Ethanol Induces Autophagy Regulated by Mitochondrial ROS in *Saccharomyces cerevisiae*^S

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Ethanol accumulation inhibited the growth of *Saccharomyces cerevisiae* during wine fermentation. Autophagy and the release of reactive oxygen species (ROS) were also induced under ethanol stress. However, the relation between autophagy and ethanol stress was still unclear. In this study, expression of the autophagy genes *ATG1* and *ATG8* and the production of ROS under ethanol treatment in yeast were measured. The results showed that ethanol stress very significantly induced expression of the *ATG1* and *ATG8* genes and the production of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$). Moreover, the *atg1* and *atg8* mutants aggregated more H_2O_2 and $O_2^{\cdot-}$ than the wild-type yeast. In addition, inhibitors of the ROS scavenging enzyme induced expression of the *ATG1* and *ATG8* genes by increasing the levels of H_2O_2 and $O_2^{\cdot-}$. In contrast, glutathione (GSH) and N-acetylcystine (NAC) decreased *ATG1* and *ATG8* expression by reducing H_2O_2 and $O_2^{\cdot-}$ production. Rapamycin and 3-methyladenine also caused an obvious change in autophagy levels and simultaneously altered the release of H_2O_2 and $O_2^{\cdot-}$. Finally, inhibitors of the mitochondrial electron transport chain (mtETC) increased the production of H_2O_2 and $O_2^{\cdot-}$ and also promoted expression levels of the *ATG1* and *ATG8* genes. In conclusion, ethanol stress induced autophagy which was regulated by H_2O_2 and $O_2^{\cdot-}$ derived from mtETC, and in turn, the autophagy contributed to the elimination H_2O_2 and $O_2^{\cdot-}$.

Keywords: Autophagy, ethanol stress, fermentation, hydrogen peroxide, superoxide anion, reactive oxygen species

Introduction

In order to remove dysfunctional and unneeded constituents and recycle intracellular nutrients, macro-autophagy (hereinafter referred to as autophagy) encapsulates and transfers cytoplasmic material to the vacuole or lysosome for degradation [1]. Autophagy-related genes (ATGs) originally were identified in the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) [2]. The most important of these ATGs was the ubiquitin-like protein ATG8 that attached to lipid phosphatidylethanolamine (PE) on the outside of emerging phagophores. The ATG8-PE adduct contributed to expand and seal the vesicle and recruit specific cargo [3, 4]. Therefore, upregulation of *ATG8* expression is implicated in the induction of autophagy [5]. Moreover, the initiation of autophagy is regulated by Ser/

Thr kinase ATG1 and its accessory regulator ATG13 in fungi [6–10]. It has been proven that the expression of *ATG1* is also an indicator of autophagy levels [11].

During the early stage of fermentation, sufficient nitrogen helps the proliferation and growth of yeast. The further consumption of nitrogen then results in sugar fermentation. Therefore, nitrogen limitation has been very common in winemaking. It was also well known that nitrogen starvation could induce autophagy [12, 13]. However, autophagy was induced early in wine fermentation in a nitrogen-replete environment [14]. About the function of autophagy during fermentation, it has been reported that recycling of iron via autophagy is critical for the transition from glycolytic to respiratory growth [15]. Therefore, the function of autophagy and the exact factors inducing it are still unknown during fermentation.

More and more studies showed that reactive oxygen species (ROS) were associated with fermentation [16–19]. ROS were a possible part of the yeast response to a variety of stress factors occurring during most fermentations [20]. ROSs, mainly including the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are generated by cells during normal metabolism [21]. Many studies have shown that ROS play a vital role in regulating autophagy [21–23]. However, which molecule of ROS regulated autophagy was still being debated. For example, it had been proven that H_2O_2 had the ability to activate autophagy [21]. However, Chen *et al.* showed that $O_2^{\cdot-}$ could induce autophagy [24]. In addition, other ROS except $O_2^{\cdot-}$ possibly induced autophagy [12]. Therefore, the exact ROS molecule regulating autophagy still needed to be researched in the future.

Ethanol production is likely to remain one of the most important biotechnological products well into the future, with the continued manufacture of spirits, wine, sake, beer, and so on [25]. But ethanol accumulation in the culture broth is still a significant stress factor during fermentation. Although *S. cerevisiae* is highly ethanol tolerant high ethanol inhibits cell growth and viability [26–28], and the mechanism of ethanol tolerance is still unclear. The aim of the current study was to obtain new insight into the response of yeast under ethanol stress conditions and to clarify the complicated relationship between ROS and autophagy during fermentation. To achieve these aims, ROS production and the expression of *ATG8* or *ATG1* genes were evaluated in wild type and mutant yeast strains grown on a medium with ethanol.

Materials and Methods

Strain and Maintenance Medium

The wine yeast strain BY4742 (*S. cerevisiae*) was supplied by Pro. Zhiwei Huang of East China University. The mutants *atg1* and *atg8* were purchased from Invitrogen (Carlsbad, CA). The mutants were constructed by hygromycin stripe homologous replacement of the mutation genes of *ATG1* or *ATG8* and selected by yeast peptone dextrose agar (YPD) medium with 200 µg/ml G418. Wild-type yeasts could not grow on YPD medium containing G418 whereas mutant cells could survive. The mutants were preserved on YPD medium containing G418 in order to prevent back mutation. The wild-type yeast maintained at 4°C on slants of YPD medium contained (g/L): glucose 20, peptone 10, yeast extract 5, and agar 20. Fresh cells grown on YPD slants for 24 h were used in all experiments.

Fermentation Conditions

For all experiments, starter cultures were prepared by growing the yeast cells overnight in 250-ml flasks containing 100 ml of YPD

medium. The flasks were incubated at 30°C in an orbital shaker set at 180 rpm. The experimental cultures were inoculated with 5×10^5 CFU/ml of starter culture. Fermentation was carried out in 500-ml flasks filled to two-thirds of their volume and maintained at 30°C in an orbital shaker at 180 rpm, according to the methodology [12]. With distilled water as a control, 10% ethanol made by adding absolute ethyl alcohol was used as ethanol stress. 100 mM 2-methoxyestradiol (2-ME) was stored in dimethylsulfoxide (DMSO) and final concentration was 100 µM. 1 M 3-amino-1,2,4-triazole (3-AT) was stored in double distilled water and final concentration was 2 mM. 10 mM rapamycin (Rapa) dissolved in ethanol and stored in TritonX-100 and final concentration was 5 µM. 3-methyladenine (3-MA) was stored in PBS solution and final concentration was 10 µM. 2.5 mM glutathione (GSH) and 10 mM N-acetyl-cysteine (NAC) were both dissolved in double distilled water. 100 mM antimycin A (Anti A) and rotenone (Rote) were both dissolved in DMSO and final concentrations for both were 5 mM. Each flask was closed with a rubber stopper. Prior to sampling, the flasks were stirred to ensure homogeneity.

Assessment of Cell Death

Cell death was assessed by propidium iodide (PI) (Sigma, USA) vital staining as described elsewhere [29] with minor adaptations. Briefly, yeast (10^6 cells/ml) was stained by 20 µM PI for 10 min at 37°C and then observed by fluorescence microscope. The fluorescence intensity was measured by fluorescence 96-well microplate (excitation wavelength 540 nm, emission wavelength 590 nm). The death of cells was with high red fluorescence.

Assessment of Intracellular H_2O_2 and $O_2^{\cdot-}$

H_2O_2 was monitored with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma) essentially as described elsewhere [30]. DCFH-DA didn't fluoresce but could transmembrane freely. The dye could be hydrolyzed by esterase to 2, 7-dichlorofluorescein (DCFH) which was arrested in an actively respiring cell. DCFH was oxidized by ROS to a fluorescent compound DCF in cytoplasm. To detect $O_2^{\cdot-}$, dihydroethidium (DHE) (Sigma) was used as a probe. Briefly, yeast (10^6 cells/ml) was stained by 4 µM DHE for 10 min at 30°C in dark. The fluorescences of DCF and DHE were detected by fluorescence microscope and Fluorescence-activated cell sorter (FACS) analysis.

FACS analysis was carried out with an FACSCalibur Flow Cytometer (Becton, Dickinson and Company, USA) equipped with an argon ion laser emitting a 488-nm beam at 15 mW. The green and red fluorescence were collected through a 488-nm blocking filter. Green and red fluorescence were used for detection by a 550-nm/long-pass dichroic mirror with a 525-nm/band-pass filter and a 590-nm/long-pass with a 620-nm/band-pass filter, respectively. An acquisition protocol was defined to measure green fluorescence (FL1 log) and red fluorescence (FL2 log) on a 4-decade logarithmic scale. Data (20,000 cells per sample) were analyzed with the cell quest pro included in the System II acquisition software for the Flow Jo software.

qRT-PCR

Wild-type yeast was treated with a different reagent from ethanol. RNA was obtained from the sample which was periodically collected. RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) standard procedures. Heat shock treatment (15 min at 42°C followed by 3 min at 95°C) was for cellular disruption. Total RNA (250 ng) was reverse transcribed using a SuperScript III Platinum Two-Step Real-Time Quantitative RT-PCR Kit with SYBR green from Invitrogen. One microliter of the reverse-transcribed RNA was used as a template to amplify the genes, using primers to the *ATG8* gene (sense, 5-TTGCTGACAGGTCA AGAATAGG-3; antisense, 5-ATCAACGCCGACAGTAGGTG-3), *ATG1* gene (sense, 5-TACTGTGCTCTTGGGGACCTA-3; antisense, 5-CGGACGCTAACTGCTGTAAATA-3) and the *ACT1* gene (sense, 5-GGATTCTGAGGTTGCTGCTTT-3; antisense, 5-TGACCCATA CCGACCATGATAC-3). The expression of the *ATG8* and *ATG1* genes was assessed by qRT-PCR in a StepOnePlus system (ABI). Results were normalized to the reference gene *ACT1*. The data were analyzed by applying the Livak method or the $2^{-\Delta\Delta CT}$ method [31]. The method was as follows: $\Delta\Delta C_T = (C_T(\text{target gene}) - C_T(\text{reference gene}))_{\text{eth}} - (C_T(\text{target gene}) - C_T(\text{reference gene}))_{\text{con}}$, where C_T is the threshold cycle.

Statistical Analysis

Data were reported as mean values of at least three independent assays and presented as means $\pm$ standard deviations (SD). Statistical analyses were carried out using a Student's *t*-test. *P* values of less than 0.05 or 0.01 were considered statistically significant, or very significant which were shown as “*” and “**” at the tops of the columns in the figures.

Fig. 1. Ethanol-induced expression of the *ATG1* and *ATG8* genes. Normalized fold expression levels of the *ATG1* and *ATG8* genes were evaluated by qRT-PCR in *S. cerevisiae* treated with 10% ethanol for 4 h. *ACT1*, encoded actin, was used as internal reference. Values indicated mean $\pm$ standard deviation ($n = 3$). Statistical significance (**, $p < 0.01$) was determined by a Student's *t*-test and was shown as the statistical significance between ethanol with control. Con: control, Eth: treated with ethanol.

Results

Ethanol Stress Induced Autophagy

Ethanol was the main metabolite of *S. cerevisiae* during the fermentation process [25]. In order to confirm the effect of ethanol stress on autophagy, the expression levels of the *ATG1* and *ATG8* genes were measured by qRT-PCR. Based on the preliminary experiment, the peak expression value of *ATG8* and *ATG1* was at 4 h. Therefore, expression of the *ATG8* and *ATG1* genes was detected at 4 h. The results showed that the expression levels of the *ATG1* and *ATG8* genes were very significantly increased during ethanol stress ($p < 0.01$) (Fig. 1). In detail, the expression levels of

Fig. 2. Cell death of mutants was higher than that of wild type under ethanol stress. Wild type, *atg1* and *atg8* treated with 10% ethanol for 2 h were stained with PI. Then all strains were analyzed by fluorescence microscope (A) and fluorescence microplate reader (B). Values indicated mean $\pm$ standard deviation from ($n = 6$). Statistical significance (**, $p < 0.01$) was determined by a Student's *t*-test and was shown as the statistical significance between ethanol with control. Con: control, Eth: treated with ethanol.

ATG1 and *ATG8* were increased to 153.5% and 252.6% by ethanol stress, respectively (Fig. 1). Therefore, we confirmed that ethanol stress indeed induced autophagy.

Autophagy Contributed to Survival of *S. cerevisiae* from Ethanol Stress

The above results revealed that ethanol stress markedly induced autophagy. In order to clarify the role of autophagy during ethanol stress, the death rates of the wild type, *atg1* and *atg8* strains were assessed by PI staining. The results showed that there was no difference between the wild type and the mutant strains during their growth on YPD medium (Fig. 2). However, ethanol stress increased the cell death rate of all strains (Fig. 2). Compared with wild-type yeast, the cell death of *atg1* and *atg8* mutants was dramatically enhanced by ethanol stress. Therefore, this indicated that autophagy protected yeast cells from ethanol stress.

Ethanol Induced Production of H_2O_2 and $O_2^{\cdot -}$ in *S. cerevisiae*

To evaluate whether ROS accumulation was accompanied by ethanol stress, ROS production was measured by fluorescence staining and flow cytometry. Production of H_2O_2 and $O_2^{\cdot -}$ reached highest at 2 h according to the preliminary experiment. Therefore, production of H_2O_2 and $O_2^{\cdot -}$ was detected at 2 h. The production of H_2O_2 and $O_2^{\cdot -}$ was induced in both cases by ethanol stress for 2 h (Fig. 3A). H_2O_2 production in *atg1*, *atg8* mutants was higher than that in wild type yeast under ethanol treatment for 24 h. In addition, the *atg1* mutant had the highest H_2O_2 concentration (Fig. 3B). Similarly, the $O_2^{\cdot -}$ content in mutant cells was also higher than that in wild type yeast cells (Fig. 3C). However, the difference in $O_2^{\cdot -}$ production between wild type and mutants was not greatly different than that observed in H_2O_2 . Therefore, these results demonstrated that ethanol stress contributed to the accumulation of ROS and autophagy had the ability to eliminate ROS.

Fig. 3. Ethanol induced more H_2O_2 and $O_2^{\cdot -}$ in mutants than in wild type.

Production of H_2O_2 and $O_2^{\cdot -}$ in wild type treated with 10% ethanol for 2 h were stained by DCFH or DHE (A). Production of H_2O_2 (B) and $O_2^{\cdot -}$ (C) in wild type, *atg1* and *atg8* treated with 10% ethanol for 24 h were stained by DCFH (B) or DHE (A). All strains were analyzed by flow cytometry. Black bars represent 10 μ m.

Autophagy Induced by Ethanol Stress Depended on ROS

The above results show that ethanol not only induced the expression of *ATG1* and *ATG8* but also enhanced the production of H_2O_2 and $\text{O}_2^{\cdot-}$. In addition, accumulating evidence shows that moderate ROS as signal molecules regulated autophagy [12, 21, 23]. Therefore, in order to clarify the relationship between autophagy and ROS, reductants were used to change ROS levels. Obviously, both GSH and NAC reductants decreased very significantly the production of H_2O_2 and $\text{O}_2^{\cdot-}$ under ethanol stress (Figs. 4A and 4B). The production of H_2O_2 was reduced to 55.4% and 44.0% by GSH and NAC (Fig. 4B). GSH and NAC decreased the production levels of $\text{O}_2^{\cdot-}$ to 43.5% and 29.2% (Fig. 4B). Simultaneously, GSH and NAC markedly decreased the gene expression of *ATG1* and *ATG8* (Fig. 4C). For example, expression of *ATG1* and *ATG8* was decreased to 54.7% and 38.8% by GSH. NAC lowered *ATG1* and *ATG8* expression to 55.6% and 19.3%. Moreover, in wild type, GSH and NAC also decreased the production of H_2O_2 and $\text{O}_2^{\cdot-}$ in *atg1* and *atg8* mutants (Fig. S1).

In spite of moderate ROS acting as a signal, excess ROS oxidized nearby biological macromolecules including DNA, proteins and lipids [32–36]. In order to protect themselves from oxidative damage, cells used enzymes to eliminate ROS, such as catalase (CAT) in cytoplasm and manganese superoxide dimutase (Mn-SOD) in mitochondria [37–39]. Therefore, 2-ME and 3-AT were used as the inhibitors of Mn-SOD and CAT, to increase of $\text{O}_2^{\cdot-}$ and H_2O_2 levels in cells. The results showed that 2-ME increased the production of H_2O_2 and $\text{O}_2^{\cdot-}$ (Figs. 5A and 5B). 3-AT only induced the levels of $\text{O}_2^{\cdot-}$ to 159.0% but had no effect on the levels of H_2O_2 (Fig. 5B). 2-ME and 3-AT both activated the expression of *ATG1* and *ATG8* genes under ethanol stress (Fig. 5C). In detail, the expression of *ATG1* and *ATG8* was elevated to 216% and 183.9% by 2-ME. 3-AT increased the expression levels of *ATG1* and *ATG8* to 185.6% and 152.1%, respectively. The production of H_2O_2 and $\text{O}_2^{\cdot-}$ in *atg1* and *atg8* mutants was also induced by 2-ME and 3-AT (Fig. S2).

To further verify that ROS regulated autophagy in yeast under ethanol stress, the inhibitor of autophagy (3-MA) and the inducer of autophagy (Rapa) were added in the medium under ethanol stress. The results showed that Rapa very significantly promoted the production of H_2O_2 and $\text{O}_2^{\cdot-}$ in wild-type yeast (Figs. 6A and 6B) and evoked the expression of *ATG1* and *ATG8* (Fig. 6C). On the contrary, 3-MA dramatically decreased only the content of H_2O_2 (Figs. 6A and 6B) and reduced the expression of *ATG1* and *ATG8* (Fig. 6C). Therefore, autophagy was dependent on levels of H_2O_2 under ethanol stress. In mutants, Rapa

Fig. 4. GSH and NAC decreased production of H_2O_2 and $\text{O}_2^{\cdot-}$ and expression of *ATG1* and *ATG8* genes in *S. cerevisiae* under ethanol stress.

(A) Production of H_2O_2 and $\text{O}_2^{\cdot-}$ in wild type treated with 10% ethanol for 2 h was stained by DCFH or DHE, respectively. Black bars represent 10 μm . Relative fluorescence of DCFH and DHE is shown in (B). Values indicated mean $\pm$ standard deviation ($n = 6$). GSH or NAC decreased expression of *ATG1* and *ATG8* (C). Normalized fold expression levels of *ATG1* and *ATG8* were evaluated by qRT-PCR in *S. cerevisiae* under ethanol stress for 4 h. *ACT1*, encoded actin, was used as internal reference. Values indicated mean $\pm$ standard deviation ($n = 3$). Statistical significance (**, $p < 0.01$) was determined by a Student's *t*-test.

Fig. 5. 2-ME and 3-AT increased production of H_2O_2 and $O_2^{\cdot -}$ and expression of *ATG1* and *ATG8* genes in *S. cerevisiae* under ethanol stress.

(A) Production of H_2O_2 and $O_2^{\cdot -}$ in wild type treated with 10% ethanol for 2 h was stained by DCFH or DHE, respectively. Black bars represent 10 μ m. Relative fluorescence of DCFH and DHE is shown in (B). Values indicated mean $\pm$ standard deviation ($n = 6$). 2-ME and 3-AT increased expression of *ATG1* and *ATG8* (C). Normalized fold expression levels of *ATG1* and *ATG8* were evaluated by qRT-PCR in *S. cerevisiae* under ethanol stress for 4 h. *ACT1*, encoded actin, was used as internal reference. Values indicated mean $\pm$ standard deviation ($n = 3$). Statistical significance (**, $p < 0.01$) was determined by a Student's *t*-test.

Fig. 6. Rapa and 3-MA regulated production of H_2O_2 and $O_2^{\cdot -}$ and expression of *ATG1* and *ATG8* genes in *S. cerevisiae*.

(A) Production of H_2O_2 and $O_2^{\cdot -}$ in wild-type yeast with 10% ethanol for 2 h was stained by DCFH or DHE, respectively. Black bars represent 10 μ m. Relative fluorescence of DCFH and DHE is shown in (B). Values indicated mean $\pm$ standard deviation ($n = 6$). Rapa and 3-MA regulated expression of *ATG1* and *ATG8* (C). Normalized fold expression levels of *ATG1* and *ATG8* were evaluated by qRT-PCR in *S. cerevisiae* under ethanol stress for 4 h. *ACT1*, encoded actin, was used as internal reference. Values indicated mean $\pm$ standard deviation ($n = 3$). Statistical significance (**, $p < 0.01$) was determined by a Student's *t*-test.

and 3-MA both promoted the production of H_2O_2 and O_2^- under ethanol stress (Fig. S3). Rapa and 3-MA did not change the levels of autophagy in the mutants. Accumulation of more H_2O_2 and O_2^- by Rapa and 3-MA may be attributed to their alternative function.

ROS Induced by Ethanol Stress Mainly Originated from Mitochondria

Since we found that ROS were implicated in autophagy under ethanol stress, the source of ROS was also explored in this study. The respiratory complexes I, II, and III of the mitochondrial electron transport chain (mtETC) were reported to be the major production sites of O_2^- [40, 41]. Anti A and Rote, as the inhibitors of complex III and complex I of the mtETC, were used to increase ROS in mitochondria. The results showed that Anti A and Rote both undoubtedly increased production of H_2O_2 and O_2^- (Figs. 7A and 7B) and promoted the expression of *ATG1* and *ATG8* genes under ethanol stress (Fig. 7C). In detail, production of H_2O_2 was increased to 212.8% and 257.4% by Rote and Anti A, respectively. Rote and Anti A raised production of O_2^- to 225.0% and 299.2%, respectively. Expression of *ATG1* and *ATG8* was increased to 496.4% and 431.9% by Anti A. Rote raised *ATG1* and *ATG8* expression to 348.5% and 299.3%. These findings indicated that ROS derived from the mtETC-regulated autophagy under ethanol stress. Therefore, ROS accumulation in yeast attributable to H_2O_2 and O_2^- derived from the mtETC. In the mutants, Anti A and Rote also increased production of H_2O_2 and O_2^- (Fig. S4). The results further proved that production of H_2O_2 and O_2^- was mainly caused by the mtETC under ethanol stress.

Discussion

The budding yeast had to escape from nitrogen deficient conditions during most alcoholic fermentations. And it was well known that autophagy could be induced by deficient nitrogen during fermentation [12, 13, 42]. However, Piggott et al. had demonstrated that autophagy was induced at the early stage of wine fermentation in a nitrogen-replete environment [14]. Therefore, it was suggested that autophagy might be triggered by other factors that arose during the early stage of fermentation. Besides that, it had been proven that autophagy was induced in second-fermentation yeasts during sparkling wine production [43].

Cost-effective ethanol production depended on rapid and high-yielding conversion of carbohydrate to ethanol. Therefore, ethanol accumulation in the culture broth

Fig. 7. Rote and Anti A increased production of H_2O_2 and O_2^- and expression of *ATG1* and *ATG8* genes in *S. cerevisiae*.

(A) Production of H_2O_2 and O_2^- in wild-type yeast with 10% ethanol for 2 h was stained by DCFH or DHE, respectively. Black bars represent 10 μm . Relative fluorescence of DCFH and DHE is shown in (B). Values indicated mean $\pm$ standard deviation ($n = 6$). Rote and Anti A increased expression of *ATG1* and *ATG8* (C). Normalized fold expression levels of *ATG1* and *ATG8* were evaluated by qRT-PCR in *S. cerevisiae* under ethanol stress for 4 h. *ACT1*, encoded actin, was used as internal reference. Values indicated mean $\pm$ standard deviation ($n = 3$). Statistical significance (**, $p < 0.01$) was determined by a Student's *t*-test.

appeared at the early stage of fermentation. It was well known that the yeast had the property of ethanol tolerance. However, the mechanism of the ethanol tolerance was not clear. In the current study, the results showed that autophagy was induced by ethanol stress (Fig. 1). And compared with *atg1* and *atg8*, wild type contributed to cell survival from ethanol stress (Fig. 2). Therefore, yeast was likely surviving ethanol injury by means of increasing the levels of autophagy to clear away the damaged organelles.

Ethanol Stress Induced ROS in *S. cerevisiae*

CTT1, encoding a kind of cytosolic CAT, was found to be highly expressed only in ethanol-tolerant sake yeast mutants or after exposure to ethanol [44]. Therefore, eliminating of ROS by CTT1 helped sake yeast survive ethanol stress. Otherwise, ROS were normally induced during fermentation [18, 19]. However, the exact relationship between ROS and ethanol stress was still unclear. In this study, ROS production in wild-type yeast (Figs. 4–7) and mutant cells (Figs. S1, S2, and S4) was induced by ethanol stress. However, the role of ROS was still unknown under ethanol stress.

Compared to wild-type yeast, the *atg1* and *atg8* mutants had higher production of H_2O_2 and $O_2^{\cdot-}$ under ethanol stress (Fig. 3). These results were in agreement with previous reports. For instance, it had been shown that the mutants *atg2* and *atg5* accumulated high levels of H_2O_2 [45]. Thus, autophagy was in favor of eliminating ROS under ethanol stress. In addition, *atg1* and *atg8* mutants had a higher cell death rate than wild type yeast (Fig. 2). These findings suggest that wild-type yeast markedly decreased cell death under ethanol by eliminating ROS by autophagy.

ROS Derived from mtETC Regulated Autophagy under Ethanol Stress

Mounting evidence suggested that ROS might play a role in the control of autophagy [21–23, 46, 47]. And we had proven that ethanol activated production of H_2O_2 and $O_2^{\cdot-}$ (Fig. 3A) and autophagy (Fig. 1). In addition, GSH and NAC decreased autophagy by decreasing the release of H_2O_2 and $O_2^{\cdot-}$ under ethanol stress (Fig. 4). In agreement, NAC had been reported to reduce ethanol-induced autophagy [48] and also to decrease salinomycin-induced autophagy [49]. On the contrary, in our study 2-ME induced autophagy by means of increasing production of H_2O_2 and $O_2^{\cdot-}$ whereas 3-AT increased autophagy probably just through production of $O_2^{\cdot-}$ (Fig. 5). Therefore, our results revealed that H_2O_2 and $O_2^{\cdot-}$ regulated autophagy levels in the yeasts under ethanol stress.

Moreover, Rapa promoted the production of H_2O_2 and $O_2^{\cdot-}$ and increased the expression of the *ATG1* and *ATG8* genes (Fig. 6). However, 3-MA decreased the levels of H_2O_2 and lowered the expression of the *ATG1* and *ATG8* genes (Fig. 6). The difference was that both Rapa and 3-MA enhanced production of H_2O_2 and $O_2^{\cdot-}$ in *atg1* and *atg8* mutants under ethanol stress (Fig. S3). Rapa and 3-MA did not affect the level of autophagy in mutants. Alternative functions of Rapa or 3-MA maybe associated with the increased accumulation of H_2O_2 and $O_2^{\cdot-}$.

Although ROS have been previously reported to be implicated in autophagy, specifically which ROS were playing this crucial regulatory role was still disputed. Chen *et al.* considered that $O_2^{\cdot-}$ was the main kind of ROS inducing autophagy [24]. However, Mendes-Ferreira *et al.* reported that all kinds of ROS except $O_2^{\cdot-}$ had vital roles in regulating autophagy [12]. There were also some reports showing that autophagy was induced by H_2O_2 [21, 46]. In our study, H_2O_2 and $O_2^{\cdot-}$ both participated in regulating autophagy in yeast under ethanol stress.

Mitochondrion was the main source of ROS [40, 41]. In the current study, Anti A and Rote also increased the production of H_2O_2 and $O_2^{\cdot-}$ in *atg1* and *atg8* mutants under ethanol (Fig. S4). The results proved that H_2O_2 and $O_2^{\cdot-}$ were mainly derived from the mtETC under ethanol. Anti A and Rote enhanced the production of H_2O_2 and $O_2^{\cdot-}$ and activated the expression of the *ATG1* and *ATG8* genes in wild type (Fig. 7). Therefore, ROS derived from mitochondria had vital roles in regulating autophagy under ethanol stress. Many studies have shown that ROS from mitochondria regulated autophagy or mitophagy [39, 50–52]. For example, MITA expression modulated autophagy flux through enhancing mitochondrial ROS by increasing complex-I activity [51]. In addition, mitochondrial complex I inhibition triggered a mitophagy-dependent ROS increase [52].

Overall, ethanol stress induced autophagy and increased the production of H_2O_2 and $O_2^{\cdot-}$ which were originated from the mtETC, although other sources of ROS are not excluded. Under ethanol stress, the high levels of H_2O_2 and $O_2^{\cdot-}$ markedly induced autophagy. Subsequently, the autophagy contributed to the elimination of H_2O_2 and $O_2^{\cdot-}$. Ultimately, the autophagy assisted yeast in surviving ethanol stress during fermentation.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

- Kanki T, Furukawa K, Yamashita SI. 2015. Mitophagy in yeast: molecular mechanisms and physiological role. *Biochim. Biophys. Acta* **1853**: 2756-2765.
- Tsukada M, Ohsumi Y. 1993. Isolation and characterization of autophagy defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* **333**: 169-174.
- Fracchiolla D, Sawa-Makarska J, Zens B, Ruiter A, Zaffagnini G, Brezovich A, *et al.* 2016. Mechanism of cargo-directed Atg8 conjugation during selective autophagy. *Elife* **5** (pii): e18544.
- Weiergräber OH, Schwarten M, Strodel B, Willbold D. 2017. Investigating structure and dynamics of Atg8 family proteins. *Methods Enzymol.* **587**: 115-142.
- Klionsky DJ, Abeliovich H, Agostinis P, Agrawal D. 2008. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4**: 151-175.
- Reggiori F, Klionsky DJ. 2013. Autophagic processes in yeast: mechanism, machinery and regulation. *Genetics* **194**: 341-361.
- Wong PM, Puente C, Ganley IG, Jiang X. 2013. The ULK1 complex: sensing nutrient signals for autophagy activation. *Autophagy* **9**: 124-137.
- Xiong Y, McCormack M, Li L, Hall Q, Xiang C, Sheen J. 2013. Glucose-TOR signalling reprograms the transcriptome and activates meristems. *Nature* **496**: 181-186.
- Chew LH, Lu S, Liu X, Li FK, Yu AY, Klionsky DJ, *et al.* 2015. Molecular interactions of the *Saccharomyces cerevisiae* Atg1 complex provide insights into assembly and regulatory mechanisms. *Autophagy* **11**: 891-905.
- Perpetuini G, Di Gianvito P, Arfelli G, Schirone M, Corsetti A, Tofalo R, *et al.* 2016. Biodiversity of autolytic ability in flocculent *Saccharomyces cerevisiae* strains suitable for traditional sparkling wine fermentation. *Yeast* **33**: 303-312.
- Suzuki H, Osawa T, Fujioka Y, Noda NN. 2017. Structural biology of the core autophagy machinery. *Curr. Opin. Struct. Biol.* **43**: 10-17.
- Mendes-Ferreira A, Sampaio-Marques B, Barbosa C, Rodrigues F, Costa V, Mendes-Faia A, *et al.* 2010. Accumulation of non-superoxide anion reactive oxygen species mediates nitrogen-limited alcoholic fermentation by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **76**: 7918-24.
- Tesnière C, Brice C, Blondin B. 2015. Responses of *Saccharomyces cerevisiae* to nitrogen starvation in wine alcoholic fermentation. *Appl. Microbiol. Biotechnol.* **99**: 7025-7034.
- Piggott N, Cook MA, Tyers M, Measday V. 2011. Genome-wide fitness profiles reveal a requirement for autophagy during yeast fermentation. *G3* **1**: 353-367.
- Horie T, Kawamata T, Matsunami M, Ohsumi Y. 2017. Recycling of iron via autophagy is critical for the transition from glycolytic to respiratory growth. *J. Biol. Chem.* **292**: 8533-8543.
- Gibson BR, Lawrence SJ, Boulton CA, Box WG, Graham NS, Linforth S, *et al.* 2008. The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. *FEMS Yeast Res.* **8**: 574-585.
- Landolfo S, Politi H, Angelozzi D, Mannazzu I. 2008. ROS accumulation and oxidative damage to cell structures in *Saccharomyces cerevisiae* wine strains during fermentation of high-sugar-containing medium. *Biochim. Biophys. Acta* **1780**: 892-898.
- Cheng Y, Du Z, Zhu H, Guo X, He X. 2016. Protective effects of arginine on *Saccharomyces cerevisiae* against ethanol stress. *Sci. Rep.* **6**: 31311.
- Charoenbhakdi S, Dokpiku T, Burphan T, Techo T, Auesukaree C. 2016. Vacuolar H⁺-ATPase protects *Saccharomyces cerevisiae* cells against ethanol-induced oxidative and cell wall stresses. *Appl. Environ. Microbiol.* **82**: 3121-3130.
- Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **9**: 249-279.
- Song C, Mitter SK, Qi X, Beli E, Rao HV, Ding J, *et al.* 2017. Oxidative stress-mediated NFκB phosphorylation upregulates p62/SQSTM1 and promotes retinal pigmented epithelial cell survival through increased autophagy. *PLoS One* **12**: e0171940.
- Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. 2007. Reactive oxygen species are essential for autophagy and specifically regulate the activity of *atg4*. *EMBO J.* **26**: 1749-1760.
- Xua J, Wua Y, Lu G, Xie S, Ma Z, Chen Z, Shen HM, *et al.* 2017. Importance of ROS-mediated autophagy in determining apoptotic cell death induced by physapubescin B. *Redox. Biol.* **12**: 198-207.
- Chen SY, Chiu LY, Maa MC, Wang JS, Chien CL, Lin WW. 2011. zVAD-induced autophagic cell death requires c-Src-dependent ERK and JNK activation and reactive oxygen species generation. *Autophagy* **7**: 217-28.
- Demain AL. 2009. Biosolutions to the energy problem. *J. Ind. Microbiol. Biotechnol.* **36**: 319-332.
- Galeote VA, Blondin B, Dequin S, Sablayrolles, JM. 2001. Stress effects of ethanol on fermentation kinetics by stationary-phase cells of *Saccharomyces cerevisiae*. *Biotechnol.*

- Lett.* **23**: 677-681.
27. Aguilera F, Peinado RA, Millan C, Ortega JM, Mauricio JC. 2006. Relationship between ethanol tolerance, H⁺-ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. *Int. J. Food Microbiol.* **110**: 34-42.
 28. Walker ME, Nguyen TD, Liccioli T, Schmid F, Kalatzis N, Sundstrom JF, *et al.* 2014. Genome-wide identification of the Fermentome; genes required for successful and timely completion of wine-like fermentation by *Saccharomyces cerevisiae*. *BMC Genomics* **15**: 552.
 29. Almeida B, Sampaio-Marques B, Carvalho J, Silva MT, Leao C, Rodrigues F, *et al.* 2007. An atypical active cell death process underlies the fungicidal activity of ciclopirox olamine against the yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **7**: 404-412.
 30. Ludovico P, Rodrigues F, Almeida A, Silva MT, Barrientos A, Corte-Real M. 2002. Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. *Mol. Bio. Cell* **13**: 2598-2606.
 31. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* **3**: 1101-1108.
 32. Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* **7**: 405-410.
 33. Barja G. 2004. Free radicals and aging. *Trends Neurosci.* **27**: 595-600.
 34. Yin D, Chen K. 2005. The essential mechanisms of aging: irreparable damage accumulation of biochemical side-reactions. *Exp. Gerontol.* **40**: 455-465.
 35. Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C. 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bio. Essays.* **28**: 1091-2101.
 36. Chen HJ, Huang CS, Huang GJ, Chow TJ, Lind YH. 2013. NADPH oxidase inhibitor diphenyleneiodonium and reduced glutathione mitigate ethephon-mediated leaf senescence, H₂O₂ elevation and senescence-associated gene expression in sweet potato (*Ipomoea batatas*). *J. Plant Physiol.* **170**: 1471-1483.
 37. Rattanawong K, Kerdsoomboon K, Auesukaree C. 2015. Cu/Zn-superoxide dismutase and glutathione are involved in response to oxidative stress induced by protein denaturing effect of alachlorin *Saccharomyces cerevisiae*. *Free Radic. Bio. Med.* **89**: 963-971.
 38. Magri A, Di Rosa MC, Tomasello MF, Guarino F, Reina S, Messina A, Pinto VD. 2016. Overexpression of human SOD1 in VDAC1-less yeast restores mitochondrial functionality modulating beta-barrel outer membrane protein genes. *Biochim. Biophys. Acta* **1857**: 789-798.
 39. Knuppertz L, Warnsmann V, Hamann A, Grimm C, Osiewacz HD. 2017. Stress-dependent opposing roles for mitophagy in aging of the ascomycete *Podospora anserina*. *Autophagy* **13**: 1037-1052.
 40. Murphy MP. 2009. How mitochondria produce reactive oxygen species. *Biochem. J.* **417**: 1-13.
 41. Huang S, Aken OV, Schwarzländer M, Belt K, Millar AH. 2016. The roles of mitochondrial reactive oxygen species in cellular signaling and stress response in plants. *Plant Physiol.* **171**: 1551-1559.
 42. Li D, Song JZ, Li H, Shan MH, Liang Y, Zhu J, *et al.* 2015. Storage lipid synthesis is necessary for autophagy induced by nitrogen starvation. *FEBS Lett.* **89**: 269-276.
 43. Cebollero E, Gonzalez R. 2006. Induction of autophagy by second-fermentation yeasts during elaboration of sparkling wines. *Appl. Environ. Microb.* **72**: 4121-4127.
 44. Ogawa Y, Nitta A, Uchiyama H, Imamura T, Shimoi H, Ito K. 2000. Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. *J. Biosci. Bioeng.* **90**: 313-320.
 45. Yoshimoto K, Jikumaru Y, Kamiya Y, Kusano M, Consonni C, Panstruga R, *et al.* 2009. Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in *Arabidopsis*. *Plant Cell* **21**: 2914-2927.
 46. Pérez-Pérez ME, Zaffagnini M, Marchand CH, Crespo JL, Lemaire SD. 2014. The yeast autophagy protease Atg4 is regulated by thioredoxin. *Autophagy* **10**: 1953-1964.
 47. Navarro-Yepes J, Burns M, Anandhan A, Khalimonchuk O, del Razo LM, Quintanilla-Vega B, *et al.* 2014. Oxidative stress, redox signaling, and autophagy: cell death versus survival. *Antioxid. Redox. Signal* **21**: 66-85.
 48. Zhu Z, Huang Y, Lv L, Tao Y, Shao M, Zhao C, *et al.* 2017. Acute ethanol exposure-induced autophagy-mediated cardiac injury via activation of the ROS-JNK-Bcl-2 pathway. *J. Cell Physiol.* **233**: 924-935.
 49. Kim KY, Park KI, Kim SH, Yu SN, Lee D, Kim YW, *et al.* 2017. Salinomycin induces reactive oxygen species and apoptosis in aggressive breast cancer cells as mediated with regulation of autophagy. *Anticancer Res.* **37**: 1747-1758.
 50. Shiroma S, Jayakody LN, Horie K, Okamoto K, Kitagakia H. 2014. Enhancement of ethanol fermentation in *Saccharomyces cerevisiae* sake yeast by disrupting mitophagy function. *Appl. Environ. Microbiol.* **80**: 1002-1012.
 51. Basit F, van Oppen LM, Schöckel L, Bossenbroek HM, van Emst-de Vries SE, Hermeling JC, *et al.* 2017. Mitochondrial complex I inhibition triggers a mitophagy-dependent ROS increase leading to necroptosis and ferroptosis in melanoma cells. *Cell Death Dis.* **8**: e2716.
 52. Bhatelia K, Singh K, Prajapati P, Sripada L, Roy M, Singh R. 2017. MITA modulated autophagy flux promotes cell death in breast cancer cells. *Cell Signal* **35**: 73-83.