FEBS Letters 581 (2007) 2935–2942

Ethanol-induced death in yeast exhibits features of apoptosis mediated by mitochondrial fission pathway

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Received 18 April 2007; revised 15 May 2007; accepted 15 May 2007

Available online 29 May 2007

Edited by Vladimir Skulachev

Abstract **Cell death in yeast (*Saccharomyces cerevisiae*) involves several apoptotic processes. Here, we report the first evidence of the following processes, which are also characteristic of apoptosis, in ethanol-induced cell death in yeast: chromatin condensation and fragmentation, DNA cleavage, and a requirement for de novo protein synthesis. Mitochondrial fission protein, Fis1, appears to mediate ethanol-induced apoptosis and ethanol-induced mitochondrial fragmentation. However, mitochondrial fragmentation in response to elevated ethanol levels was not correlated with cell death. Further, in the presence of ethanol, generation of reactive oxygen species was elevated in mutant *fis1*Δ cells. Our characterization of ethanol-induced cell death in yeast as being Fis1-mediated apoptosis is likely to pave the way to overcoming limitations in large-scale fermentation processes, such as those employed in the production of alcoholic beverages and ethanol-based biofuels.**

Keywords: Ethanol; Yeast; Apoptosis; Mitochondria; Fragmentation

1. Introduction

Ethanol is the most important fermentation product on earth. It is utilized in many aspects of human life, including consumption of alcohol beverages and use of ethanol-based biofuels. However, efficient ethanol production faces a bottle neck in that the yeast *Saccharomyces cerevisiae* commonly used for fermentation dies when ethanol levels exceed a certain concentration. For these reasons, then, elucidation of ethanol-induced cell death in yeast is likely to have a significant impact on the fermentation industry.

Cell death can be divided into two main categories, necrosis and apoptosis [35]. Whereas necrosis is a catastrophic "death by assault", apoptosis can be thought of as an altruistic death, in which a cell positively executes death. Multicellular organisms utilize apoptosis for a variety of biological programs, including in development and disease prevention, and defects in apoptotic programs in multicellular organisms can lead to diseases like cancer. Although the yeast *S. cerevisiae* is an unicellular organism, there are compelling evidences that yeast can undergo altruistic programmed cell death similar to apoptosis [1–4,33,34]. To date, the stimuli reported to induce this type of cell death in yeast include hydrogen peroxide, acetic acid [5,6], high osmotic shock [7], viruses [8], pheromone [9,40] and defects in *N*-glycosylation [10].

In yeast, ethanol has an effect on many different cellular behaviors and processes related to cell death, including the stress responses [11], changes in membrane fluidity [12], protein structure [13] and mRNA export from the nucleus [14]. Despite the importance of ethanol-induced death to the fermentation industry, no previous report has explored possibility that ethanol-induced cell death in yeast is carried out via apoptosis program. Here, we provide evidences that ethanol-induced cell death exhibits features in common with apoptosis. Moreover, we show that ethanol-induced yeast cell death is mediated by mitochondrial fission protein Fis1.

2. Materials and methods

2.1. Yeast strains

S. cerevisiae strains BY4743 (MATa/α *his3* Δ *1/his3* Δ *1 leu2* Δ *0/leu2* Δ *0 MET15/met15* Δ *0 LYS2/lys2* Δ *0 ura3* Δ *0/ura3* Δ *0*) and its disruptants were obtained from Euroscarf. Cells expressing mitochondria-targeted GFP (mito-GFP) [15,16] were grown in SD-Leu. Other cells were incubated in YPD containing 2% yeast peptone, 1% bactopeptone and 2% glucose. Unless otherwise indicated, cells were inoculated from an overnight culture to a fresh medium at OD_{660} = 0.1, and then incubated at 30 °C for 24 h with shaking before being harvested for specific assays.

2.2. Survival assay

Yeast cells were incubated overnight in YPD, inoculated to a fresh YPD medium at OD_{660} of 0.1, incubated at 30 °C for 24 h, treated with 21% or 23% of ethanol, diluted in sterile deionized water at 10^{-4}, spread onto YPD plates, and colony forming unit (cfu) was determined. Cfus of the ethanol-treated cells were compared with those just before ethanol treatment and expressed as survival (cfu%). The survival ratios were expressed as mean values with standard error of mean (S.E.M.) of at lease three independent experiments.

2.3. Microscopy

Digital fluorescence and differential interference contrast (DIC) images of cells were acquired using a Nikon Eclipse E800 microscope (Nikon) equipped with DIC optics with epifluorescence capabilities,

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Abbreviations: mito-GFP, mitochondria-targeted GFP; S.E.M., standard error of mean; cfu, colony forming unit; TUNEL, TdT-mediated dUTP-biotin Nick End Labeling; DAPI, 4,6′-diaminidino-2-phenylindole; RT, room temperature; PFGE, pulse field gel electrophoresis; PI, propidium iodide; ROS, reactive oxygen species; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacatate

0014-5793/$32.00
doi:10.1016/j.febslet.2007.05.048

and a Nikon Plan-Apo 100×/1.4D Oil DIC objective. Nikon filter sets (excitation/beamsplitter/emission) were BP 460-500/FT 505/BP 510-560 for GFP single imaging; and BP 340-380/FT 400/BP 435-485 for 4,6′-diaminidino-2-phenylindole (DAPI, Wako) imaging, respectively. Images were captured using a Hamamatsu ORCA-ER monochrome digital camera and processed using the Hamamatsu Aqua Lite 1.2 processing and analysis system. At least 200 cells were counted to determine the mitochondrial morphology.

2.4. Reactive oxygen species (ROS) detection

For ROS detection, ethanol-treated cells were harvested by centrifugation at 5 krpm for 5 min, resuspended in sterile deionized water, centrifuged at 5 krpm for 5 min, resuspended in 10 μg/ml 2′,7′-dichlorodihydrofluorescein diacatate (H_2DCF-DA, Molecular Probes) dissolved in sterile deionized water from a stock solution of 2.5 mg/ml in ethanol so that the cell concentration becomes approximately 5×10^6 cells in 1 ml and incubated for 2 h at room temperature (RT) in the dark (modified from [5,39,40]). FACS analysis was performed using a Becton Coulter Epics Elite ESP equipped with a 488 nm argon laser, and emission signals were 525–550 nm.

2.5. Pulse field gel electrophoresis (PFGE)

Yeast cells were fixed with 3.7% (v/v) formaldehyde for 1 h and suspended in 1 ml of 50 mM EDTA (pH 8.0) to 10^9 cells/ml. This suspension was mixed with 1 ml of 250–500 μg/ml zymolyase 100 T (Seikagaku kogyo) and 0.5 M EDTA (pH 8.0) and 2 ml of 2% low melting agarose (BMC InCert), 125 mM EDTA (pH 8.0) and 0.5 M Tris–HCl (pH 7.5). The mixture was allowed to solidify in a Plug Mold (BIO RAD). The gels were incubated in 3.7 ml of 0.5 M EDTA, 50 mM Tris–HCl (pH 7.5)and 0.3 ml of 7.5% (v/v) β-mercaptoethanol for 3 h at 37 °C to turn to protoplast and successively in 4 ml of 0.5 M EDTA, 50 mM Tris–HCl (pH 7.5) containing 1% *N*-lauroylsarconic acid and 0.1 mg/ml Proteinase K at 50 °C for 3 h. The buffer was replaced with a fresh buffer and the sample was incubated at 50 °C for another 12–16 h. The gel was incubated at 50 °C for 1 h in 50 volumes of buffer containing 50 mM EDTA, 10 mM Tris–HCl (pH 7.6) and 40 μg/ml phenylmethylsulfonyl fluoride. The buffer was replaced and the gel was incubated for another 1 h at 50 °C. The buffer was replaced again and incubated at room temperature for 1 h. The gel was set in the 1% agarose gel, and PFGE was performed with CHEF-DRII (BIO RAD). The conditions for the electrophoresis was initial A time – 60 s, final A time – 60 s, START RATIO – 1, RUN TIME – 15 h, mode – 10, initial A time – 90 s, final A time – 90 s, RUN TIME – 8 h, mode – 10, 200 V, 23 h. The gel was stained with 50 μM ethidium bromide for 30 min and visualized by UV.

2.6. Test of apoptotic markers

TdT-mediated dUTP-biotin Nick End Labeling (TUNEL) staining was performed essentially as previously described [17]. Briefly, cells were fixed with 3.7% (v/v) formaldehyde for 1 h, treated with Zymolyase 20 T (650 μg/ml, Seikagaku kogyo) and 15 μl/200 μl β-glucuronidase (Roche diagnostics) at 37 °C for 40 min in 200 μl sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM potassium phosphate buffer (pH 7.3)), washed twice with PBS, applied to poly-lysin coated glass slide, permeablized with 0.1% Triton X-100 and 0.1% citric acid on ice for 15 min, washed with PBS three times, incubated with TUNEL staining reaction mixture (in situ cell death detection kit, fluorescein, Roche diagnostics) at 37 °C for 60 min in the dark, washed six times with PBS and observed under fluorescent microscope. DAPI staining was performed by staining cells with 0.3 μg/ml DAPI at RT in the dark for 10 min, washing with PBS and observing under fluorescent microscope. Propidium iodide (PI) staining was performed by staining cells with 5 μg/ml PI for 10 min and observing under fluorescent microscope.

2.7. Cycloheximide and caspase inhibitor experiments

Cycloheximide inhibition experiment was performed by incubating cells with 21% ethanol in the presence or absence of cycloheximide to a final concentration of 40 μg/ml from 20 mg/ml stock solution in dimethylsulfoxide, incubated for 3 h, diluted, spread onto YPD plates and incubated at 30 °C for 2 days. Numbers of colonies were counted and expressed as cfu. Cfus of cells incubated with both ethanol and cycloheximide and those incubated with ethanol but not with cycloheximide were compared each time. Caspase inhibition experiment was performed by incubating cells with 21% ethanol in the presence or absence of a caspase inhibitor DEVD (Calbiochem, Caspase-3 inhibitor I, cell permeable) to a final 40 μM from 20 mM stock solution diluted in 1:1 DMSO to ethanol for 3 h, diluted, spread onto YPD plates and incubated at 30 °C for 2 days. Numbers of colonies were counted and expressed as cfu. Cfus of cells incubated with both ethanol and caspase inhibitor and those incubated with ethanol but not with caspase inhibitor were compared each time.

3. Results and discussion

3.1. Ethanol-induced yeast cell death has features common to apoptosis

We first searched for the optimal condition to investigate apoptotic features in ethanol-induced cell death of yeast. It has been reported that apoptotic features in yeast become evident when the survival rate is 10–20% approximately 3 h after the stimuli [5,6]. Therefore, we searched for the ethanol-induced death condition that causes this outcome. Since mitochondrial pathway plays the central pathway of apoptosis in mammalian cells [33–37] and in pheromone [29,40], virus [30] and acetic acid [6]-induced apoptosis in yeast, we adopted cells in the post-diauxic phase to investigate apoptosis in yeast, at which point mitochondria are fully developed. As a result, we found that treating yeast cells with 23% ethanol results in 19.8 ± 5.3% of survival 3 h after the treatment (Fig. 1A). Thus, these conditions were deemed appropriate for our study and were used for all subsequent analyses unless otherwise indicated.

To ask if ethanol-induced cell death of yeast is consistent with apoptosis, we looked at the morphology of the chromatin after treatment with ethanol. As shown in Fig. 1B, we found that like other cell types undergoing apoptosis, ethanol-treated cells have chromatin condensation and fragmentation. Apoptotic stimuli have also been reported to cause DNA cleavage in the nucleus. Thus, we next asked if ethanol-induced yeast cells are associated with DNA cleavage, which can be revealed by TUNEL staining and PFGE. We observed TUNEL staining in 50.6 ± 2.0% of the nucleus of ethanol-treated cells, which stands for apoptotic cells (Fig. 1C and D), while 33.2 ± 5.1% of the cells showed PI staining, which stands for necrotic cells [7], indicating that apoptosis rather than necrosis is the major event in this condition. Moreover, DNA breakdown was observed in ethanol-treated cells as visualized by PFGE (Fig. 1E), similar to hydrogen peroxide, acetic acid and glucose-induced apoptosis [18]. Together, these results indicate that DNA cleavage has occurred in the nucleus of these cells.

Another feature of apoptosis is that it requires de novo protein synthesis. To test this, we investigated the effect of inhibition of protein synthesis via cycloheximide on ethanol-induced death. After incubating the cells from $OD_{660} = 0.1$ for 24 h at 30 °C, cells were incubated with 21% ethanol in the existence or absence of 40 μg/ml cycloheximide for 3 h, and cfus were determined. Cfus of cycloheximide-treated and untreated samples were compared each time. Consistent with the existence of apoptotic programs [5,6], ethanol- and cycloheximide-treated cells showed an increased number of cfu (123.5 ± 8.7% ($n = 5$)) as compared to ethanol-treated but cycloheximide-untreated cells, suggesting that de novo protein synthesis partially contributes to cell death in response to ethanol. Taken together, these results indicate that ethanol-induced death exhibits several features of apoptosis.

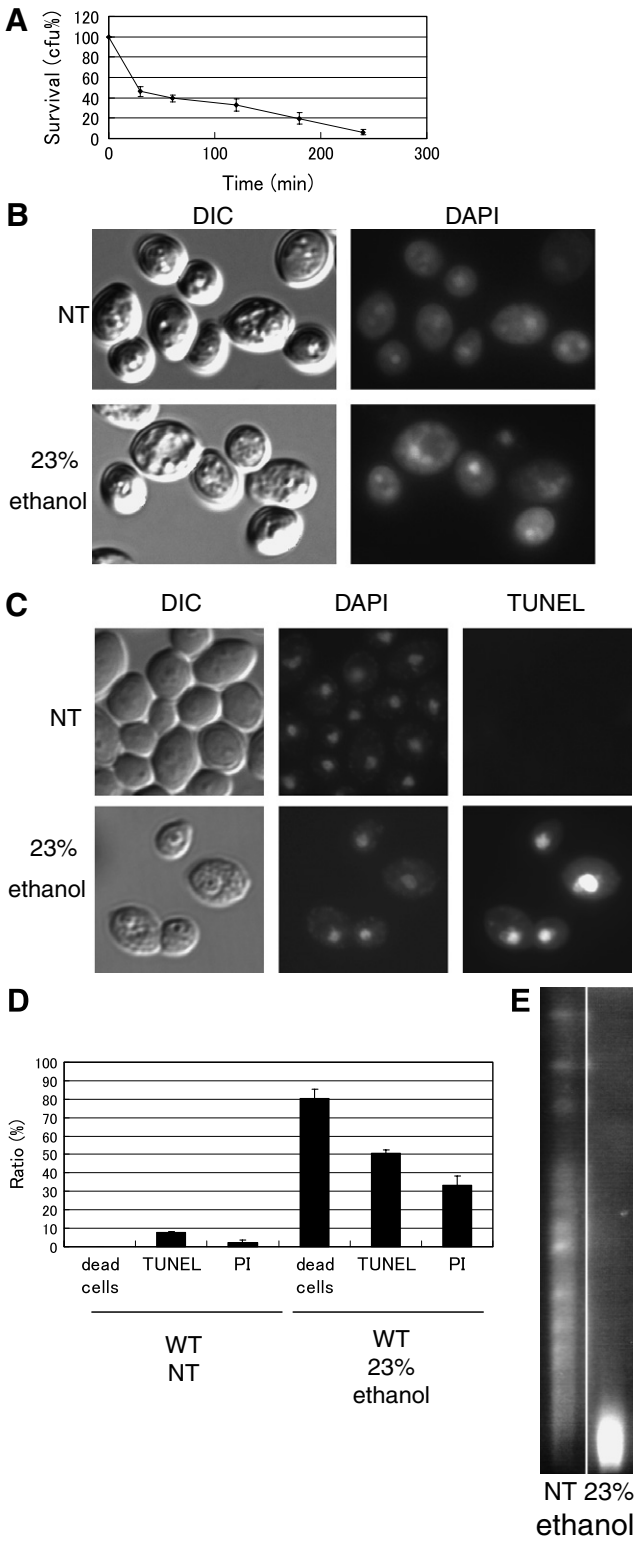

Fig. 1. Ethanol-induced yeast cell death exhibits features of apoptosis. (A) Survival curve of WT cells treated with 23% ethanol for various time periods. (B) DAPI staining of WT cells incubated for 3 h in the presence or absence of 23% ethanol. (C) TUNEL staining of WT cells incubated for 3 h in the presence or absence of 23% ethanol. (D) The ratio of dead, TUNEL- and PI-positive cells of WT cells cell incubated for 3 h in the presence or absence of 23% ethanol. Dead cells were determined by cfu. Mean values ± S.E.M., or representatives of at least three independent experiments are shown. (E) PFGE analysis of the chromosomes of cells treated with or without 23% ethanol for 3 h.

3.2. Fis1 inhibits apoptotic cell death by ethanol

We next asked if factors known to be involved in apoptosis caused by other death stimulus [1] are also involved in ethanol-induced cell death. To do this, we investigated the possibility that mutations in the following genes affect ethanol-induced cell death: *YCA1*, which encodes a mammalian caspase homolog [19]: *AIF1*, which encodes a yeast homolog of mammalian apoptosis-inducing factor [20]: *CYC3*, which encodes cytochrome *c* heme-lyase. This protein is required for the covalent binding of the heme group to isoforms 1 and 2 of apocytochrome *c* to form mature cytochrome *c* [22], which is released from mitochondria to cause apoptosis [6]. *MDV1*, *DNM1* and *FIS1*, which encode mitochondrial fission proteins [21,31,32]: *NUC1*, which encodes a yeast homolog of endonuclease G responsible for execution of apoptosis [41]. Cells were incubated in YPD for 24 h, treated with 21% ethanol for 3 h, and cfus were determined. The ratio of the cfu of cells after ethanol treatment relative to that just before ethanol treatment was expressed as the survival (cfu%). With a notable exception of *fis1*Δ and *nuc1*Δ, none of the mutations had a significant effect on sensitivity to ethanol (Fig. 2A). For the exception, *fis1*Δ and *nuc1*Δ exhibited significantly increased sensitivity to ethanol as compared to WT. The survival rate of *fis1*Δ cells treated with 21% ethanol after 3 h of incubation was 11.1 ± 2.6% (Fig. 2B), which is consistent with the survival rate for apoptosis [5,6], suggesting that *fis1*Δ dies from ethanol-induced apoptosis. Indeed, *fis1*Δ cells treated with ethanol at 21% for 3 h exhibited several apoptotic features, including chromatin condensation (Fig. 2C) fragmentation and DNA cleavage (50.7 ± 4.8% TUNEL-positive) (Fig. 2D and E), and DNA breakdown (data not shown). To determine if de novo protein synthesis is involved in ethanol-induced death in *fis1*Δ cells, *fis1*Δ cells were incubated from OD_{660} = 0.1 at 30 °C for 24 h, treated with 21% ethanol in the existence or absence of 40 μg/ml cycloheximide for 3 h and cfus were determined. Cfus of cycloheximide-treated and untreated samples were compared each time. Ethanol- and cycloheximide-treated cells showed higher rate of survival relative to ethanol-treated but cycloheximide-untreated cells (cycloheximide-treated cells exhibited 257.3 ± 93.4% (n = 4) increased colony formation relative to cycloheximide-untreated cells), suggesting that de novo protein synthesis is involved in ethanol-induced death of *fis1*Δ in this condition. To determine if caspase is involved in ethanol-induced death of *fis1*Δ, *fis1*Δ cells treated with 21% ethanol for 3 h in the existence or absence of the 40 μM caspase inhibitor DEVD for mammalian effecter caspase and cfus were determined. Cfus of DEVD-treated and untreated samples were determined and compared each time. Ethanol- and DEVD-treated cells showed higher rate of survival relative to ethanol-treated but DEVD-untreated cells (DEVD-treated cells exhibited 186.5% ± 21.8% (n = 3) increased colony formation relative to DEVD-untreated cells). These results suggest that ethanol-induced death of *fis1*Δ requires caspase activity. Taken together, these results indicate that Fis1 inhibits ethanol-induced apoptosis. *nuc1*Δ, which is deleted for endonuclease G, was also sensitive to ethanol (Fig. 2A), which is consistent with the reported sensitivity of *nuc1*Δ to hydrogen peroxide, acetic acid and amiodarone [41], suggesting that similar apoptotic programs involving endonuclease G is shared among ethanol-, hydrogen peroxide-, acetic acid- and amiodarone-induced apoptosis.

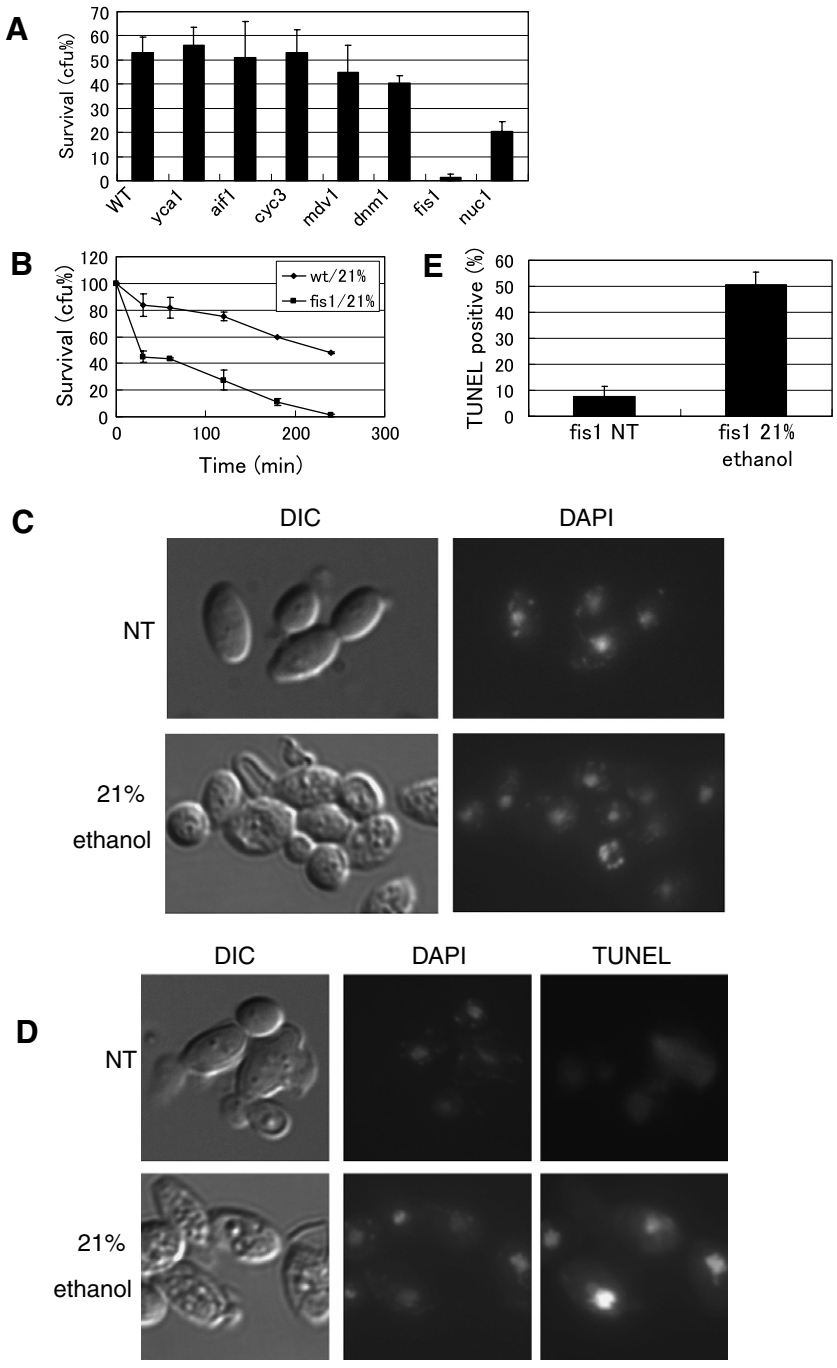

Fig. 2. The Fis1 pathway is involved in ethanol-induced yeast apoptosis. (A) Ethanol sensitivity of mutant strains with deletions in known apoptosis-related genes. Cells were treated with the 21% ethanol at RT for 3 h and the numbers of cfu were determined. The ratio of cfu after ethanol treatment relative to that before treatment is expressed as survival (cfu%). (B) Survival curve of WT and *fis1*Δ treated with 21% ethanol for various time periods. (C) DAPI staining of *fis1*Δ cells incubated for 3 h in the presence or absence of 21% ethanol. (D) TUNEL staining of *fis1*Δ cell incubated for 3 h in the presence or absence of 21% ethanol. (E) The ratio of TUNEL positive cells in *fis1*Δ incubated for 3 h in the presence or absence of 21% ethanol. Mean values ± S.E.M., or representatives of at least three independent experiments are shown.

3.3. Ethanol induces mitochondrial fragmentation

As Fis1 has been shown to be involved in mitochondrial fragmentation and mitochondrial fragmentation is an early maker of apoptosis in mammalian cells [23], we hypothesized that Fis1 mediates apoptosis in ethanol-treated yeast cells via regulation of mitochondrial fragmentation. To verify this hypothesis, mitochondria were visualized using a mitochondria-localized GFP marker (mito-GFP) [15,16]. Yeast cells with mito-GFP were treated with various concentrations of ethanol for 30 min. Using the marker, we observed that mitochondria were fragmented in response to 6%, 10% and 15% ethanol (Fig. 3A), clearly indicating that ethanol is a potent inducer of mitochondrial fragmentation.

3.4. Fis1 is required for mitochondrial fragmentation in response to ethanol

In order to help determine if Fis1 is required for mitochondrial fragmentation in response to ethanol, we next asked if mitochondrial fragmentation in response to ethanol is different from WT in the *fis1*Δ, or in mutant strains of other mitochondrial fission genes, *dnm1*Δ and *mdv1*Δ. In WT, *dnm1*Δ and *mdv1*Δ, mitochondrial fragmentation is observed after ethanol treatment (Fig. 3B). By contrast, treatment with ethanol does not cause mitochondrial fragmentation in *fis1*Δ (Fig. 3B), indicating that Fis1 is required for mitochondrial fragmentation in response to ethanol.

To explore the potential link between mitochondrial fragmentation and ethanol-induced death, the relationship between these two phenomena in this condition was investigated. Notably, treatment with 15% ethanol for 15 min is sufficient to induce mitochondrial fragmentation, but even after 60 min, 15% ethanol treatment does not result in cell death in WT or *fis1*Δ cells (Fig. 3C and D). Thus, mitochondrial fragmentation in response to ethanol is mediated by Fis1, but this mitochondrial fragmentation does not directly correlate with cell death.

3.5. Fis1 decreases ethanol-induced generation of ROS

Next, we hypothesized that Fis1 inhibits ethanol-induced apoptosis via a decrease in ROS generation, as the results of previous studies indicate that ROS generation is coupled to yeast apoptosis [6,7,29,39], Fis1 functions as a Bcl2-like protein in yeast [24,30], and Bcl2 functions to block generation of ROS in mammalian neural cells [25]. To test this, cells were treated with or without 21% or 23% ethanol for 3 h, mixed with ROS indicator H_2DCF-DA and subjected to FACS analysis (Fig. 4). The cells that were not treated with H_2DCF-DA showed background autofluorescence and thus, the difference

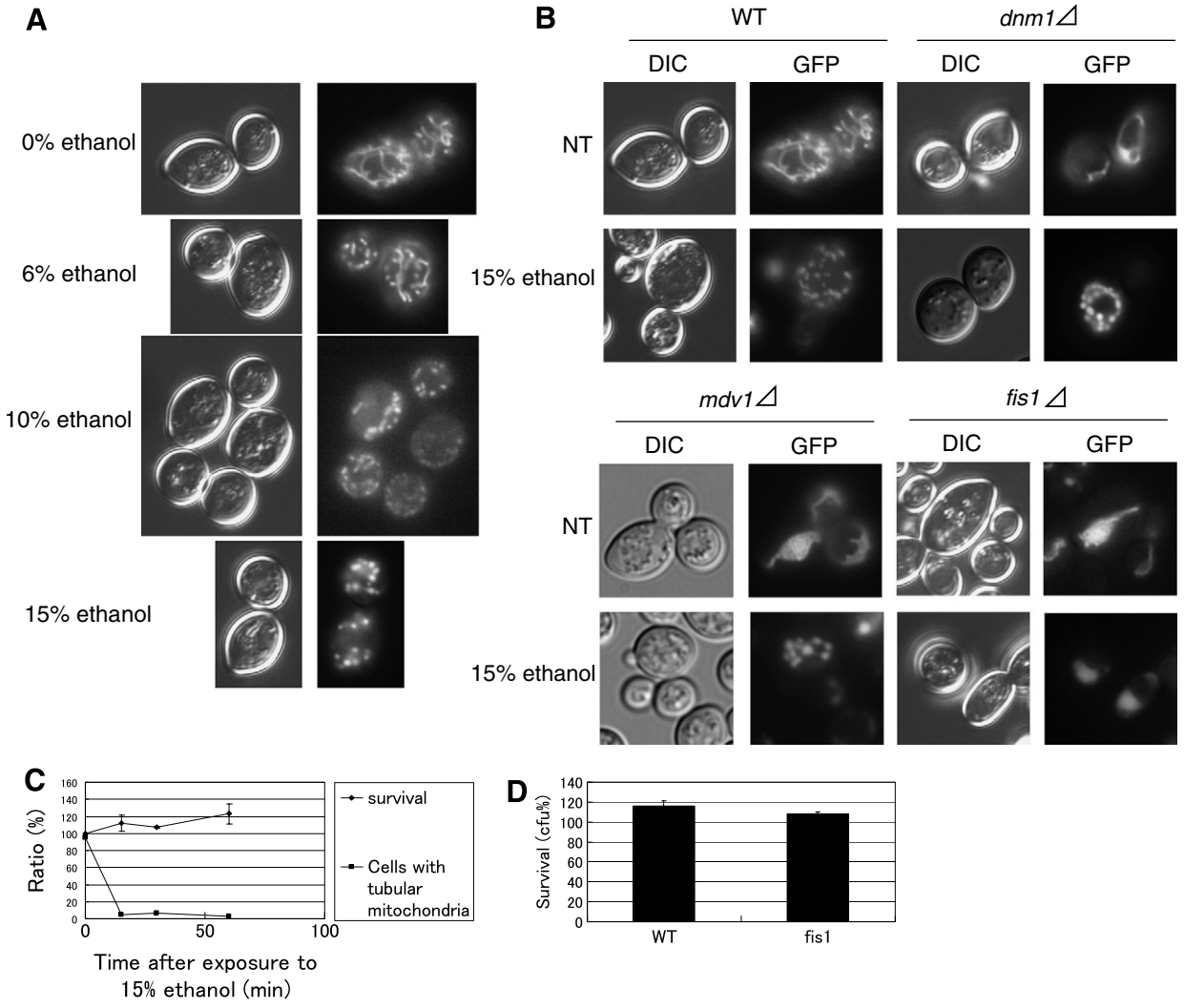

Fig. 3. Fis1 is required for mitochondrial fragmentation in response to ethanol. (A) WT cells expressing mito-GFP were treated with various concentrations of ethanol for 30 min at RT and observed under a fluorescent microscope. (B) WT, *dnm1*Δ, *mdv1*Δ and *fis1*Δ cells expressing mito-GFP were treated with 15% ethanol at RT for 30 min and observed under a fluorescent microscope. (C) WT cells expressing mito-GFP were treated with 15% ethanol for various time periods, and observed under a fluorescent microscope or the number of cfu was determined. (D) WT and *fis1*Δ cells were treated with 15% ethanol for 60 min and the number of cfu was determined. The ratio of cfu after ethanol treatment relative to that before ethanol treatment was expressed as survival (cfu%). Mean values ± S.E.M., or representatives of at least three independent experiments are shown.

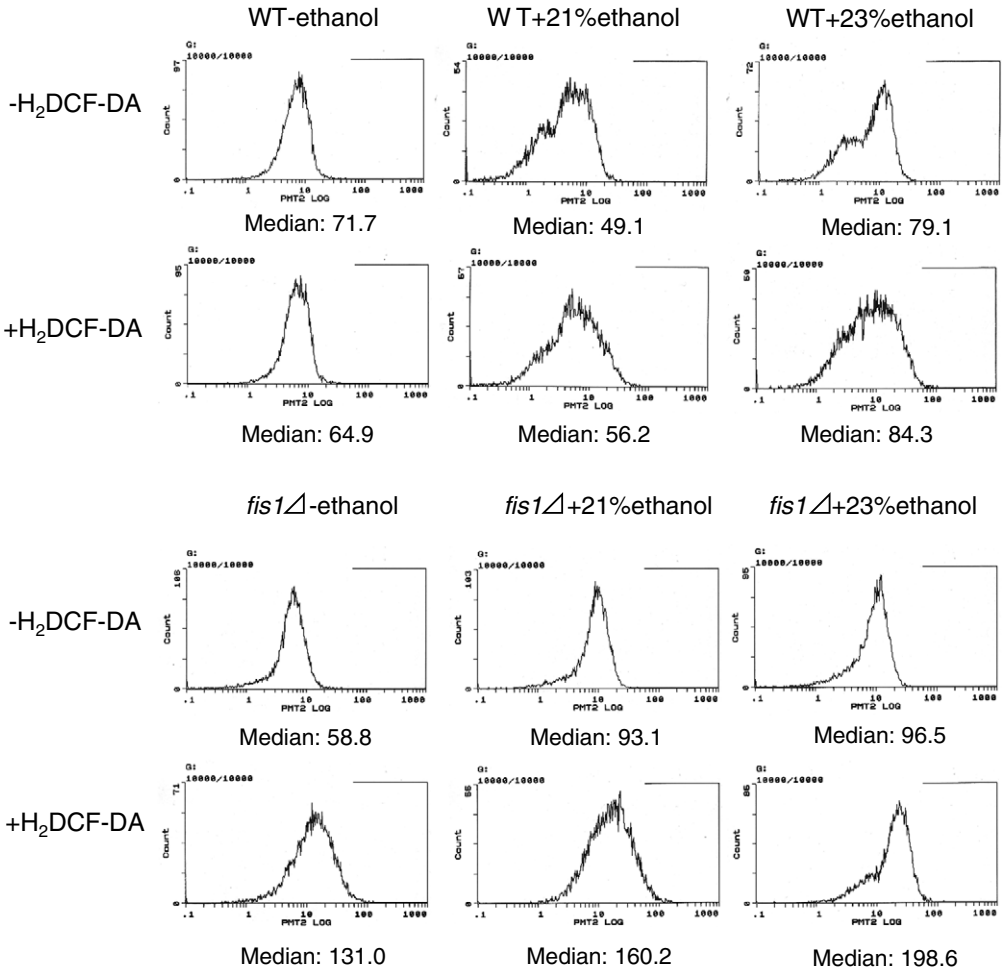

Fig. 4. Fis1 inhibits ethanol-induced generation of ROS. Cells were treated with or without H_2DCF-DA, incubated at RT for 2 h and subjected to FACS with an excitation of 488 nm and emission of 525–550 nm. Representatives of at least three independent experiments are shown.

in fluorescence between H_2DCF-DA-treated and untreated cells were used for intracellular ROS levels. Although ethanol-treated WT cells did not show a significant increase in the amount of ROS, both 21% and 23% ethanol-treated *fis1*Δ cells had significantly increased ROS amount. These results suggest that Fis1 indeed decreases the amount of intracellular ROS to inhibit apoptosis in response to ethanol. Consistent with the role of ROS in ethanol-induced apoptosis, gene disruptants for *SOD1*, *SOD2* and *GSH1*, which encode for proteins required for molecular defence against ROS (cytosolic superoxide dismutase [42], mitochondrial superoxide dismutase [43] and gamma-glutamylcysteine synthetase [44], respectively), exhibited decreased survival in response to treatment with 21% ethanol for 3 h (9.9 ± 5.6%, 17.1 ± 1.1%, and 25.7 ± 1.6%, respectively, relative to 53.0 ± 6.6% of WT). Together, these results suggest that Fis1 normally acts to reduce the level of ROS generation induced in response to ethanol and ROS generated in response to ethanol play significant roles in apoptosis.

From the results obtained in this study, the following model is proposed. As mitochondria sense the ethanol, they fragment in a Fis1-dependent manner. Eventually, ethanol induces generation of ROS, which appears to be mitigated directly or indirectly via the activity of Fis1. Finally, generated ROS induce apoptotic programs, including protein synthesis-dependent induction of chromatin condensation and fragmentation and DNA cleavage.

3.6. The mechanism of mitochondrial fragmentation in different situations

Several stimuli have been reported to cause mitochondrial fragmentation in yeast including acetic acid [24] and pheromone treatment [38], and involvement of mitochondrial pathway in yeast apoptotic program in response to these stimuli have been reported [6,29]. However, there are several discrepancies between the reported results and the results obtained in this study. For example, while this report elucidated that Fis1 is required for mitochondrial fragmentation which occurs upon treatment with 15% ethanol for 30 min, mitochondrial fragmentation which occurs upon treatment with 100 mM acetic acid for 3 h did not require Fis1 [24]. However, when we treated the *fis1*Δ cells with 100 mM acetic acid for 30 min, mitochondrial fragmentation was not observed (data not shown). This result suggests that the mechanism by which mitochondria fragment in response to apoptotic stimuli depends on the time span of treatment, and indicates that Fis1 is indeed required for mitochondrial fragmentation which

occurs within short time span (30 min) after treatment with apoptotic stimuli. Moreover, while *dnm1*Δ and *mdv1*Δ were resistant to acetic acid and heat shock in the previous report [24], the ethanol sensitivities of these mutants were not significantly altered in this report. This difference suggests that the roles of mitochondrial fragmentation machinery in apoptosis depend on the forms of apoptosis, which should be clarified in the future research.

3.7. The involvement of mitochondria in ethanol-tolerance mechanism

The results of genome-scale screens have revealed roles for a large numbers of mitochondrial genes in the ethanol tolerance of yeast [11]. However, the concrete mechanism of the involvement of mitochondria in ethanol tolerance has not previously been elucidated. The results in this report may help elucidate the involvement of mitochondrial function in ethanol tolerance. For example, one possible reason might be that mitochondrial membrane is differently affected by ethanol, as a result of the fact that the mitochondria has membrane lipid profiles that is quite different from the membranes of other organelles [26].

3.8. The involvement of ROS in ethanol-induced apoptosis

ROS have been reported to be involved in many forms of apoptosis in yeast [6,7,29,39], and our model that ROS are involved in ethanol-induced apoptosis is consistent with the results from previous reports which studied the effect of ethanol on yeast cells. For example, loss of superoxide dismutase rendered yeast cells ethanol sensitive [27], suggesting that antioxidative activity of superoxide dismutase protects cells from ROS induced by ethanol. Moreover, a previous study reported from microarray analysis that the genes involved in antioxidant defense are upregulated in response to ethanol [28]. Together with the data elucidated in this report, these evidences strengthen our model that ROS are generated in response to ethanol to cause apoptosis.

In summary, we characterized ethanol-induced cell death in yeast in comparison with apoptosis in yeast and other cell types and found that ethanol-induced apoptosis is mediated by the mitochondrial fission pathway. It seems likely that these results will provide insights into improving the fermentation process that will be highly relevant to the large alcohol beverage and bio-ethanol industries, including strategies for molecular genetic manipulation of yeast strains.

Acknowledgement: Mito-GFP (pYX142-mtGFP [15,16] was a kind gift from Dr. Koji Okamoto (University of Utah).

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