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Characterization of DNA Damage in Yeast Apoptosis Induced by Hydrogen Peroxide, Acetic Acid, and Hyperosmotic Shock

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Saccharomyces cerevisiae **has been reported to die, under certain conditions, from programmed cell death with apoptotic markers. One of the most important markers is chromosomal DNA fragmentation as indicated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. We found TUNEL staining in *S. cerevisiae* to be a consequence of both single- and double-strand DNA breaks, whereas in situ ligation specifically stained double-strand DNA breaks. Cells treated with hydrogen peroxide or acetic acid staining positively for TUNEL assay stained negatively for in situ ligation, indicating that DNA damage in both cases mainly consists of single-strand DNA breaks. Pulsed field gel electrophoresis of chromosomal DNA from cells dying from hydrogen peroxide, acetic acid, or hyperosmotic shock revealed DNA breakdown into fragments of several hundred kilobases, consistent with the higher order chromatin degradation preceding DNA laddering in apoptotic mammalian cells. DNA fragmentation was associated with death by treatment with 10 mM hydrogen peroxide but not 150 mM and was absent if cells were fixed with formaldehyde to eliminate enzyme activity before hydrogen peroxide treatment. These observations are consistent with a process that, like mammalian apoptosis, is enzyme dependent, degrades chromosomal DNA, and is activated only at low intensity of death stimuli.**

INTRODUCTION

Programmed cell death (PCD) is an active form of cell death in which the cell uses specialized cellular machinery to commit suicide. PCD is found in many different cells of diverse organisms, the purpose being removal of damaged cells or cells representing a threat to the integrity of the organism. Such cells are, for example, virus-infected cells or cancerous cells. PCD is also a normal part of development of multicellular organisms and also necessary for the maintenance of cellular homeostasis. Apoptosis, the most common form of PCD in eukaryotes, is associated with a number of characteristic markers depending on cell type and organism. The most common are cell shrinkage and development of bubble-like blebs on the surface (Kerr *et al*., 1972). The phospholipid phosphatidylserine, which is normally hidden on the inside of the plasma membrane, can be exposed on the cell surface (Fadok *et al*., 1992). There is also a two-step degradation of chromatin. The first step, sometimes referred to as higher order chromatin fragmentation, produces large fragments, 50–300 kilobases (kb) (Ucker *et al*., 1992; Oberhammer *et al*., 1993; Rusnak *et al*., 1996), perhaps reflecting chromatin higher order structure (Filipski *et al*., 1990). The second step is the formation of the characteristic internucleosomal DNA ladder of fragments differing in length by ~200 base pairs (Wyllie, 1980). A large portion of the fragments are blunt double-stranded DNA fragments (Staley *et al*., 1997; Didenko *et al*., 2003; Liu *et al*., 2003). Internucleosomal DNA laddering is often, but not always associated with apoptosis; some types of cells only undergo higher order chromatin fragmentation, without showing any internucleosomal DNA laddering (Ucker *et al*., 1992; Oberhammer *et al*., 1993).

One drawback of detecting apoptotic DNA fragmentation by gel electrophoresis is that the result is necessarily an average of a large pool of cells, loosing information on differences within the cell population. The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was developed for measuring DNA fragmentation at the single-cell level by incorporating biotylated or fluorescent dUTP at sites of free 3′-OH in DNA (Gavrieli *et al*., 1992). A *Saccharomyces cerevisiae* mutant carrying a temperature-sensitive mutation in the gene *CDC48* (Madeo *et al*., 1997) has been reported to die at nonpermissive temperature, showing many of the markers associated with apoptosis. One striking observation was positive TUNEL staining, indicating apoptotic DNA fragmentation.

However, no internucleosomal DNA laddering or other DNA fragmentation was found in the *CDC48* mutant (Madeo *et al*., 1997). The lack of specific cleavage of internucleosomal DNA was suggested to be caused by short linker DNA between nucleosomes (Lowary and Widom, 1989). The consensus among later reports on apoptosis-like cell death in yeast seems to be that apoptotic laddering or other apoptotic DNA fragmentation is not seen using normal DNA

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Abbreviations used: DSB, double-strand DNA break; ISL, in situ ligation; PBS, phosphate-buffered saline; PFGE, pulsed field gel electrophoresis; SSB, single-strand DNA break; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

agarose gel electrophoresis, although the cells are TUNEL positive (Madeo *et al*., 1997; Yamaki *et al*., 2001; Chen *et al*., 2003; Mazzoni *et al*., 2003).

TUNEL assay has been used in numerous studies to classify apoptotic death in yeast. TUNEL assay has been used to assess DNA damage in yeast as a consequence of death associated with expression of human *BAX* (Ligr *et al*., 1998); hydrogen peroxide (Madeo *et al*., 1999); acetic acid (Ludovico *et al*., 2001b); replicative ageing (Laun *et al*., 2001); overexpression of proteasomal substrate *STM1* in a proteasome-deficient strain (Ligr *et al*., 2001); high concentrations of mating pheromone (Severin and Hyman, 2002; Pozniakovsky *et al*., 2005); salt stress (Huh *et al*., 2002); UV radiation (Del Carratore *et al*., 2002); defective mRNA decapping (Mazzoni *et al*., 2003); expression of apoptotic mediator Nma111 (Fahrenkrog *et al*., 2004); chronological ageing (Herker *et al*., 2004); loss of *ubp10* (Orlandi *et al*., 2004); loss of yeast tumor suppressor homologue *SRO7/SOP1* (Wadskog *et al*., 2004); exposure to yeast killer toxins (Reiter *et al*., 2005), as a consequence of cell death in aging colonies (Vachova and Palkova, 2005); and hyperosmotic stress by 60% (wt/wt) glucose (Silva *et al*., 2005).

However, the type of DNA damage that is measured by TUNEL in *S. cerevisiae* has not been characterized. TUNEL assay is inherently unable to distinguish between nicks (single-strand breaks; SSBs) and double-strand breaks (DSBs), leaving the possibility that free 3′ ends detected by TUNEL are due to SSBs in TUNEL-positive *S. cerevisiae*. Because the TUNEL assay has turned a widely applied method for classifying yeast cell death, evaluation of the specificity of the technique is of critical importance for the meaning of TUNEL staining of *S. cerevisiae* and for the future characterization of potential apoptotic nucleases in this organism. To classify the type of DNA damage in TUNEL-positive *S. cerevisiae*, we applied the in situ ligation (ISL) assay that is based on ligation of a small fluorescent piece of double-stranded DNA to the DSBs in chromosomal DNA. Because it leaves SSBs undetected, it is a powerful method for finding DSBs in yeast. We also applied pulsed field agarose gel electrophoresis (PFGE) in order to determine potential higher order DNA fragmentation in *S. cerevisiae*. We report, for the first time, chromosome fragmentation into large fragments as a probable consequence of programmed cell death in *S. cerevisiae*. This fragmentation was evident in TUNEL-positive cells treated with hydrogen peroxide and acetic acid as well as in cells subjected to hyperosmotic shock.

MATERIALS AND METHODS

Strains

The yeast *S. cerevisiae* BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) was used throughout this work. This organism was maintained on YEPD agar slants containing glucose [2% (wt/vol)], yeast extract [1% (wt/vol)], peptone [2% (wt/vol)], and agar [2% (wt/vol)]. In experiments, the yeast cells were grown to mid-exponential phase in liquid synthetic complete (SC) medium containing 6.7 g/l yeast nitrogen base (Difco, Detroit, MI) with ammonium sulfate without amino acids, 20 g/l glucose, and 2 g/l amino acid drop-out mix. The drop-out mix was prepared by mixing 10 g of L-leucine, 0.2 g of para-aminobenzidine, and 0.5 g of L-adenine with 2 g each of L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-thronine, L-tryptophan, L-tyrosine, L-valine myo-inositol, and uracil to a total of 50.7 g. All amino acids were purchased from Sigma-Aldrich (St. Louis, MO). The experiments were performed in 50-ml flasks containing a 10:1 ratio of air to liquid phase and incubated on a mechanical shaker (200 rpm) at 30°C.

Induction of Apoptosis or Necrosis

Exponential growth-phase cells were harvested and suspended (10^7 cells ml^{-1}) in SC containing 10 mM or 150 mM H_2O_2, 175 mM acetic acid, pH 3.0, or 60% (wt/wt) glucose. The treatments were carried out for 200 min at 30°C with mechanical shaking (200 rpm) except for glucose treatment that was carried out for 10 h.

TUNEL Assay

DNA strand breaks were demonstrated by TUNEL with the In Situ Cell Death Detection kit, fluorescein, from Roche Molecular Biochemicals (Mannheim, Germany). Yeast cells were fixed with 3.7% (vol/vol) formaldehyde for 30 min at room temperature, washed three times with phosphate-buffered saline (PBS), and cell walls were digested with 24 μg/ml Zymolyase 100T (10^5 units/g; MP Biomedicals, Irvine, CA) at 37°C for 60 min. Ten microliters of the cell suspension was applied to a microscope slide and allowed to dry for 30 min at 37°C.

The slides were rinsed with PBS, incubated in permeabilization solution [0.1% (vol/vol) Triton X-100 and 0.1% (wt/wt) sodium citrate] for 2 min on ice, and rinsed twice with PBS.

For the treatment with endonucleases (DNaseI from Sigma-Aldrich; BsuRI, HhaI, and Bme1390I from MBI Fermentas, Hanover, MD; and nicking endonuclease N.BbvCIA from New England BioLabs, Beverly, MA), 30 U of the restriction enzyme were applied to the cells on microscope slides. The slides were placed in a humidified box for 1 h at 37°C. They were then washed for 15 min twice in PBS.

Slides were subsequently incubated with 10 μl of TUNEL reaction mixture, containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate dUTP, for 60 min at 37°C. Finally, the slides were rinsed three times with PBS, and a coverslip was mounted with a drop of antifading agent VECTASHIELD (Invitrogen, Carlsbad, CA). Observations were carried out using a Leitz Laborlux epifluorescence microscope equipped with a HBO-100 mercury lamp, filter set 40 (BP360/51, BP485/17, BP560/18) from Carl Zeiss (Jena, Germany), excitation filter BP 450–490 nm, beam splitter FT510 and emission filter LP520. Images were acquired with a SONY DXC-9100P camera using AXIOVISION software (Carl Zeiss).

In Situ ligation Assay

The sequence of the 21-mer fluorescent in situ ligation probe is as follows 5′-GCGCTAGACCXGGTCTAGCGC-3′. The symbol X represents a dTTP coupled to a fluorescein (fluorescein-dT). The probe was designed to form a hairpin loop by base pairing between bases 1–10 and 12–21. The probe was synthesized in 0.01 μmol of synthesis scale and purified with high-performance liquid chromatography (MWG, Ebersberg, Germany).

Microscope slides with cells were prepared in the same way as for the TUNEL assay, until and excluding addition of TUNEL reaction mixture. For Klenow enzyme treatment, to fill in 5′ overhangs, a 10-μl solution containing 70 mM Tris-HCl, pH 7.5, 70 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 mM dATP, dGTP, dCTP, and dTTP, and 5 U of Klenow enzyme (Roche Molecular Biochemicals) was added to the cells, incubated at 37°C for 30 min, and then washed in water two times for 10 min each. To remove 3′ overhangs using the Klenow enzyme, dNTPs were omitted from the reaction mixture.

Subsequently, 10 μl of the ligation buffer (66 mM of Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithioerythritol, 1 mM ATP, and 15% polyethylene glycol, mol. wt. 8000; Sigma-Aldrich) containing 14 μg/ml probe and 100 U/ml T4 DNA ligase (Roche Molecular Biochemicals) was applied to the cells. The slides were placed in a humidified box (in the dark) for 16 h at room temperature (23°C). They were then washed twice in water and once with 100 mM Tris-HCl, pH 9.0, for 10 min to increase the brightness of fluorescein before observation.

Pulsed Field Gel Electrophoresis

The methods used to prepare cells and agarose plugs were modified from a method previously published (Schwartz and Cantor, 1984). Untreated and treated cells were fixed in 3.7% (vol/vol) formaldehyde for 30 min, washed twice with 0.05 M EDTA, pH 8.0, by centrifugation and suspended at a concentration of 1.2×10^9 cells/ml in 0.05 M EDTA containing 3 mg/ml Zymolyase 100T for digestion. Plugs were formed by mixing the suspension of cells with the same volume of low melting agarose 2% (wt/wt) (SeaPlaque; Cambex Bio Science, Rockland, ME) at 40°C.

Plugs were incubated overnight in 0.45 mM EDTA, pH 8.0, and 7.5% (vol/vol) 2-mercaptoethanol at 37°C. Plugs were washed three times in TE (10 mM Tris, pH 8.0, and 1 mM EDTA, pH 8.0) and incubated overnight in 0.5 M EDTA, 10 mM Tris, pH 8.0, 1 mg/ml proteinase K (Sigma-Aldrich), and 1% (wt/wt) sodium-*N*-lauryl sarcosinate at 50°C. After washing five times, 30 min each time, with TE, pH 8.0, at room temperature, samples were stored at 4°C.

Treatment of the plugs before PFGE, with 1 U/ml S1 nuclease (MBI Fermentas), 200 U/ml nicking endonuclease N.BbvCIA (New England BioLabs), and 200 U/ml DNaseI (Sigma-Aldrich), was done in a final volume of 150 μl in the appropriate supplied buffer, for 60 min at 37°C.

PFGE was run in a CHEF-DRII Chiller System (Bio-Rad, Hercules, CA). PFGE gels were run in 0.5% Tris borate-EDTA buffer at 12°C with an angle of 120° with a voltage of 6 V/cm and switch times of 60 s for 15 h and 90 s for 7 h. Thereafter, gels were stained with 0.8% ethidium bromide solution for 45 min and distained for 20 min. Gels were visualized under UV light and analyzed using the EagleEye II Image Acquisition System (Stratagene, La Jolla, CA).

RESULTS

Death Induction by Hydrogen Peroxide, Acetic Acid, and Hyperosmotic Shock

We chose hydrogen peroxide and acetic acid for inducing PCD and TUNEL-positive phenotype in *S. cerevisiae*, to determine the nature of the DNA lesions detected by the TUNEL assay. Apoptosis-like cell death triggered by these two compounds has been extensively characterized by several laboratories (Madeo *et al*., 1999, 2002; Ludovico *et al*., 2001a, 2002; Fabrizio *et al*., 2004; Wissing *et al*., 2004; Giannattasio *et al*., 2005). Hyperosmotic shock by 60% (wt/wt) glucose has been recently described also to induce TUNEL-positive phenotype as well as other apoptotic markers (Silva *et al*., 2005). Cells were grown in preculture overnight and then for two generations in fresh medium before treatment with hydrogen peroxide, acetic acid, or 60% (wt/wt) glucose, as described in *Materials and Methods*. Samples were taken at time zero and at various time points, as indicated in Figure 1. Cells were spread on solid YPD medium and colony-forming units (c.f.u.) were counted after 3 d of incubation. The survival percentage was calculated as the number of c.f.u. obtained at each time point divided by the number of c.f.u. obtained at time 0. Treatment with 10 mM hydrogen peroxide initiated cell death (Figure 1A) without lag phase. Cell death by 175 mM acetic acid at pH 3.0 showed an initial lag phase of ~30 min, before cell death was evident (Figure 1B). Treatment for 200 min with 175 mM acetic acid at pH 3.0 or 10 mM hydrogen peroxide or 60% (wt/wt) glucose for 10 h (Figure 1C) resulted in ~10% survival, which in our and others' experience (Madeo *et al*., 1999) gives the highest yield of TUNEL-positive cells. Treatment for 20–25 min with 150 mM hydrogen peroxide (Figure 1D) gave about the same remaining survival as 10 mM for 200 min, thus representing a cell death induction approximately 10 times faster.

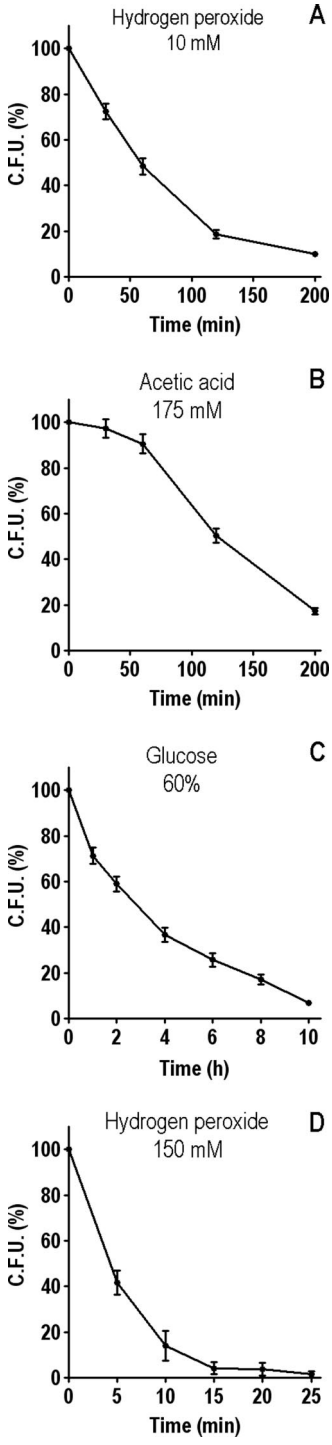

Figure 1. *S. cerevisiae* BY4741 was incubated with 10 mM hydrogen peroxide (A) or 175 mM acetic acid, pH 3.0 (B), 60% (wt/wt) glucose (C) or 150 mM hydrogen peroxide (D). Samples were taken at the time points indicated and plated on solid YPD media. c.f.u. were counted after 2 d of incubation at 30°C. Relative survival is plotted on the *x*-axis (100% corresponds to the number of c.f.u. at time 0). Values are mean ± SEM of three independent experiments.

TUNEL Stains Both SSBs and DSBs in S. cerevisiae

Cells grown as described in *Materials and Methods* were fixed with formaldehyde and subsequently digested with Zymolyase to remove the cell wall. Cells were treated with DNaseI to generate chromosomal DSBs. DNaseI cleaves both DNA strands at approximately the same site, producing DNA fragments with blunt ends or to a lesser extent protruding termini of one or two nucleotides. The cells were then stained with TUNEL protocol according to previously published protocols (Madeo *et al*., 1997). The nuclei was intensely stained by TUNEL in cells treated with DNaseI (Figure 2, DNaseI) This staining does not occur in non-treated cells (Figure 2, negative control). Positive TUNEL staining was also obtained by treatment with N.BbvCIA nicking endonuclease (Figure 2, N.BbvCIA). N.BbvCIA is a restriction enzyme engineered to cut one DNA strand only. This result shows that the free 3′-OH exposed in SSBs in *S. cerevisiae* is a good substrate for the terminal transferase in the TUNEL reaction. Cells treated with hydrogen peroxide or acetic acid (as described in *Materials and Methods*), but no added restriction enzymes, did also show TUNEL staining (Figure 2, hydrogen peroxide and acetic acid). TUNEL staining is more diffuse and has slightly higher background for the hydrogen peroxide-treated cells. Nevertheless, nuclei are clearly visible against the background.

ISL Assay Specifically Stains DSBs in S. cerevisiae

Cells were also subjected to ISL staining with a fluorescent double-stranded DNA hairpin probe according to the methods published by Didenko and coworkers (Didenko and Hornsby, 1996; Didenko *et al*., 1999). A small double strand fluorescent DNA probe is ligated to sites of DSBs. It has been shown to specifically stain mammalian apoptotic cells (Didenko *et al*., 2003). The probe cannot ligate to single-

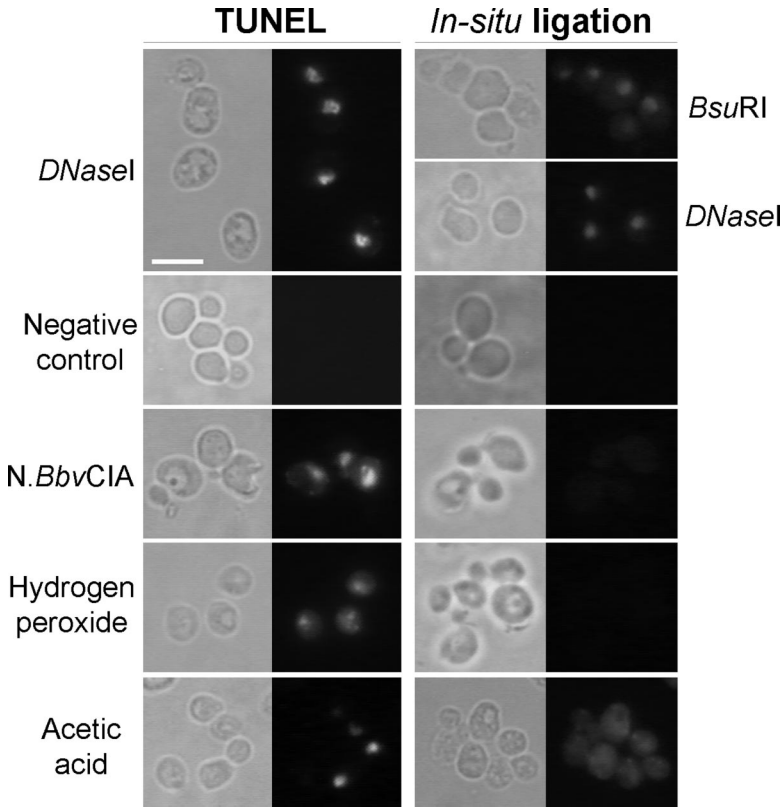

Figure 2. Bright field and epifluorescence images of TUNEL and in situ ligation-stained cells. Left column, TUNEL staining of exponentially growing cells, fixed and treated with DNaseI or N.BbvCIA nicking endonuclease. Right column, in situ ligation staining of exponentially growing cells, fixed and treated with BsuRI (blunt endonuclease) or DNaseI and N.BbvCIA (nicking endonuclease). Row marked negative control shows exponentially growing cells stained with TUNEL or in situ ligation staining. Rows marked hydrogen peroxide and acetic acid show cells incubated for 200 min with 10 mM hydrogen peroxide or 175 mM acetic acid, pH 3.0. Left, phase-contrast microscopy; right, fluorescence microscopy of the same cells. Bar, 5 μm.

stranded DNA breaks and is therefore in theory more specific for DSBs than TUNEL staining. Specific ISL staining, visually similar to staining by TUNEL, was evident in cells treated with DNaseI and the blunt restriction enzyme BsuRI (Figure 2, in situ ligation, DNaseI and BsuRI). There was no detectable staining in the absence of endonucleases (Figure 2, in situ ligation, negative control). ISL does not stain cells treated with nicking endonuclease N.BbvCIA, confirming that SSBs do not facilitate ligation of the ISL probe (Figure 2, N.BbvCIA). These results show that both TUNEL and ISL procedures are equally efficient at detecting DSBs in *S. cerevisiae* chromosomal DNA, but TUNEL assay also detects nicked DNA. Cells treated with peroxide or acetic acid did not show any ISL staining (Figure 2, hydrogen peroxide and acetic acid), indicating that these cells do not contain blunt DSBs.

Staggered DSBs Can Be Discriminated by ISL in Combination with Klenow DNA Polymerase

ISL has been used to distinguish between different types of staggered DSBs in combination with Klenow DNA polymerase (Didenko *et al.*, 2003). Theoretically, if DSBs with 5′ overhangs are present, the DNA polymerase activity of Klenow will synthesize the missing DNA in the presence of dNTPs and produce blunt ends. In contrast, if DSBs breaks with 3′ overhangs are present, the Klenow 5′-3′ exonuclease activity will degrade the overhang until the DNA is blunt.

To test whether ISL could discriminate between different types of DSBs in *S. cerevisiae*, we performed ISL on cells treated with endonucleases producing 5′ or 3′ overhang. Cells were grown, fixed, and Zymolyase treated, as described above, and subsequently treated with restriction endonucleases, creating either 1-base pair 3′ overhangs (HhaI) or 1-base pair 5′ overhangs (Bme1390I). None of the enzyme-treated samples showed any positive staining with ISL assay (Figure 3, − Klenow). This shows that ISL does not stain staggered DSBs. ISL staining is restored to cells treated with HhaI and to cells treated with Bme1390I, by treatment with Klenow DNA polymerase and Klenow DNA polymerase plus dTNPs, respectively (Figure 3, + Klenow). This means that different types of staggered double-stranded DNA ends can be discriminated by the ISL assay in yeast.

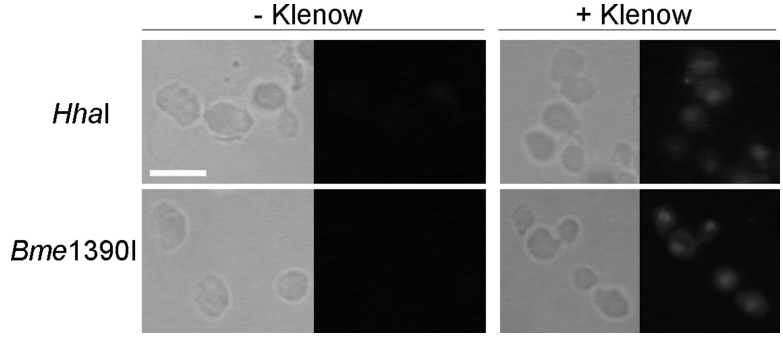

Figure 3. Bright field and epifluorescence images of in situ ligation-stained cells. Exponentially growing cells were treated with HhaI (1-base pair 3′ overhang) and Bme1390I (1-base pair 5′ overhang) endonucleases. The cells in the right column were treated with Klenow (+ Klenow, HhaI) or Klenow + dNTPs (+ Klenow, Bme1390I) before in situ ligation staining. Left, phase-contrast microscopy; right, fluorescence microscopy of the same cells. Bar, 5 μm.

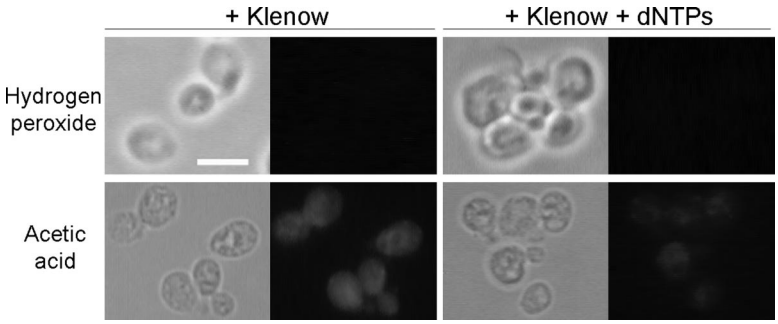

Figure 4. Bright field and epifluorescence images of in situ ligation-stained cells. In situ ligation staining of cells incubated for 200 min with 10 mM hydrogen peroxide or 175 mM acetic acid, pH 3.0. The cells were treated with Klenow (left column) or Klenow + dNTPs (right column) before in situ ligation staining. Left, phase-contrast microscopy; right, fluorescence microscopy of the same cells, Bar, 5 μm.

The intensity is lower than for the DNaseI or BsuRI treatment (Figure 2), probably due to incomplete transformation of staggered DSBs.

We reasoned that the lack of ISL staining of cells treated with hydrogen peroxide or acetic acid (Figure 2, hydrogen peroxide, acetic acid) may be that they contain staggered DSBs that are not detectable by ISL. We added Klenow or Klenow and dNTPs to cells treated with hydrogen peroxide or acetic acid in a manner similar to the experiment described in Figure 3, but the ISL staining was still negative (Figure 4). This indicates that hydrogen peroxide- or acetic acid-treated cells do not contain staggered DSBs with 1-base pair overhangs.

Chromosomal DNA Is Damaged in Cells Treated with Hydrogen Peroxide, Acetic Acid, or High Concentrations of Glucose

In our hands, ISL assay together with Klenow or Klenow + dNTPs is limited in the overhang length of staggered DSBs that the method can overcome. We treated cells with enzymes creating four-base pair overhangs with subsequent Klenow transformation to blunt DSBs, and we noted a considerable decrease in the ISL signal (our unpublished data) compared with the treatments with HhaI and Bme1390I (Figure 3). This led to the hypothesis that cells treated with hydrogen peroxide or acetic acid may have staggered DSBs with long overhangs, undetectable by ISL.

To overcome this difficulty, we analyzed chromosomal DNA from cells treated with hydrogen peroxide, acetic acid, or glucose-induced hyperosmotic shock using PFGE (Figure 5A). Samples were taken at six time points between zero and 200 min (hydrogen peroxide, acetic acid) or 10 h (hyperosmotic shock). DNA from cells treated with hydrogen peroxide after 200 min (Figure 5A, hydrogen peroxide, lane 200 min) was completely degraded to a smear of fragments slightly shorter than *S. cerevisiae* chromosome I (225 kb) but still of a considerable size. The DNA from acetic acid-treated cells showed less degradation but still a visible smear and chromosomal bands of lower intensity (Figure 5A, acetic acid, lanes 60–200 min). DNA from cells killed by hyperosmotic shock showed degradation over 10 h comparable to that of hydrogen peroxide-treated cells (Figure 5A, glucose 60%, lanes 6–10 h). This is the first time that clear DNA degradation has been shown in *S. cerevisiae* associated with PCD.

DNA Fragmentation by Hydrogen Peroxide Requires Active Enzymes

Cells were fixed with 3.7% (vol/vol) formaldehyde before treatment with 10 mM hydrogen peroxide for 200 min or 150 mM for 25 min (Figure 5B) to test whether the presence of active enzymes is necessary for DNA fragmentation to proceed. Fixing cells with formaldehyde preserves the structure of cells and enzymes, but it eliminates enzymatic activity. The fixed cells treated with hydrogen peroxide show no DNA damage, indicating that the DNA fragmentation requires some enzymatic activity within the cell and that fragmentation is not due to a direct chemical reaction between hydrogen peroxide and DNA (Figure 5B). In addition, purified yeast chromosomes were not degraded by 10 mM hydrogen peroxide in vitro for 200 min (our unpublished data).

DNA Fragmentation by Hydrogen Peroxide Does Not Occur after Treatment with High Concentrations of Hydrogen Peroxide

A common observation regarding PCD is that specific markers of PCD usually only occur within a rather limited window of treatment intensity. At too high intensity of the treatment, the cell is presumed to die from complete breakdown (necrosis) before any PCD process can be initiated. Chromosomal DNA was fragmented during 200-min treatment with 10 mM hydrogen peroxide (Figure 5A). During this process, the fraction of viable cells decreases from 100 to ~5–10% (Figure 1A). The DNA in *S. cerevisiae* cells treated with 150 mM hydrogen peroxide remained intact (Figure 5C) for the 25 min necessary for a decrease from 100 to ~1% surviving cells (Figure 1C). This result shows that cell death is only associated with DNA fragmentation for treatment of relatively low intensity.

DNA Damage in Hydrogen Peroxide- or Acetic Acid-treated Cells Is Primarily Made Up of SSBs

Lack of ISL staining of cells treated with hydrogen peroxide or acetic acid combined with the finding that TUNEL assay is not specific for DSBs led us to the hypothesis that the main type of DNA damage detected by TUNEL in our experiments consists of SSBs. This hypothesis can be tested by treatment of the DNA with S1 nuclease that will preferably attack at sites of single-stranded DNA breaks, reducing SSBs to DSBs, causing the DNA to migrate faster on the gel. Figure 5D shows a PFGE gel with DNA from cells treated with hydrogen peroxide or acetic acid, with and without treatment with S1 nuclease. Untreated DNA (Figure 5D, lane control) shows little degradation with S1 nuclease treatment (Figure 5D, lane control S1), indicating that no or few single-stranded breaks are present in untreated cells. Comparing the same time points for hydrogen peroxide- and acetic acid-treated cells without (Figure 5A) and with S1 treatment (Figure 5D, lanes marked +), there is an enhanced degradation upon S1 treatment. We conclude that the main form of DNA damage in hydrogen peroxide- and acetic acid-treated cells consists of single-stranded DNA breaks.

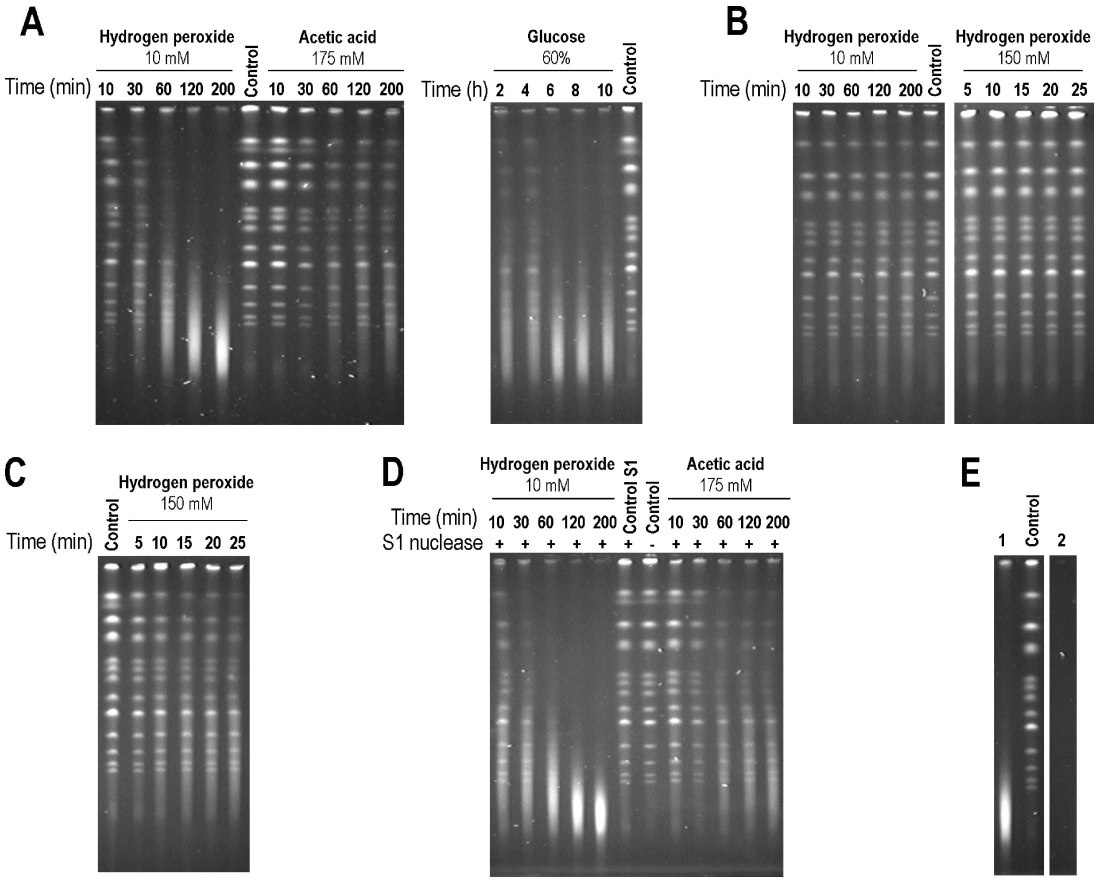

Figure 5. Genomic DNA analyzed by PFGE from viable cells (A and C–E) or fixed *S. cerevisiae* cells (B). Cells were exposed to 10 mM hydrogen peroxide, 175 mM acetic acid, pH 3.0, 60% (wt/wt) glucose, or 150 mM hydrogen peroxide as indicated in the figure. Lanes marked as control were loaded with DNA isolated from exponentially growing cells without further treatment. Samples were collected after various periods as indicated (minutes) except for treatment with 60% (wt/wt) glucose, where time points are indicated in hours. (D) Cells were exposed to 10 mM hydrogen peroxide or 175 mM acetic acid, pH 3.0, and isolated chromosomal DNA was subsequently treated with S1 nuclease to degrade nicked DNA. Lane marked control S1 is similar to control lanes, but the DNA was treated with S1 nuclease before PFGE. (E) Lanes 1 and 2 show chromosomal DNA from nontreated cells, digested with N.BbvCIA nicking endonuclease (1) or DNaseI (2).

N.BbvCIA Nicking Endonuclease and Hydrogen Peroxide Treatment Yield Similar DNA Damage

It is evident from the images showing DNA damage (Figure 5A) that although the chromosomes are broken down to the point of not being visible, the resulting fragments are still several hundreds of kilobases and do not seem to break down into smaller fragments. We treated isolated DNA with DNaseI and N.BbvCIA nicking endonuclease (Figure 5E). Isolated DNA treated with DNaseI broke down into fragments too small to be visible on the gel (Figure 5E, lane 2), whereas DNA treated with N.BbvCIA (Figure 5E, lane 1) revealed DNA fragmentation with the same visual appearance on the gel as cells treated with hydrogen peroxide for 200 min (Figure 5A, lane 200 min). This observation is consistent with the conclusion that DNA damage in hydrogen peroxide-treated cells are primarily a consequence of accumulation of SSBs.

DISCUSSION

Apoptotic chromosomal DNA fragmentation is often an integral part of apoptosis in higher organisms. This fragmentation can be detected by gel electrophoresis, but also by in situ by DNA staining methods such as TUNEL, nick translation, or ISL. On the contrary, DNA fragmentation in *S. cerevisiae* undergoing apoptosis-like PCD has so far only been evident by TUNEL-positive phenotype. We detected large DNA fragments (several hundred kilobases) in cells dying from treatment with acetic acid, hydrogen peroxide, or from hyperosmotic shock. These fragments are too large to be detected by normal DNA agarose electrophoresis.

No DNA fragmentation was found in fixed cells treated with hydrogen peroxide, ruling out unspecific chemical or other degradation of DNA by hydrogen peroxide. Treatment of cells with 150 mM hydrogen peroxide (Figure 1D) results in cell killing approximately 10 times faster than treatment with 10 mM hydrogen peroxide (Figure 1A). DNA breakdown is nearly undetectable for cells killed with 150 mM hydrogen peroxide between 100 and ~1% survival (Figure 5C). This means that cells treated with 150 mM hydrogen peroxide die without DNA fragmentation, supporting that this process only functions at low intensities of death stimuli. This difference between high and low intensity treatment has been reported on numerous occasions (Madeo *et al.*, 1999; Ludovico *et al.*, 2001b, 2002; Huh *et al.*, 2002; Reiter *et al.*, 2005) and is a further support for the concept of an active cell death process.

The relatively long DNA fragments (Figure 5A) are likely formed through the accumulation of SSBs, because no blunt

fragments could be detected by ISL. The formation of these fragments could be analogous to the high-molecular-weight DNA (50- to 300-kb) fragmentation in mammalian cells (Oberhammer *et al*., 1993), because the latter has been described as a gradual accumulation of SSBs in apoptotic thymocytes (Walker *et al*., 1997), especially after hydrogen peroxide treatment (Bai and Konat, 2003; Konat, 2003).

We did not find blunt DSBs in *S. cerevisiae*, normally associated with apoptosis in mammalian cells (Staley *et al*., 1997; Didenko *et al*., 2003; Liu *et al*., 2003) by using ISL. DNA degradation associated with yeast PCD could be of a different nature than DNA degradation in higher organisms. However, our results do not rule out the existence of DSBs in *S. cerevisiae* challenged with other death stimuli or conditions not tested in this work.

Future work will focus on the identification of the nuclease(s) involved in the DNA fragmentation in *S. cerevisiae*. Our preliminary results preclude the involvement of the candidate genes *APN1* and *RAD1,10*, which encode the nucleases involved in the base excision and the nucleotide excision repair pathways, respectively. The gene *TAT-D* was proposed as a candidate for an apoptotic nuclease in *S. cerevisiae*, but because TUNEL-positive phenotype is stronger in the absence of *TAD-D* it is not an obvious candidate (Qiu *et al*., 2005).

Our studies show that the phenotype of yeast PCD differs in some respects from mammalian apoptosis but also that there are striking similarities. These similarities will aid in the further analysis of *S. cerevisiae* as a model of certain aspects of apoptosis.

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REFERENCES

Bai, H., and Konat, G. W. (2003). Hydrogen peroxide mediates higher order chromatin degradation. Neurochem. Int. *42*, 123–129.

Chen, S. R., Dunigan, D. D., and Dickman, M. B. (2003). Bcl-2 family members inhibit oxidative stress-induced programmed cell death in *Saccharomyces cerevisiae*. Free Radic. Biol. Med. *34*, 1315–1325.

Del Carratore, R., Della Croce, C., Simili, M., Taccini, E., Scavuzzo, M., and Sbrana, S. (2002). Cell cycle and morphological alterations as indicative of apoptosis promoted by UV irradiation in *S. cerevisiae*. Mutat. Res. *513*, 183–191.

Didenko, V. V., Boudreaux, D. J., and Baskin, D. S. (1999). Substantial background reduction in ligase-based apoptosis detection using newly designed hairpin oligonucleotide probes. Biotechniques *27*, 1130–1132.

Didenko, V. V., and Hornsby, P. J. (1996). Presence of double-strand breaks with single-base 3′ overhangs in cells undergoing apoptosis but not necrosis. J. Cell Biol. *135*, 1369–1376.

Didenko, V. V., Ngo, H., and Baskin, D. S. (2003). Early necrotic DNA degradation: presence of blunt-ended DNA breaks, 3′ and 5′ overhangs in apoptosis, but only 5′ overhangs in early necrosis. Am. J. Pathol. *162*, 1571–1578.

Fabrizio, P., Battistella, L., Vardavas, R., Gattazzo, C., Liou, L. L., Diaspro, A., Dossen, J. W., Gralla, E. B., and Longo, V. D. (2004). Superoxide is a mediator of an altruistic aging program in *Saccharomyces cerevisiae*. J. Cell Biol. *166*, 1055–1067.

Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. *148*, 2207–2216.

Fahrenkrog, B., Sauder, U., and Aebi, U. (2004). The *S. cerevisiae* HtrA-like protein Nma111p is a nuclear serine protease that mediates yeast apoptosis. J. Cell Sci. *117*, 115–126.

Filipski, J., Leblanc, J., Youdale, T., Sikorska, M., and Walker, P. R. (1990). Periodicity of DNA folding in higher order chromatin structures. EMBO J. *9*, 1319–1327.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. *119*, 493–501.

Giannattasio, S., Guaragnella, N., Corte-Real, M., Passarella, S., and Marra, E. (2005). Acid stress adaptation protects *Saccharomyces cerevisiae* from acetic acid-induced programmed cell death. Gene *354*, 93–98.

Herker, E., Jungwirth, H., Lehmann, K. A., Maldener, C., Frohlich, K. U., Wissing, S., Buttner, S., Fehr, M., Sigrist, S., and Madeo, F. (2004). Chronological aging leads to apoptosis in yeast. J. Cell Biol. *164*, 501–507.

Huh, G. H., Damsz, B., Matsumoto, T. K., Reddy, M. P., Rus, A. M., Ibeas, J. I., Narasimhan, M. L., Bressan, R. A., and Hasegawa, P. M. (2002). Salt causes ion disequilibrium-induced programmed cell death in yeast and plants. Plant J. *29*, 649–659.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239–257.

Konat, G. W. (2003). H2O2-induced higher order chromatin degradation: a novel mechanism of oxidative genotoxicity. J. Biosci. *28*, 57–60.

Laun, P., Pichova, A., Madeo, F., Fuchs, J., Ellinger, A., Kohlwein, S., Dawes, I., Frohlich, K. U., and Breitenbach, M. (2001). Aged mother cells of *Saccharomyces cerevisiae* show markers of oxidative stress and apoptosis. Mol. Microbiol. *39*, 1166–1173.

Ligr, M., Madeo, F., Fröhlich, E., Hilt, W., Fröhlich, K. U., and Wolf, D. H. (1998). Mammalian Bax triggers apoptotic changes in yeast. FEBS Lett. *438*, 61–65.

Ligr, M., Velten, I., Frohlich, E., Madeo, F., Ledig, M., Frohlich, K. U., Wolf, D. H., and Hilt, W. (2001). The proteasomal substrate Stm1 participates in apoptosis-like cell death in yeast. Mol. Biol. Cell *12*, 2422–2432.

Liu, Q. Y., Ribecco-Lutkiewicz, M., Carson, C., Testolin, L., Bergeron, D., Kohwi-Shigematsu, T., Walker, P. R., and Sikorska, M. (2003). Mapping the initial DNA breaks in apoptotic Jurkat cells using ligation-mediated PCR. Cell Death Differ. *10*, 278–289.

Lowary, P. T., and Widom, J. (1989). Higher-order structure of *Saccharomyces cerevisiae* chromatin. Proc. Natl. Acad. Sci. USA *86*, 8266–8270.

Ludovico, P., Rodrigues, F., Almeida, A., Silva, M. T., Barrientos, A., and Corte-Real, M. (2002). Cytochrome c Release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. Mol. Biol. Cell *13*, 2598–2606.

Ludovico, P., Sansonetty, F., and Corte-Real, M. (2001a). Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. Microbiology *147*, 3335–3343.

Ludovico, P., Sousa, M. J., Silva, M. T., Leao, C., and Corte-Real, M. (2001b). Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid. Microbiology *147*, 2409–2415.

Madeo, F., Fröhlich, E., and Fröhlich, K. U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. J. Cell Biol. *139*, 729–734.

Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., and Fröhlich, K. U. (1999). Oxygen stress: a regulator of apoptosis in yeast. J. Cell Biol. *145*, 757–767.

Madeo, F., *et al*. (2002). A caspase-related protease regulates apoptosis in yeast. Mol. Cell *9*, 911–917.

Mazzoni, C., Mancini, P., Verdone, L., Madeo, F., Serafini, A., Herker, E., and Falcone, C. (2003). A truncated form of KlLsm4p and the absence of factors involved in mRNA decapping trigger apoptosis in yeast. Mol. Biol. Cell *14*, 721–729.

Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R., and Sikorska, M. (1993). Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J. *12*, 3679–3684.

Orlandi, I., Bettiga, M., Alberghina, L., and Vai, M. (2004). Transcriptional profiling of ubp10 null mutant reveals altered subtelomeric gene expression and insurgence of oxidative stress response. J. Biol. Chem. *279*, 6414–6425.

Pozniakovsky, A. I., Knorre, D. A., Markova, O. V., Hyman, A. A., Skulachev, V. P., and Severin, F. F. (2005). Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. J. Cell Biol. *168*, 257–269.

Qiu, J., Yoon, J. H., and Shen, B. (2005). Search for apoptotic nucleases in yeast: role of Tat-D nuclease in apoptotic DNA degradation. J. Biol. Chem. *280*, 15370–15379.

Reiter, J., Herker, E., Madeo, F., and Schmitt, M. J. (2005). Viral killer toxins induce caspase-mediated apoptosis in yeast. J. Cell Biol. *168*, 353–358.

Rusnak, J. M., Calmels, T. P., Hoyt, D. G., Kondo, Y., Yalowich, J. C., and Lazo, J. S. (1996). Genesis of discrete higher order DNA fragments in apoptotic human prostatic carcinoma cells. Mol. Pharmacol. *49*, 244–252.

Schwartz, D. C., and Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell *37*, 67–75.

Severin, F. F., and Hyman, A. A. (2002). Pheromone induces programmed cell death in *S. cerevisiae*. Curr. Biol. *12*, R233–R235.

Silva, R. D., Sotoca, R., Johansson, B., Ludovico, P., Sansonetty, F., Silva, M. T., Peinado, J. M., and Corte-Real, M. (2005). Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. Mol. Microbiol. *58*, 824–834.

Staley, K., Blaschke, A. J., and Chun, J. (1997). Apoptotic DNA fragmentation is detected by a semiquantitative ligation-mediated PCR of blunt DNA ends. Cell Death Differ. *4*, 66–75.

Ucker, D. S., Obermiller, P. S., Eckhart, W., Apgar, J. R., Berger, N. A., and Meyers, J. (1992). Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic T lymphocytes. Mol. Cell. Biol. *12*, 3060–3069.

Vachova, L., and Palkova, Z. (2005). Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia. J. Cell Biol. *169*, 711–717.

Wadskog, I., Maldener, C., Proksch, A., Madeo, F., and Adler, L. (2004). Yeast lacking the SRO7/SOP1-encoded tumor suppressor homologue show increased susceptibility to apoptosis-like cell death on exposure to NaCl stress. Mol. Biol. Cell *15*, 1436–1444.

Walker, P. R., Leblanc, J., and Sikorska, M. (1997). Evidence that DNA fragmentation in apoptosis is initiated and propagated by single-strand breaks. Cell Death Differ. *4*, 506–515.

Wissing, S., *et al*. (2004). An AIF orthologue regulates apoptosis in yeast. J. Cell Biol. *166*, 969–974.

Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature *284*, 555–556.

Yamaki, M., Umehara, T., Chimura, T., and Horikoshi, M. (2001). Cell death with predominant apoptotic features in *Saccharomyces cerevisiae* mediated by deletion of the histone chaperone ASF1/CIA1. Genes Cells *6*, 1043–1054.