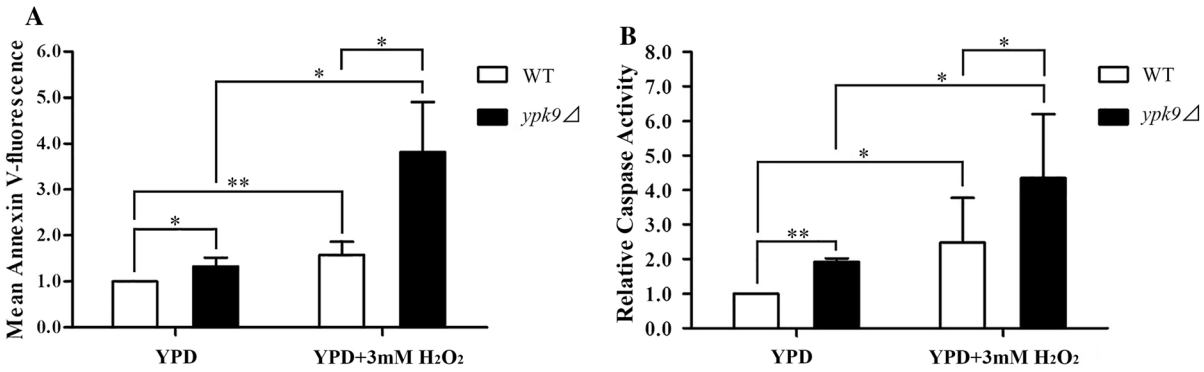

Fig. 5 *YPK9* deficiency leads to early apoptosis. wild-type yeast cells and *ypk9Δ* yeast cells in the exponential phase were stressed or not stressed with 3 mM H_2O_2 for 1 h. The cell walls were then digested, and the cells were stained with FITC-labeled annexin V for apoptosis assessment (**A**). Total yeast protein was extracted and subjected to a caspase activity assay (**B**). Differences between groups were analyzed by Student's t test, and a p value less than 0.05 was considered to indicate statistical significance. $^*p < 0.05$, and $^{**}p < 0.01$

YPK9 deficiency leads to shortened RLS

Accumulating evidence suggests that there is crosstalk between apoptosis and aging in model organisms, including *Drosophila melanogaster* (*D. melanogaster*), nematodes and *S. cerevisiae* (Laun et al. 2008). For example, caloric restriction (CR), a method of lifespan elongation that is effective in many model organisms for aging research, can attenuate age-related apoptosis and extend lifespan (Zhang and Herman 2002). However, the relationship between apoptosis and lifespan remains unclear.

Budding yeast has two different paradigms of aging: RLS, which is defined as the number of divisions an individual yeast cell undergoes before dying (Jazwinski 1990), and chronological life span (CLS), which is defined as the length of time a population of yeast cells remains viable in a nondividing state following nutrient deprivation (MacLean et al. 2001).

Given the observed early apoptosis of *ypk9Δ* yeast cells, we monitored RLS and found that the RLS of the *ypk9Δ* yeast strain (mean RLS = 15, cells = 160) was 32% shorter than that of the wild-type yeast strain (mean RLS = 22, cells = 80) ($p < 0.05$) (Fig. 6).

At least 17 proapoptotic genes and 4 antiapoptotic genes have been reported thus far in budding yeast. Deletion or overexpression of these genes could impact CLS or RLS. The majority of related studies have focused on the connection between apoptosis and chronological aging, and the impact of deleting just a few genes on RLS has been examined (Laun et al. 2012; MacLean et al. 2001; Rockenfeller and Madeo 2008). For example, previous studies have reported that deletion of the antiapoptotic gene *MMI1* results in increased RLS (Rinnerthaler et al. 2006). In addition, deletion of *DNM1* can extend RLS, possibly by increasing cellular resistance to apoptosis (Scheckhuber et al. 2007). However, very few papers have been published that deal explicitly with the relationship between aging and apoptosis in single-celled organisms, and whether apoptosis or related processes are causes or consequences of aging still needs further research and discussion. On the other hand, apoptosis is well accepted as an anti-aging mechanism in multicellular animals because it can remove damaged cells from tissues and is critical for tissue repair and tissue homeostasis (Schmitt et al. 2007).

Overexpression of *CTA1* can reverse the phenotypic abnormalities of the *ypk9Δ* yeast strain

As mentioned in the previous section, the catalase-encoding gene *CTA1* was downregulated in the *ypk9Δ* strain compared to the wild-type control strain under unstressed conditions. There are two kinds of catalases in budding yeast that catalyze the intracellular degradation of H_2O_2 into H_2O and O_2: peroxisomal/mitochondrial catalase A (Cta1p) and cytosolic catalase T (Ctt1p) (Alugoju et al. 2020). Both *CTA1* and *CTT1* are involved in the oxidative stress response and longevity of *S. cerevisiae* (Mesquita et al. 2010).

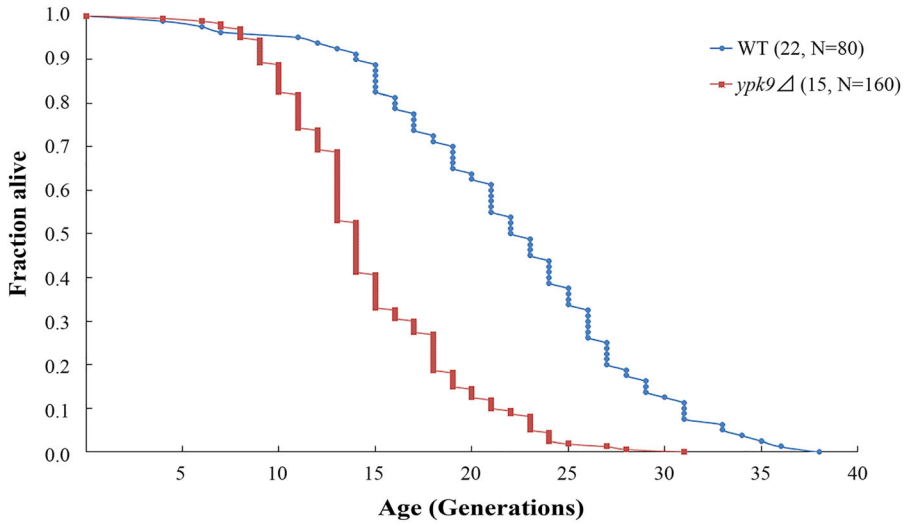

Fig. 6 *YPK9* deficiency leads to shortened RLS. The mean RLS (under 2% glucose conditions) and total number of daughter cells are shown in parentheses; the total number of daughter cells is denoted by "N". The values were analyzed for statistical significance using the Wilcoxon rank-sum test. $p < 0.05$ was considered to indicate statistical significance

We generated the *ypk9Δ CTA1* OX strain (in which *CTA1* was overexpressed by using a high-copy-number vector, and both RT-qPCR (Fig. 7A) and catalase activity assays (Fig. 7B) confirmed that *CTA1* was overexpressed in *ypk9Δ* yeast cells) and investigated whether overexpression of *CTA1* could eliminate defects associated with *YPK9* deficiency.

We obtained several findings. ①There were no significant differences between the *ypk9Δ CTA1* OX and *ypk9Δ* strains under unstressed conditions, but under H_2O_2-stressed conditions, *ypk9Δ CTA1* OX cells grew faster than *ypk9Δ* cells (Fig. 7C, D). ② The intracellular ROS levels were lower in *ypk9Δ CTA1* OX cells than in *ypk9Δ* cells under both H_2O_2-stressed conditions and unstressed conditions (Fig. 7E). ③ MMP was higher in *ypk9Δ CTA1* OX yeast cells than in *ypk9Δ* yeast cells under both H_2O_2-stressed conditions and unstressed conditions (Fig. 7F). ④ Caspase activity (Fig. 7G) and FITC-labeled annexin V fluorescence intensity (Fig. 7H), which are early markers of apoptosis, were lower in *ypk9Δ CTA1* OX cells than in *ypk9Δ* cells under both H_2O_2-stressed conditions and unstressed conditions. ⑤ More importantly, the RLS of *ypk9Δ CTA1* OX cells was obviously higher than that of *ypk9Δ* cells and even reached a level similar to that of wild-type cells (Fig. 7I). These findings strongly suggest that overexpression of *CTA1* can eliminate defects associated with *YPK9* deficiency.

Notably, previous research has reported that *CTA1* deletion does not influence the lifespan of wild-type yeast (W303a strain) grown on glucose but decreases the lifespan of wild-type yeast grown on ethanol (Van Zandycke et al. 2002). In contrast, our observations suggest that downregulation of *CTA1* expression may play a role in shortening RLS in *ypk9Δ* cells grown on glucose and imply the existence of an interaction between *CTA1* and *YPK9*.

CR increased the RLS of *ypk9Δ*

Previous reports have suggested that CR could extend yeast RLS by increasing the rate of yeast mitochondrial respiration (Lin et al. 2002; Schleit et al. 2013). In yeast, mitochondrial function is important for both RLS and CLS (Delaney et al. 2013). CR-mediated pathways could influence mitochondrial segregation and inheritance, nuclear-mitochondrial communication, and mitochondrial quality control, ultimately affecting RLS (Ruetenik and Barrientos 2015).

Altered mitochondrial metabolism is one hallmark of aging (Lin and Beal 2006). The aging process is always accompanied by decreased efficiency of the mitochondrial respiratory chain, reduced MMP and ATP generation, and increased electron leakage (Amigo et al. 2016). Since we observed that *YPK9* deficiency leads to mitochondrial dysfunction,

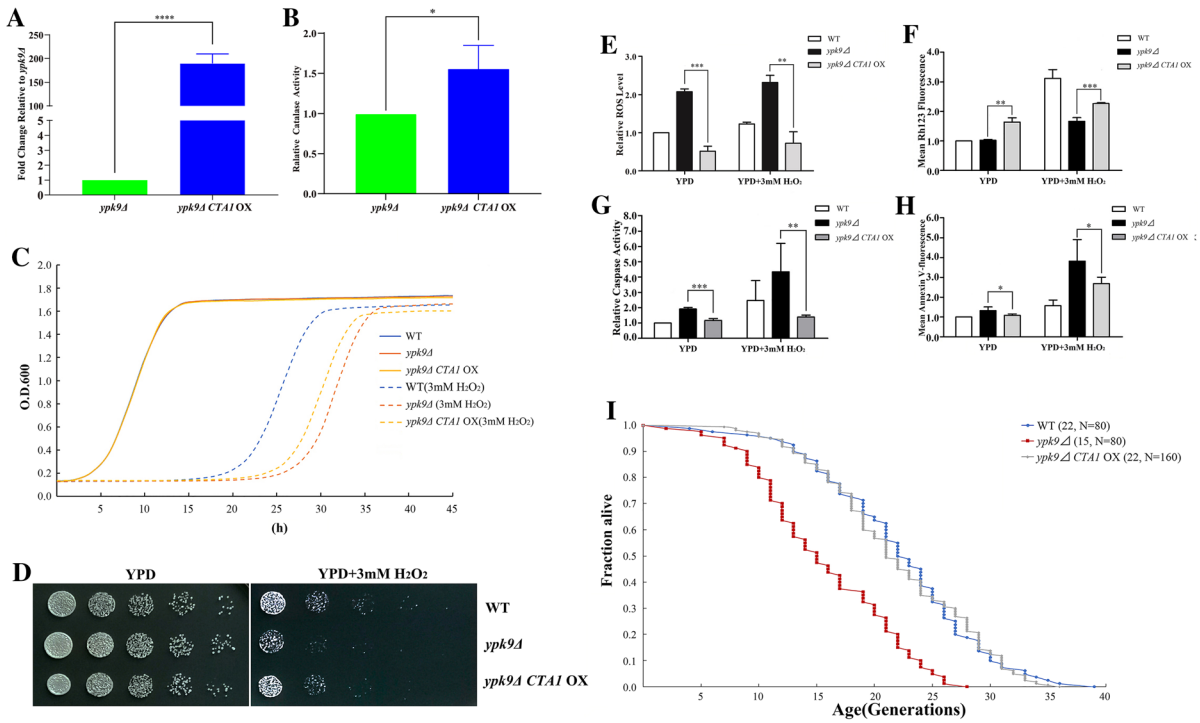

Fig. 7 Overexpression of *CTA1* eliminates the phenotype associated with *YPK9* deficiency. (**A**) RT-qPCR and (**B**) catalase activity assays confirmed that *CTA1* was overexpressed in *ypk9Δ* yeast cells. (**C**) The growth curves of the wild-type strain, the *ypk9Δ* strain and the *ypk9Δ CTA1* OX strain were assessed with a Bioscreen C machine under H_2O_2-stressed and unstressed conditions. The results represent the averages from three separate experiments. The Friedman test was applied for statistical significance analysis, and a *p*-value less than 0.05 was considered to indicate statistical significance. (**D**) The exponential-phase wild-type, *ypk9Δ*, and *ypk9Δ CTA1* OX cells were fivefold serially diluted with PBS and were spotted onto YPD or H_2O_2-added solid YPD plates, which were kept at 30 °C until colonies formed. Wild-type cells, *ypk9Δ* cells and *ypk9Δ CTA1* OX cells were stressed or not stressed with 3 mM H_2O_2 for 1 h and then subjected to a ROS assay (**E**), an MMP assay (**F**), a caspase activity assay (**G**) and a FITC-labeled annexin V apoptosis assay (**H**). The results are displayed as the mean ± SEM value. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$. (**I**) Overexpression of *CTA1* restores the RLS of the *ypk9Δ* strain. The mean RLS and total number of daughter cells are shown in parentheses; the total number of daughter cells is denoted by "N". The Wilcoxon rank-sum test was applied for statistical significance analysis, and $p < 0.05$ was considered to indicate statistical significance

including an abnormally expressed mt-nucleoid gene profile, decreased MMP and a high rate of respiration-deficient petite rate under H_2O_2 treatment, we wished to determine whether CR could extend the RLS of *ypk9Δ* cells.

Glycerol is a widely used dietary supplement capable of mimicking CR by shifting metabolism away from glycolysis and toward oxidative phosphorylation (Wei et al. 2009). Given this, we determined the RLS of *ypk9Δ* under the CR conditions (3% glycerol). We found that the RLS of *ypk9Δ* yeast cells was 19 generations under the CR conditions and was 26.7% higher than the RLS under standard conditions (2% glucose), while, the RLS of wild-type BY4742 cells (23 generations) was not obviously changed under the 3% glycerol conditions (Schleit et al. 2013) (Fig. 8). This result suggested that the shortened RLS of *ypk9Δ* cells with dysfunctional mitochondria could be rescued by CR. This observation was in accord with previous studies (Schleit et al. 2013) that growth on the nonfermentable carbon source, 3% glycerol, could extend the RLS of *phbΔ* (with mitochondrial dysfunction and decreased RLS under 2% glucose conditions).

In conclusion, we have demonstrated, for the first time, that *YPK9* deficiency leads to oxidative stress accompanied by abnormal mitochondrial function and an increased incidence of early apoptosis in budding yeast, which may have contributed to the observed shortened RLS of *ypk9Δ* yeast in this study. More

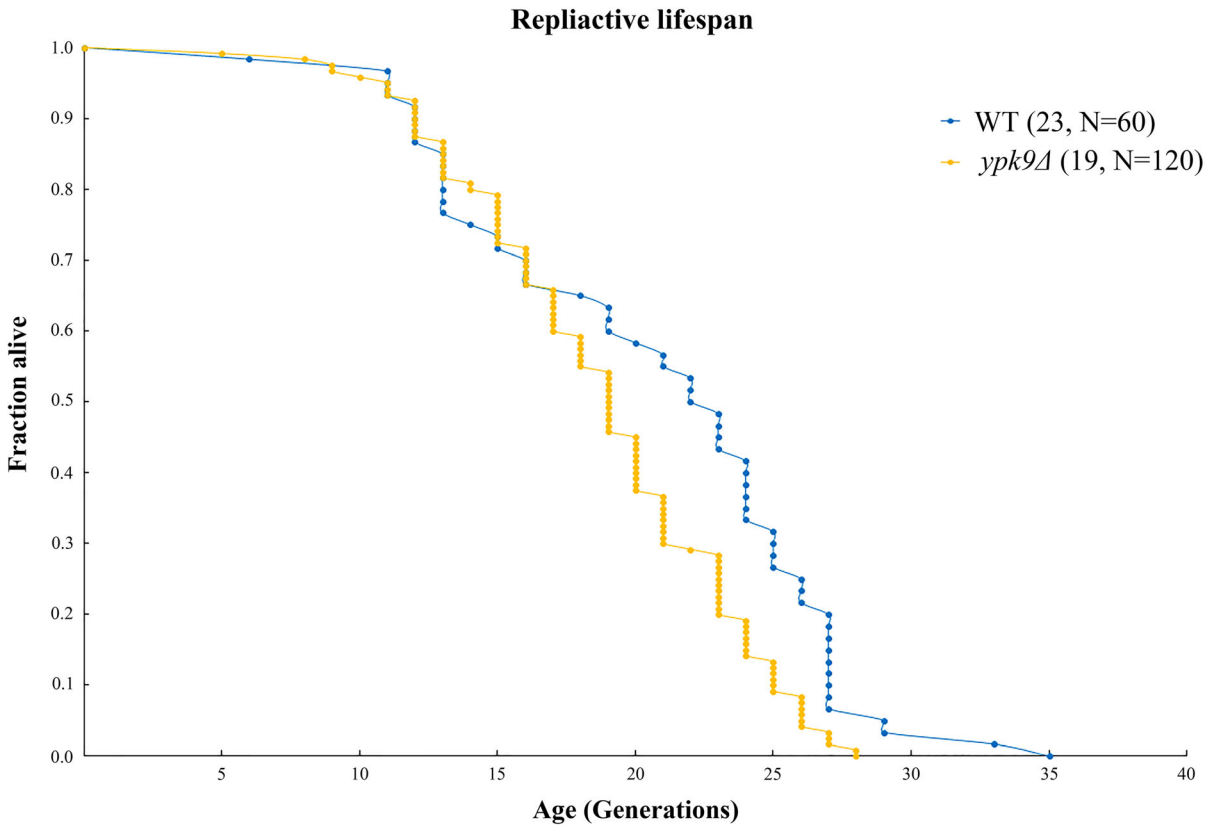

Fig. 8 CR increased the RLS of *ypk9Δ*. The mean RLS (under 3% glycerol conditions) and total number of daughter cells are shown in parentheses; the total number of daughter cells is denoted by "N". The values were analyzed for statistical significance using the Wilcoxon rank-sum test. $p < 0.05$ was considered to indicate statistical significance

importantly, we found that these senescence-related phenotypes can be eliminated by overexpression of the catalase-encoding gene *CTA1*. These findings highlight the involvement of Ypk9p in the oxidative stress response and modulation of RLS.

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Author contributions Xinguang Liu and Jie Ruan designed the experiments. Fang Guo, Lingyue Kong, Jiaxin Liu, Xiaoshan Hong, Zhiwen Jiang, Haochang Song and Xiaojing Cui performed experiments. Wei Zhao wrote the manuscript. All authors discussed the results and commented on the manuscript.

Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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