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The Proteasomal Substrate Stm1 Participates in Apoptosis-like Cell Death in Yeast

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We have identified the yeast gene *STM1* in an overexpression screen for new proteasomal substrates. Stm1 is unstable in wild-type cells and stabilized in cells with defective proteasomal activity and thus a bona fide substrate of the proteasome. It is localized in the perinuclear region and is required for growth in the presence of mutagens. Overexpression in cells with impaired proteasomal degradation leads to cell death accompanied with cytological markers of apoptosis: loss of plasma membrane asymmetry, chromatin condensation, and DNA cleavage. Cells lacking Stm1 display deficiency in the apoptosis-like cell death process induced by treatment with low concentrations of H_2O_2. We suggest that Stm1 is involved in the control of the apoptosis-like cell death in yeast. Survival is increased when Stm1 is completely missing from the cells or when inhibition of Stm1 synthesis permits proteasomal degradation to decrease its amount in the cell. Conversely, Stm1 accumulation induces cell death. In addition we identified five other genes whose overexpression in proteasomal mutants caused similar apoptotic phenotypes.

INTRODUCTION

Multicellular organisms are in the state of dynamic equilibrium, sustained by the mutually opposing processes of cell division and cell death. The importance of programmed cell death to maintain the integrity of metazoans is widely appreciated, but is there a place for this process in the life cycle of single cell organisms?

The existence of programmed cell death in bacteria is now firmly established (Engelberg-Kulka and Glaser, 1999). Recently we have identified a translation-dependent programmed cell death process also in the unicellular eukaryote *Saccharomyces cerevisiae* (Fröhlich and Madeo, 2000). We observed that yeast cells underwent cell death due to presence of the *cdc48-S565G* mutation (Madeo *et al.*, 1997), overexpression of the mammalian apoptotic cell death regulator Bax (Ligr *et al.*, 1998), or exposure to oxidative conditions (Madeo *et al.*, 1999). This process resembled apoptosis, a form of programmed cell death indispensable for development and homeostasis of metazoan organisms (Webb *et al.*, 1999). The occurrence of cytological markers of metazoan apoptosis in yeast, such as loss of plasma membrane asymmetry, chromatin condensation and margination, fragmentation of DNA, and membrane blebbing, as well as the identification of reactive oxygen species as a common regulator (Madeo *et al.*, 1999), led us to suggest that the basic mechanism of apoptosis is present already in this unicellular eukaryote (Fröhlich and Madeo, 2000). This view is further supported by recent reports that the orthologues of Cdc48 regulate the apoptotic pathways of *Caenorhabditis elegans* (Wu *et al.*, 1999) and humans (Shirogane *et al.*, 1999).

Cdc48 is an ATPase of the AAA family associated with a variety of cellular activities. Notably, Cdc48p is emerging as a factor involved in the regulation of the evolutionary conserved ubiquitin-proteasome system (Ghislain *et al.*, 1996; Dai *et al.*, 1998; Koegl *et al.*, 1999; Meyer *et al.*, 2000). Substrates to be degraded by this pathway are first covalently tagged with the small protein ubiquitin by an enzymatic cascade consisting of ubiquitin activating and conjugating enzymes, in most cases in cooperation with additional substrate-specific recognition elements. Polyubiquitylated proteins are recognized and degraded by the 26S proteasome, a multisubunit multicatalytic protease (Hilt and Wolf, 1996). In mammals, inhibition of proteasome-dependent proteolysis leads to either repression or induction of apoptosis, depending on the proliferative status of the particular cell type (Drexler, 1998). It has been suggested that in proliferating cells the proteasome continuously degrades an activator of

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Table 1. Yeast strains

Strain	Genotype	Reference/Source
WCG4/a	*MAT***a** *his3-11,15 leu2-3,112 ura3*	Heinemeyer *et al.*, 1993
YHI29-1	*MAT***a** *pre1-1*	Heinemeyer *et al.*, 1991
YHI29-14	*MAT***a** *pre1-1 pre4-1*	Hilt *et al.*, 1993
YML1	*MAT***a** *pre1-1 cyh2* [pML1]	This study
YML2	*MAT***a** *pre1-1 pre4-1 cyh2* [pML1]	This study
YIV2	*MAT***a** *stm1-Δ1::kanMX*	This study
YL280	*MAT***a** *STM1::IRS*	This study
YL286	*MAT***a** *STM1::IRS pre1-1 pre4-1*	This study

All strains are isogenic with WCG4/a.

apoptosis. Curbing proteasomal activity is thought to result in accumulation of this hypothetical regulator and thereby activation of the apoptotic cell death cascade (Drexler, 1997).

Does proteasomal degradation play a similar role in the apoptosis-like cell death process in yeast? To answer this question, we screened for genes that cause this type of death when overexpressed in cells with defective proteasomes.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Media

To construct plasmid pML1, a *PRE1*-containing *Bam*HI-*Xho*I fragment of p13/*PRE1* (a gift of W. Heinemeyer) was ligated into *Bam*HI-*Xho*I sites of pRS318 (*CYH2 LEU2 CEN6*; Sikorski and Boeke, 1991). The integrative plasmid pL090 was assembled from the *Nhe*I-*Mlu*I fragment of pYES2 (Invitrogen, San Diego, CA), a polymerase chain reaction (PCR) fragment of the *STM1* terminator (flanked by *Sph*I and *Mlu*I sites), and the *STM1* open reading frame (ORF) flanked by *Nhe*I at the 5′-end and the IRS sequence (Luo *et al.*, 1996) followed by *Sph*I site at the 3′-end. The *STM1* terminator region was amplified from yeast chromosomal DNA with the use of primers AAAAGCATGCAAGCCTTATATATGAATAATTCCAACTG and AAAAACGCGTCGAACGGAAGAAGTGAATGG. The *STM1* ORF was amplified with the use of primers AAAAGCTAGCATGTCCAACCCATTTGATTTG and AAAAGCATGCCTAAGAACGAATATAACGAGCCAAAGATGGCAAGTTAG, with an *STM1* cDNA library plasmid as the template. Plasmid pL092 (P_{GAL1}*::STM1::IRS URA3* 2μ) was made by inserting the *Nco*I-*Xba*I fragment of pYES2 (containing P_{GAL1} and 2μ sequences) between *Nco*I and *Nhe*I sites of pL090. All PCR and molecular cloning steps were done under standard conditions (Ausubel *et al.*, 1989).

S. cerevisiae strains used in this study are listed in Table 1. The strains YML1 and YML2 were constructed in two steps. First, YHI29-1 and YHI29-14 were selected for spontaneous mutations in the *CYH2* gene on YPD plates containing 10 $\mu g \cdot cm^{-3}$ cycloheximide (Sikorski and Boeke, 1991). Cyh^r clones were isolated and transformed with plasmid pML1, yielding strains YML1, and YML2, respectively. Complementation of the *pre1-1* mutation was confirmed by the restoration of proteasomal chymotrypsin-like activity, assayed by a substrate overlay test as described previously (Hilt and Wolf, 1999). Strains YL280 and YL286 were generated by pop-in/pop-out allele replacement with the use of plasmid pL090 linearized with *Cla*I. The growth of YL280 was indistinguishable from wild type on YPD plates supplemented with 12 mM caffeine or 10 $\mu g \cdot cm^{-3}$ bleomycin, proving that the Stm1-IRS construct was fully functional.

Yeast cells were grown at 30°C if not stated otherwise and liquid cultures were agitated at 200 rpm. Rich growth medium (YPD) contained 1% yeast extract, 2% Bacto-peptone, and 2% D-glucose. Synthetic complete (SC) medium (0.67% nitrogen base without amino acids and nucleotide bases) was lacking the appropriate auxotrophic factors for selection and contained either 2% glucose or 2% galactose as required. Yeast transformations were carried out as described previously (Gietz *et al.*, 1995).

High Expression Lethality Screen

A pYES2-based cDNA library (Espinet *et al.*, 1995) was transformed into YML1 and YML2 strains pregrown on YPD. Transformants were selected on SC glucose medium lacking leucine and uracil (SC $ura^- leu^-$). After 3 d of growth, colonies were replica plated onto SC glucose medium lacking uracil (SC ura^-) to enable loss of plasmid pML1. After an additional 2 d of growth the colonies were replica plated onto SC glucose medium lacking uracil supplemented with 10 $\mu g \cdot cm^{-3}$ cycloheximide (SC $ura^- cyh^+$). This step was repeated after 2 d of growth to ensure that colonies consisted of cells that had lost the plasmid pML1 complementing the *pre1-1* mutation. Loss of plasmid pML1 carrying *PRE1* was further confirmed by test for absence of the chymotrypsin-like activity (Hilt, unpublished results). Two days later, the colonies were replica plated onto SC galactose medium lacking uracil (SCgal ura^-). At the same time the original colonies from SC $ura^- leu^-$ plates ("wild type") were also replica plated onto the SCgal ura^- medium to induce expression of the library genes. After 2 d the two sets of plates were compared and screened for clones able to grow on galactose in the presence but not in the absence of plasmid pML1. To confirm the phenotype, candidates showing such features were picked from the original plates (SC glucose $ura^- leu^-$ or SC glucose $ura^- cyh^+$) onto SCgal ura^-. Plasmid DNA from positive clones (cured of pML1) was isolated and a restriction analysis was performed to ensure homogeneity of the colonies and to estimate the size of the cDNA inserts. Plasmids obtained by these means were transformed into the strains WCG4/a, YHI29–1, and YHI29–14 and retested for the ability of their encoded cDNAs to cause high expression growth arrest in cells with impaired proteasome by streaking onto SCgal ura^- plates.

Gene Disruption

The *STM1* ORF was disrupted with a PCR-mediated method with the use of the kanamycin resistance gene as a selection marker (Güldener *et al.*, 1996). PCR was performed with the use of plasmid pUG6 as a template and primers designed to amplify the kanamycin cassette flanked by 40 base sequences corresponding to immediate down- and upstream region of the *STM1* ORF. Yeast cells were transformed with the PCR product and integrants were selected on YPD plates containing geneticin G418 (Life Technologies, Rockville, MD) at 0.2 $mg \cdot cm^{-3}$. Correct integration was confirmed by Southern blotting with the kanamycin cassette as a probe.

Analysis of DNA

Sequencing was performed with the use of dideoxy sequencing (T7 Sequencing Kit; Pharmacia Biotech, Uppsala, Sweden) and the Sequi-Gen GT Nucleic Acid Electrophoresis Cell (Bio-Rad, Hercules, CA). For Southern blotting the semidry system and the Southern Gen Image kit (Amersham Pharmacia Biotech, Piscataway, NJ) were used.

Immunofluorescence Microscopy

Cells growing in logarithmical phase were fixed for 30 min (3.7% formaldehyde, 0.1 M PO_4^{3-}, pH 6.5) and then washed three times in SP buffer (1.2 M sorbitol, 0.1 M PO_4^{3-}, pH 6.5). The cell wall was digested with 15 $U \cdot cm^{-3}$ Zymolyase 100T (Seikagaku, Tokyo, Japan) in 1.2 M sorbitol, 20 mM β-mercaptoethanol, 0.1 M PO_4^{3-}, pH 6.5, at 30°C for 30 min. After washing three times in SP buffer spheroplasts

were bound on poly-L-lysine-coated slides, washed three times with phosphate-buffered saline (PBS; 53 mM NaH_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl), and then incubated for 20 min at room temperature in PBT (1% bovine serum albumin, 0.1% Triton X-100 in PBS). The IRS-specific monoclonal antibody (BabCO, Richmond, CA) was diluted 1:100 in PBT and applied to the samples for 2 h at room temperature in a humid chamber. The slides were washed five times in PBT and incubated with goat anti-mouse immunoglobulin G-AlexaFluor 594 conjugate (Molecular Probes, Eugene, OR) diluted 1:250 in PBT for 90 min in a dark humid chamber. The antibody was removed and the samples were washed five times with PBT and five times with PBS. A coverslip was mounted with 90% glycerol and 22.5 ng·cm^{-3} 4′,6-diamidino-2-phenylindole in PBS.

Chromosome Spreads

Immunostaining of spread chromosomes was performed as described earlier (Bishop, 1994) with modifications. Spheroplasts were prepared (see the previous section) and resuspended in ice-cold 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, 1 mM EDTA, 0.5 mM $MgCl_2$, and 1 M sorbitol. Twenty microliters of this suspension were placed on a glass slide and mixed with 40 μl of 4% paraformaldehyde in 3.4% sucrose. Afterward 80 μl of 1% Lipsol were added, and then after a few seconds 80 μl of 4% paraformaldehyde in 3.4% sucrose were added. The mixture was spread over the slide with a glass rod and allowed to dry overnight. The slide was submerged in PBS for 10 min and blocked for 10 min in 1% bovine serum albumin in PBS. The reaction with antibodies and mounting of the slides was performed as described above.

Annexin V Assay

Externalization of phosphatidylserine was detected essentially as described previously (Ligr *et al.*, 1998). Cells were resuspended in digestion buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM PO_4^{3-}, pH 6.8) and incubated for 2 h at 30°C with 15 U·cm^{-3} Zymolyase 100T (Seikagaku) and 5.5% Glusulase (NEN, Boston, MA). After cell wall digestion the cells were washed in binding buffer containing sorbitol (1.2 M sorbitol, 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The protoplasts were resuspended in 38 μl of binding buffer and incubated with 2 μl of green fluorescence protein (GFP)-annexin V (Clontech, Palo Alto, CA) and 2 μl of propidium iodide (50 μg·cm^{-3}) in the dark for 20 min at room temperature. The cells were mounted on a slide and examined under the fluorescence microscope.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End-labeling (TUNEL)

The TUNEL assay for detection of fragmented nuclear DNA in yeast was used as previously described (Ligr *et al.*, 1998). Cells were fixed in 3.7% formaldehyde for 1 h and the cell walls were removed as described above. The protoplasts were then applied to polylysine-coated slides. The In Situ Cell Death Detection Kit POD (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer's instructions. After mounting a coverslip with a drop of Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) the cells were examined under the light microscope.

Electron Microscopy

Yeast cells were fixed with phosphate-buffered glutardialdehyde, the cell walls were removed, and the cells were postfixed with osmium tetroxide and uranyl acetate and dehydrated as described for stationary-phase cells (Byers and Goetsch, 1991). After the 100% ethanol washes, the cells were washed with 100% acetone, infiltrated with 50% acetone/50% Epon for 30 min and with 100% Epon for 20 h. The cells were transferred to fresh 100% Epon, incubated at 56°C for 48 h, and thereafter cut into thin sections and stained with lead acetate.

Promoter Shut-off and Cycloheximide-Chase Analysis and Western Blotting

Strains expressing plasmid-encoded IRS-tagged *STM1* under the control of *GAL1* promoter were grown on SC glucose medium until $A_{600} \sim 1$ and then transferred to SC galactose to the final density of $A_{600} \sim 0.5$. After the culture reached $A_{600} \sim 1.5$ glucose and cycloheximide were added to the final concentration of 2% and 0.5 mg·cm^{-3}, respectively. Strains expressing Stm1-IRS from chromosome were grown on YPD until $A_{600} \sim 1.5$, and cycloheximide was added to the final concentration of 0.5 mg·cm^{-3}. The cells (5 A_{600} U) were harvested and lysed in 0.25 M NaOH and 1% β-mercatoethanol. The proteins were precipitated with 5.8% trichloroacetic acid, pelleted, and washed with acetone. The dry pellet was resuspended in urea buffer (8 M urea, 5% SDS, 0.1 M EDTA, 0.02% bromphenol blue, 1% β-mercaptoethanol, 40 mM Tris/HCl, pH 6.8). The proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. IRS-tagged Stm1 was detected with monoclonal anti-IRS antibody and the ECL kit (Amersham).

RESULTS

A Screen for Yeast cDNAs That Causes Growth Arrest when Overexpressed in Cells with Impaired Proteasome-mediated Proteolysis

We developed a screen to search for proteins whose degradation by the ubiquitin-proteasome system is required for viability or growth. We reasoned that overexpression of such a protein should cause little effect in wild-type cells with fully functioning proteasomes but cause a growth defect in cells in which proteasomal function is impaired. Proteasomal activity could not be eliminated completely, because knock-outs of proteasomal subunits are lethal. Cells with the *pre1-1* mutation residing in a β-type subunit of the proteasome are defective in chymotrypsin-like activity and show a significant defect in growth but only slightly impaired protein degradation. The *pre4-1* mutation locating in another β-type subunit causes loss of the PGPH-like activity of the proteasome, but cells otherwise behave phenotypically like wild type. When *pre1-1* and *pre4-1* mutations are combined, proteasomal protein degradation is significantly slowed down, and cells grow at a reduced rate (Hilt *et al.*, 1993). A *pre1-1 pre4-1* strain was selected for spontaneous recessive mutations in the *CYH2* locus conferring cycloheximide resistance (Sikorski and Boeke, 1991). Plasmid pML1 carrying wild-type *PRE1* and *CYH2* genes was introduced into this strain to complement the defect in the chymotrypsin-like activity of the proteasome. The resulting strain, which was phenotypically wild type concerning proteasome-dependent proteolysis and cycloheximide sensitive because of the presence of *CYH2,* was transformed with a 2μ-based cDNA library under the control of the *GAL1* promoter (Espinet *et al.*, 1995). Transformants were plated on selective medium with glucose and replicas were made on cycloheximide-containing plates to select for cells that had lost the *PRE1*-encoding plasmid pML1. Original plates containing wild type cells and their copies containing clones with a *pre1-1 pre4-1* background were then replica plated onto medium containing galactose to induce expression of the plasmid-encoded cDNAs. Library plasmids that caused growth arrest in *pre1-1 pre4-1* mutants but not in the *PRE1*

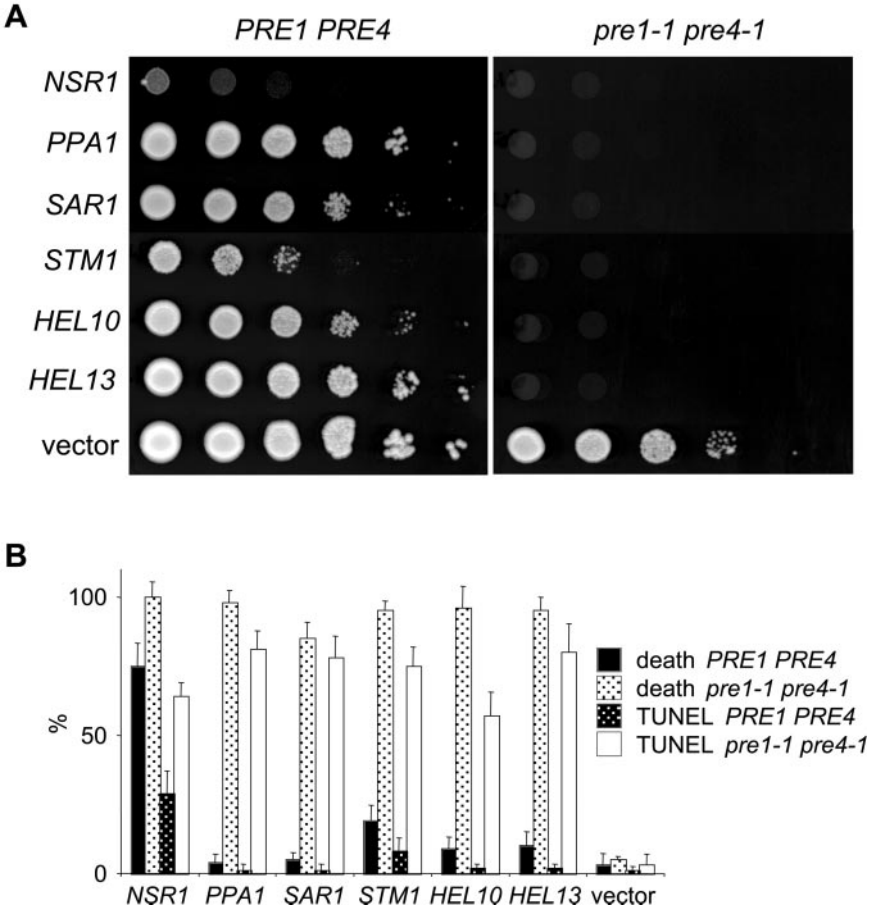

Figure 1. Several *HEL* genes cause growth arrest and cell death when overexpressed in proteasomal mutants. Wild-type and *pre1-1 pre4-1* cells carrying pYES2-encoded, P_{GAL1}-driven cDNAs of *HEL* genes (as indicated) were cultivated overnight in SC ura$^-$. Cells were harvested, and washed in water. (A) To test for growth arrest, 10-fold serial dilutions were spotted on SCgal ura$^-$ plates. Plates were incubated for 3 d at 30°C. (B) After 8 h of induction in SCgal ura$^-$ liquid medium, cell death rate was scored as 100% − (plating efficiency on YPD medium). Frequency of cells with TUNEL positive nuclei was determined by microscopic inspection. Results were averaged from two experiments.

pre4-1 background (wild type) were isolated and their ability to induce growth arrest in cells with defective proteasomes was confirmed after retransformation of the isolated plasmids into the wild type, *pre1-1*, and *pre1-1 pre4-1* cells (Figure 1A). Library plasmids (n = 125) conferring the expected phenotype were isolated and sequenced, revealing 62 individual ORFs causing high expression lethality (*HEL* genes).

Overexpression of Distinct HEL Genes Causes Cell Death and Apoptotic Phenotypes in Proteasomal Mutants

We noticed that overexpression of some *HEL* genes did not only halt growth but also led to decreased survival. Therefore, we examined all isolated cDNAs for their ability to induce apoptosis-like cell death in *pre1-1 pre4-1* mutants.

In both *S. cerevisiae* and mammalian cells, 90% of phosphatidylserine is found under normal conditions in the inner leaflet of the plasma membrane, facing the cytosol (Cerbón and Calderón, 1991). Early during apoptosis in mammals (Martin *et al.*, 1995) and during the apoptosis-like cell death process in yeast (Madeo *et al.*, 1997; Ligr *et al.*, 1998) the asymmetric distribution of phosphatidylserine is lost. This effect can be detected by binding of annexin V to the cell surface. We observed GFP-annexin V binding to yeast protoplasts derived from *pre1-1 pre4-1* cells that overexpressed six of the 62 *HEL* genes identified in the screen (Figure 2). Integrity of protoplasts was assessed by counterstaining with propidium iodide to exclude cells with GFP-annexin V bound to the cytosolic face of the plasma membrane (not shown). The highest rates of staining (~40% of the cells) were observed in strains that overexpressed the *PPA1* or the *YOR309C* gene. Lower but significant rates of annexin staining (15–20% of the cells) were found for *pre1-1 pre4-1* clones overexpressing *NSR1*, *SAR1*, *STM1*, or *YNL208W*. No staining was observed in *pre1-1 pre4-1* cells carrying an empty vector.

Another hallmark of apoptosis is DNA fragmentation (Collins *et al.*, 1997) that can be detected in situ by the TUNEL test. This assay detects the increased presence of free 3′-ends of DNA generated by fragmentation of chromosomes and visualizes them via attachment of labeled nucleotides by terminal deoxynucleotidyl transferase. The 62 cDNAs identified in the high expression lethality screen were analyzed for their capacity to cause DNA fragmentation when overexpressed in *pre1-1 pre4-1* cells. Six cDNAs were detected that induced TUNEL staining in a significant fraction of nuclei in the respective cells (Figure 1B). Significantly, these were the same *HEL* genes that were identified to cause a positive signal in the annexin V test. Thus, the loss

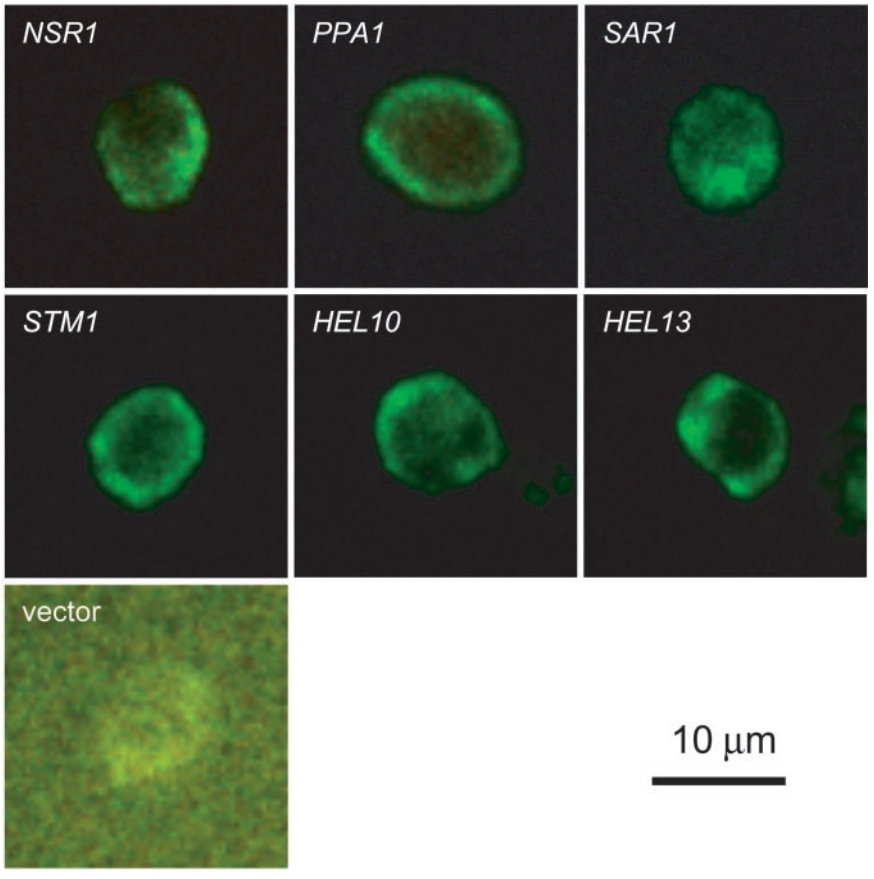

Figure 2. Overexpression of *HEL* genes in proteasomal mutants leads to exposure of phosphatidylserine. *pre1-1 pre4-1* cells that carried pYES2-encoded cDNAs (as indicated) under control of the *GAL1* promoter were pregrown on SC ura$^-$ until logarithmic phase. Expression of cDNAs was then induced by incubation in SCgal ura$^-$ medium for 8 h at 30°C. Only protoplasts excluding propidium iodide (and therefore intact) are shown.

of plasma membrane asymmetry as indicated by annexin V staining was always associated with DNA fragmentation detected by TUNEL assay and vice versa. These results indicate that the identified genes (Table 2) were able to trigger an apoptosis-like process when overexpressed in *pre1-1 pre4-1* mutant cells.

To further support this interpretation, we analyzed the terminal phenotypes of *pre1-1 pre4-1* strains overexpressing one of the six detected cDNAs, each (listed in Table 2) with electron microscopy. In every one of these six strains cells were found that had abnormal nuclei with condensed and marginalized chromatin (Figure 3) as typically seen during mammalian apoptosis (Kerr *et al.*, 1972) and apoptosis-like cell death in yeast (Madeo *et al.*, 1997; Ligr *et al.*, 1998).

In addition, we confirmed that the appearance of apoptotic phenotypes in *pre1-1 pre4-1* cells overexpressing one of the six detected *HEL* genes is associated not only with growth arrest (Figure 1A) but also with cell death (Figure 1B). Overexpression of *NSR1* caused a strong growth defect already in wild-type cells. However, the survival rate was still 20% and therefore we included it in further analyses. Moreover, because growth defects, reduction of survival, and appearance of apoptotic markers were significantly enhanced when *NSR1* was overexpressed in proteasomal mutant cells, *NSR1* was grouped together with the remaining five detected *HEL* genes.

Previously we have shown that the apoptosis-like process in yeast triggered in *cdc48-S565G* mutant cells depends on production of reactive oxygen species. We performed tests to detect oxygen radicals in *pre1-1 pre4-1* strains overexpressing every single one of the six cDNAs inducing apoptotic phenotypes as described before (Madeo *et al.*, 1999). In no case was any significant increase in reactive oxygen species production observed.

Table 2. Overexpressed ORFs causing apoptosis in *pre1-1 pre4-1* cells

ORF	Protein function (Costanzo *et al.*, 2000)
NSR1	Nucleolar protein involved in processing 20–18S rRNA
SAR1	Component of COPII coat of vesicles involved in endoplasmic reticulum to Golgi transport; GTP-binding protein of the arf family
STM1	Protein with specific affinity for G-rich quadruplex nucleic acids; multicopy suppressor of *pop2* and *tom1*
PPA1	Proteolipid of the vacuolar H^+-ATPase (V-ATPase)
HEL 10 (YNL208W)	Unknown
HEL13 (YOR309C)	Unknown

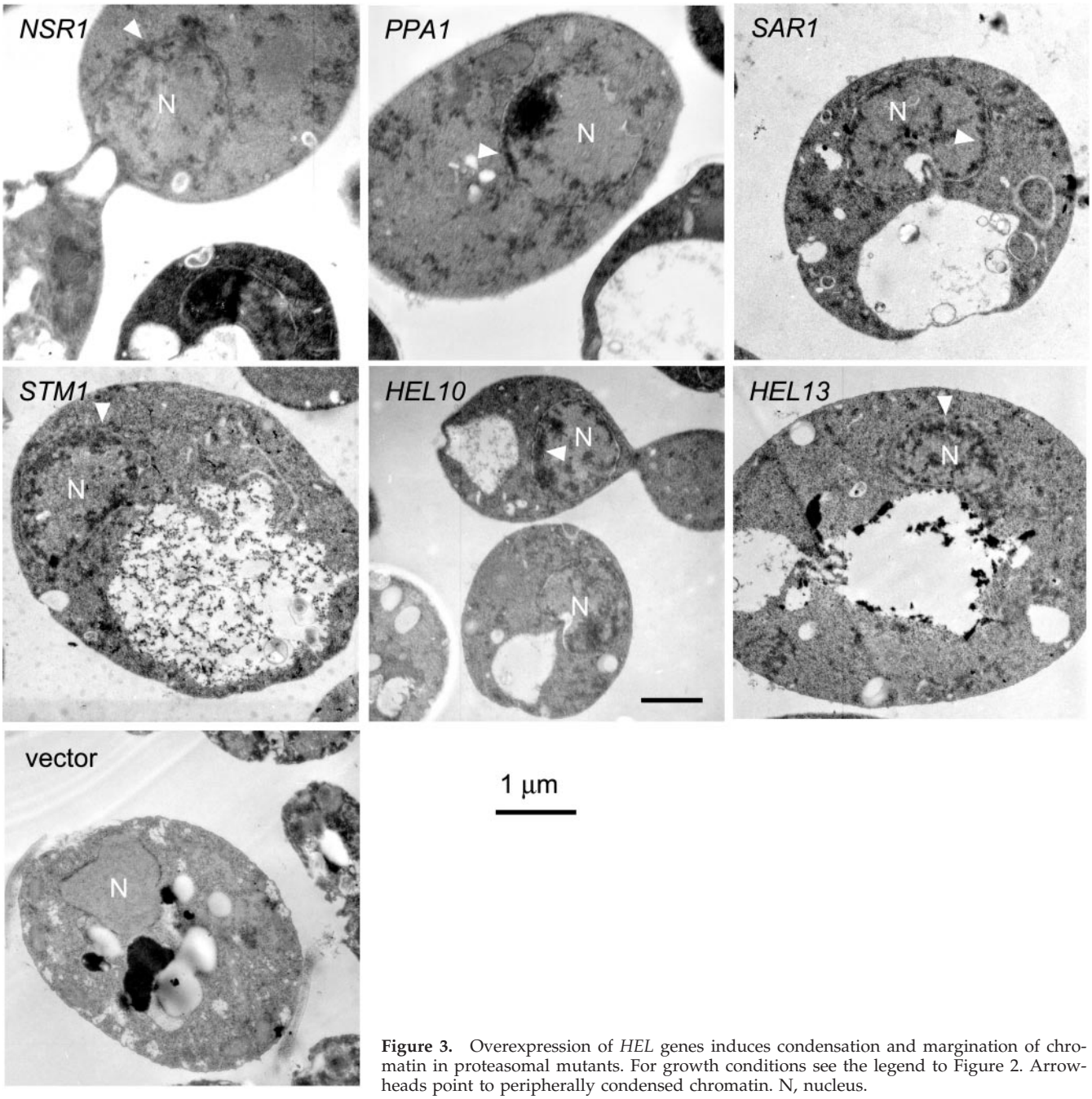

Figure 3. Overexpression of *HEL* genes induces condensation and margination of chromatin in proteasomal mutants. For growth conditions see the legend to Figure 2. Arrowheads point to peripherally condensed chromatin. N, nucleus.

Stm1 Is Degraded by the Proteasome

We were interested to see whether the toxic effect of overexpressed *STM1* in *pre1-1 pre4-1* proteasomal mutant was due to proteolytic stabilization and thereby accumulation of the gene product. To this end, wild-type and proteasomal mutant cells were transformed with a multicopy plasmid carrying the *STM1* ORF C-terminally tagged with a single IRS epitope under the control of the *GAL1* promoter. The Stm1-IRS construct proved to be functional (see MATERIALS AND METHODS). After inducing expression of *STM1::IRS* on galactose the synthesis of Stm1-IRS was stopped by repressing the *GAL1* promoter by addition of glucose and blocking protein synthesis by application of cycloheximide. In wild-type cells the Stm1-IRS was rapidly degraded, whereas in *pre1-1 pre4-1* cells Stm1-IRS was completely stabilized (Figure 4, top). Similar results were obtained by cycloheximide chase analysis of C-terminally tagged Stm1 protein expressed from its endogenous chromosomal promoter (Figure 4, bottom), demonstrating that Stm1 is a natural substrate of the proteasome.

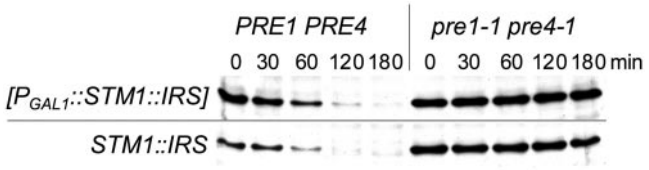

Figure 4. Stm1-IRS is stabilized in proteasome mutant cells. Top, expression of pL092-encoded Stm1-IRS was induced on SCgal ura$^-$ medium. Stm1-IRS synthesis was blocked by addition of glucose and cycloheximide. Bottom, synthesis of chromosomally encoded Stm1-IRS was stopped by addition of cycloheximide. Stm1-IRS levels were followed at indicated chase times by Western analysis. "0" is the time point when glucose and cycloheximide were added.

Stm1 Is a Perinuclear Protein Conferring Resistance to Mutagens

Analysis of the Stm1 sequence with PSORT (http://psort-.nibb.ac.jp/) algorithm (Nakai and Kanehisa, 1992) revealed a putative nuclear localization sequence at the N terminus. To find out whether this domain is functionally relevant, localization of Stm1-IRS was determined by immunofluorescence (Figure 5A). We observed an intense staining in the perinuclear region and in some cases also weak diffuse cytosolic staining, suggesting the existence of two distinct populations of Stm1 in the cell. Notably, no Stm1-IRS signal was seen in the lumen of the nucleus. To uncover whether Stm1 is associated with nuclear envelope or directly with chromatin, chromosome spreading experiments were performed. After mounting chromosomes on glass slides and with the use of a detergent to remove material not directly associated with DNA, the Stm1-IRS signal remained in a ring-shaped arrangement suggesting association of Stm1 with the periphery of nucleoids (Figure 5B).

Stm1 null mutant cells grow as wild type on rich medium at 30°C (Figure 6A) and display only marginally reduced growth at 37°C (Figure 6B; doubling time during logarithmic phase was 2.5 h as compared with 2.1 h for wild type). Cells lacking Stm1 are also sensitive to caffeine. On plates containing 12 mM caffeine *stm1-Δ1* mutant cells showed ~100-fold reduced plating efficiency compared with wild-type cells (Figure 6C). Sensitivity to caffeine is often associated with defects in the protein kinase C (PKC)-mitogen-activated protein kinase pathway. However, staurosporine, a specific inhibitor of Pkc1, had no effect on *stm1-Δ1* cells in a halo assay, arguing against direct involvement of Stm1 in the PKC pathway.

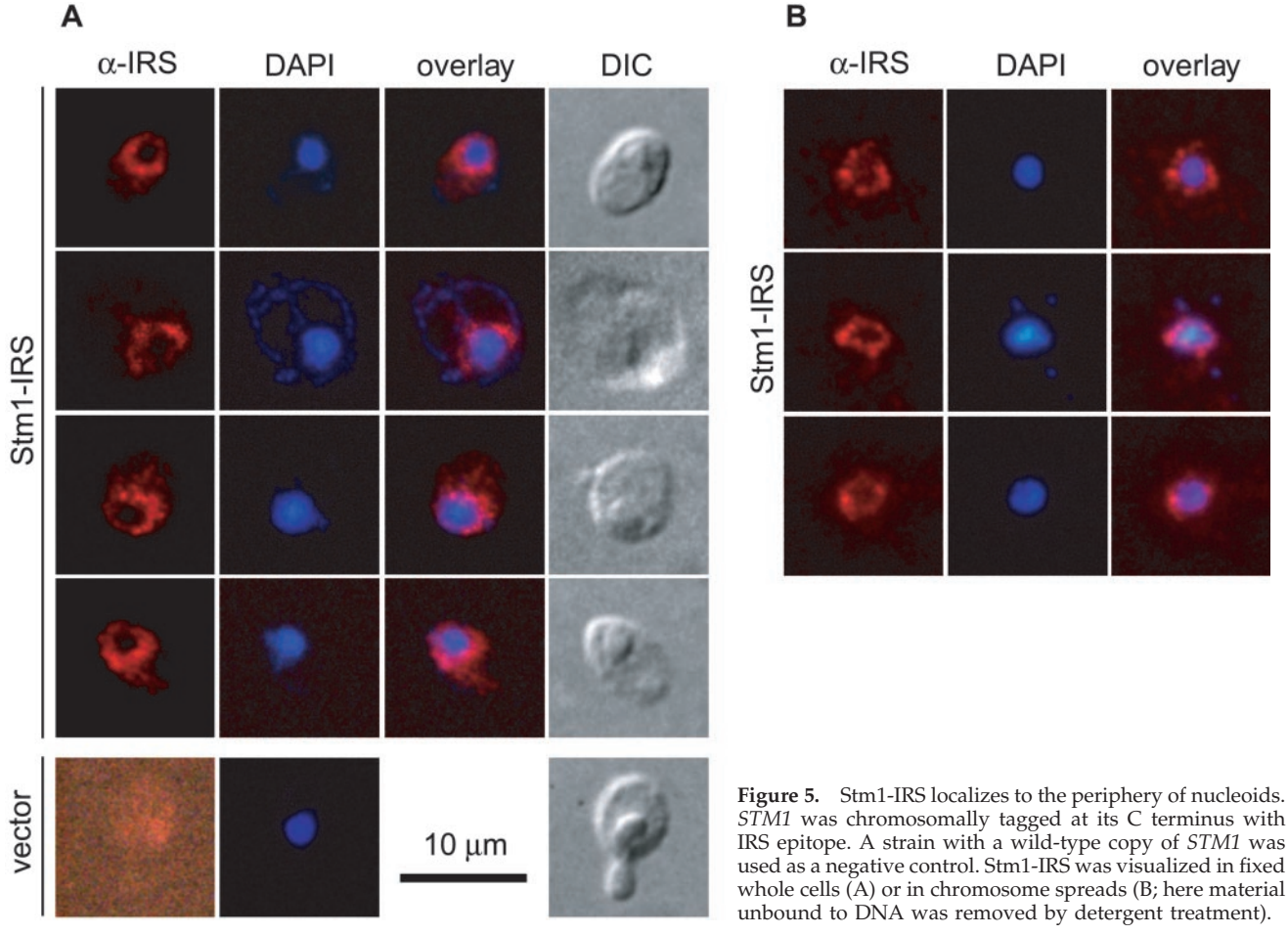

Figure 5. Stm1-IRS localizes to the periphery of nucleoids. *STM1* was chromosomally tagged at its C terminus with IRS epitope. A strain with a wild-type copy of *STM1* was used as a negative control. Stm1-IRS was visualized in fixed whole cells (A) or in chromosome spreads (B; here material unbound to DNA was removed by detergent treatment).

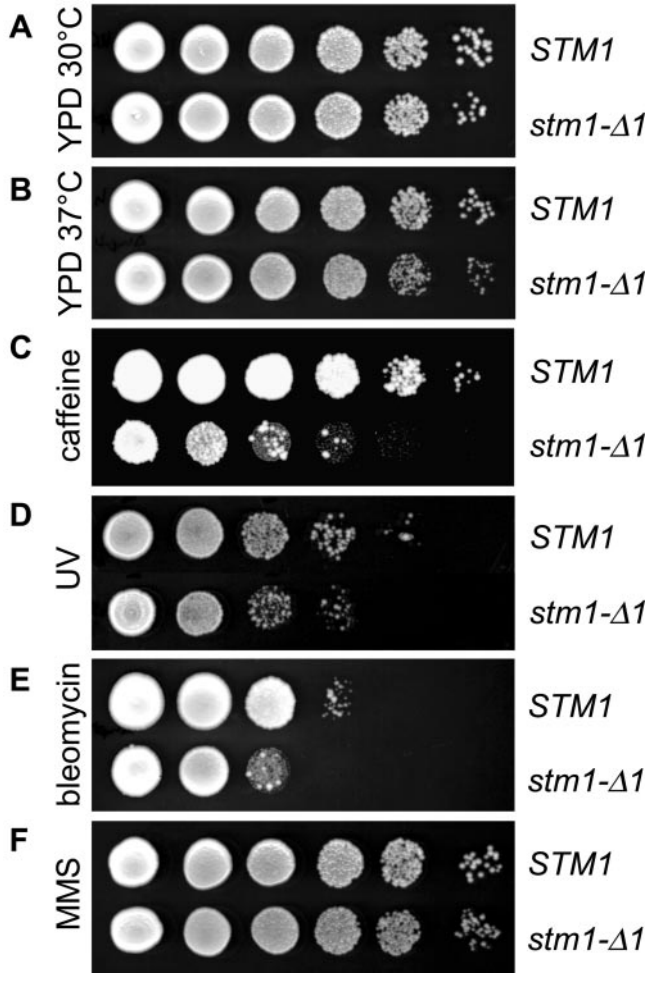

Figure 6. *stm1-Δ1* cells are sensitive to caffeine and DNA-damaging agents. Cells were grown on liquid YPD to saturation and 5 μl of 10-fold serial dilutions were spotted on agar medium. Cells on YPD plates were incubated at 30°C (A) and 37°C (B) for 2 d. (C) Cells were spotted on YPD plates containing 12 mM caffeine and incubated at 30°C for 5 d. (D) Cells plated on YPD were exposed to UV light for 45 s and incubated in dark for 1.5 d at 30°C. (E) Cells were incubated for 4 d at 37°C on YPD plates containing 10 $\mu g \cdot cm^{-3}$ bleomycin. (F) Cells were grown for 5 d at 30°C on YPD plates containing 0.02% MMS.

Given that caffeine is a purine analogue, we explored the possibility that the sensitivity of *stm1-Δ1* cells to this substance may reflect a role of Stm1 in nucleic acid metabolism. We observed that mutant cells show 10-fold enhanced UV sensitivity as compared with wild-type cells (Figure 6D). Bleomycin is a radiomimetic drug that induces single- and double-strand breaks through the production of free radicals (Hampsey, 1997). *stm1-Δ1* mutant cells displayed only a slight growth defect on YPD plates containing 10 $\mu g \cdot cm^{-3}$ bleomycin at 30°C (not shown), but their plating efficiency on the same medium dropped ~10-fold at 37°C compared with wild type (Figure 6E). In contrast, an alkylating agent, methyl methanesulfonate (MMS), did not cause any differential effect in wild-type and *stm1-Δ1* strains in a halo assay (Ligr and Hilt, unpublished results) or in a test on YPD plates containing 0.02% MMS, either at 30 or 37°C (Figure 6F).

Immunofluorescence experiments were performed to check whether treatment with caffeine or bleomycin leads to an alteration of Stm1 localization within the cell. No change in the localization pattern of Stm1-IRS was observed after 2.5 h growth of cells on YPD in the presence of 12 mM caffeine or 750 $\mu g \cdot cm^{-3}$ bleomycin at 30 and 37°C as compared with cells grown on YPD at 30°C.

Cells Lacking Stm1 Can Recover from H_2O_2 Treatment

As described in a previous section, accumulation of Stm1 leads to apoptosis-like cell death. Apoptotic phenotypes can also be induced in yeast by treatment with low concentrations of H_2O_2 (Madeo *et al.*, 1999). Therefore the question arose whether *stm1-Δ1* cells are as sensitive to H_2O_2 treatment as wild type. In a halo assay, both strains displayed the same level of sensitivity after incubation for 1.5 d. However, after 3 d *stm1-Δ1* cells started populating the zone that was up to that point devoid of any growth, thereby decreasing the size of the halo. In contrast, wild-type cells did not extend their growth significantly toward the center of the halo (Figure 7A). To address the possibility that the *stm1-Δ1* cells growing in the halo were suppressor mutants, several of them were isolated and tested again for H_2O_2 sensitivity with the use of the halo assay. No increase in H_2O_2 resistance relative to the original *stm1-Δ1* strain was observed, thereby excluding the appearance of suppressor mutations. A possible explanation for the recovery of *stm1-Δ1* in the halo zone is that a portion of *stm1-Δ1* cells survived the otherwise lethal level of H_2O_2 and resumed their growth after the decrease of H_2O_2 concentration (by diffusion/reaction with the components of the media). Therefore we analyzed plating efficiency of wild-type and *stm1-Δ1* cells after exposure to various concentrations of H_2O_2 in liquid cultures. This experiment showed that, compared with wild type, *stm1-Δ1* cells are slightly, but significantly, more resistant to treatment with low concentrations of H_2O_2, whereas higher concentrations of H_2O_2 (>1 mM) are lethal to both mutant and wild-type cells (Figure 7B). Quantification of TUNEL staining showed that the increase in survival rate of *stm1-Δ1* cells treated with a low dose of H_2O_2 (0.05 mM) is accompanied by a decrease in the number of cells showing an apoptotic phenotype (Figure 7C).

As previously shown, cycloheximide treatment leads to increased survival of yeast cells after exposure to low concentrations of H_2O_2 (Collinson and Dawes, 1992) by preventing apoptosis-like cell death (Madeo *et al.*, 1999). However, cycloheximide treatment did not further increase resistance of *stm1-Δ1* cells to apoptosis-like cell death brought about by low concentrations of H_2O_2 (Figure 7C).

DISCUSSION

The ubiquitin-proteasome system has been proposed to control mammalian apoptosis by degrading a short-lived proapoptotic protein (Drexler, 1997). To find out if the ubiquitin system plays a similar role in yeast we performed a two-layer screen. In the first step we looked for potential yeast

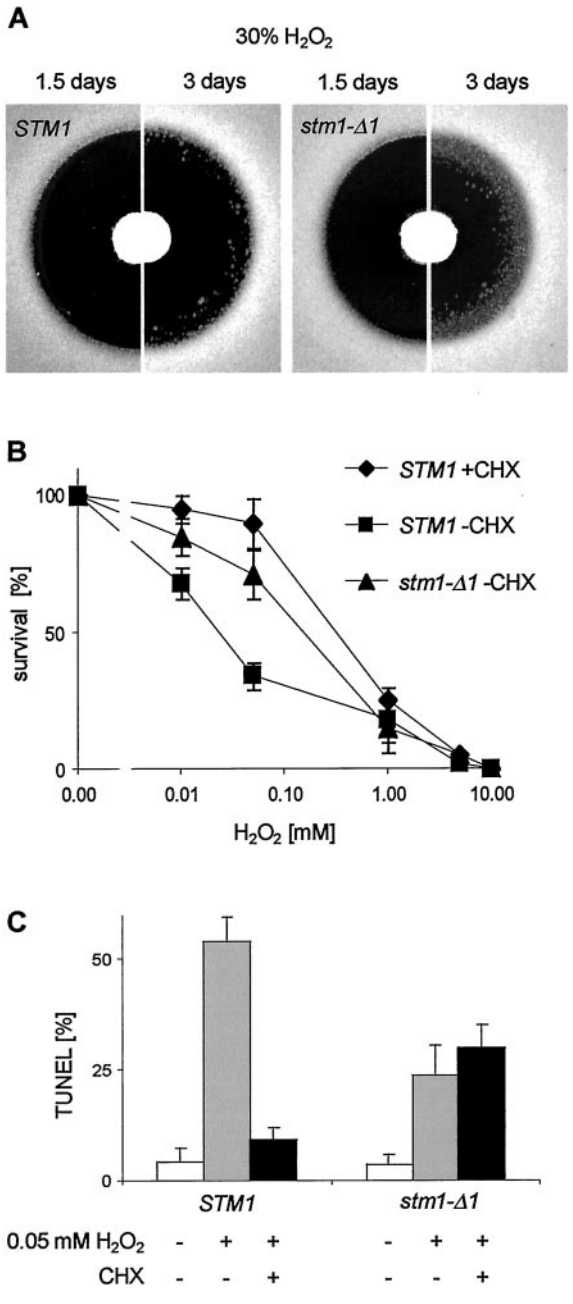

Figure 7. *stm1-Δ1* cells are resistant to low concentrations of H_2O_2. (A) Wild-type and *stm1-Δ1* cells were grown on YPD to saturation, mixed with 0.5% agar in YPD (40°C) and cast on YPD plates. Whatman 003 paper disks were soaked with 10 μl of 30% H_2O_2 and placed on the surface of the plates. Halo formation was recorded after 1.5 and 3 d of growth at 30°C. (B) Cells growing logarithmically on YPD were challenged with various concentrations of H_2O_2 for 200 min in the presence (+) or absence (−) of 15 $\mu g \cdot cm^{-3}$ cycloheximide (CHX) as indicated. Equal numbers of cells were plated on YPD and colony-forming units counted after 2 d of growth. Survival rate was expressed as a fraction of colony forming units relative to untreated cells. (C) Cells were treated with 0.05 mM H_2O_2/15 $\mu g \cdot cm^{-3}$ cycloheximide when indicated. TUNEL frequency was determined by counting cells with TUNEL-positive nuclei under a microscope. Results were averaged from three experiments.

proteasomal substrates that when overexpressed cause cells with defective proteasome to arrest. In the second step, we screened these putative substrates for their ability to cause cell death and to elicit diagnostic markers of apoptosis in yeast cells. Six proteins were found whose overexpression in the proteasomal mutant led to exposure of phosphatidylserine on the cell surface, chromatin condensation, DNA breakage, and cell death.

One of them was Stm1, a protein that was known to bind quadruplex DNA (Frantz and Gilbert, 1995) and purine-rich triplex DNA (Nelson *et al.*, 2000) in vitro. Quadruplex structures were suggested to be present at chromosome ends (Liu *et al.*, 1993). However, with the use of a one-hybrid assay for telomere-binding proteins (Bourns *et al.*, 1998) we could not detect any expression of the reporter gene that would indicate interaction of Stm1 with telomeric DNA. Consistent with its predicted ability to interact with DNA, we found that *stm1-Δ1* cells are sensitive to UV light and treatment with bleomycin, a drug that mimics the effect of ionizing radiation. They are not, however, sensitive to the alkylating agent MMS, suggesting that Stm1 might function in a specific aspect of DNA repair. Stm1 shows weak diffused cytosolic and strong perinuclear staining in fixed cells. Its localization at the periphery of spread nucleoids suggests direct interaction with DNA. These results are consistent with the detection of Stm1 in the highly enriched nuclear envelope fraction (Rout *et al.*, 2000) and with the presence of a putative nuclear localization sequence in the protein.

Stm1 is an in vivo substrate of the proteasome, as evidenced by its rapid turnover in wild-type cells and its complete stabilization in mutants with severely impaired proteasomes. Because degradation of Stm1 is blocked under nonlethal conditions (normal expression of Stm1 from its endogenous promoter), Stm1 stabilization is not a consequence of cell death. Therefore, the data strongly suggest that the lethal effect of overexpressed Stm1 in *pre1-1 pre4-1* mutants is a result of accumulation of the stabilized protein.

The conspicuous feature of the *pre1-1 pre4-1* cells killed by overexpression of Stm1 is the appearance of phenotypes found in metazoan cells undergoing apoptosis and yeast cells killed by exposure to low concentrations of H_2O_2. We tested the sensitivity of *stm1-Δ1* cells to treatment with H_2O_2 with the use of a halo assay and a survival test in liquid culture. In both cases a significant portion of *stm1-Δ1* cells survived exposure to low doses of H_2O_2 that are toxic to wild-type cells. In addition, DNA cleavage as detected by the TUNEL assay was correspondingly reduced in the *stm1* null mutant, indicating that increased survival of these mutants is due to suppression or absence of the apoptosis-like cell death. Cycloheximide treatment—and thereby blocking of protein synthesis—has a protective effect on wild-type yeast cells exposed to low levels of H_2O_2 (Collinson and Dawes, 1992), but this phenomenon was absent in *stm1-Δ1* mutants. In a recent work we proposed that cycloheximide increases survival of H_2O_2-treated cells by inhibiting a translation-dependent apoptosis-like cell death process (Madeo *et al.*, 1999). Taken together, these findings led to the idea that protection against H_2O_2-induced cell death is based, at least in part, on depletion of Stm1 activity due to deletion of the *STM1* gene (*stm1-Δ1* cells) or blocking of its synthesis (application of cycloheximide). Hence, data presented here suggest that the Stm1 protein is an activator of the cell death

process triggered by exposure of cells to low concentrations of H_2O_2. Control of its synthesis and/or degradation may be regulatory steps of H_2O_2-induced apoptosis-like cell death in yeast.

STM1 was originally identified as a multicopy suppressor of *tom1*, *htr1*, and *pop2* mutations, each of them being involved in an aspect of cell cycle control (for a summary, see Nelson *et al.*, 2000). In a genome-wide two-hybrid screen, Stm1 was found to interact with a product of a predicted gene *YJR072C* (Uetz *et al.*, 2000), which has conserved orthologues in *C. elegans* and humans. Stm1 itself has a highly conserved orthologue in *Schizosaccharomyces pombe* and a putative orthologue in *Drosophila melanogaster* (Nelson *et al.*, 2000). This hints that Stm1 may regulate or participate in an evolutionarily conserved process.

Apoptosis in mammalian cells has been tightly linked to activation of caspases (Rich *et al.*, 1999), which are missing in yeast. However, recent reports suggest that the appearance of apoptotic morphology can proceed in the absence of caspases, albeit in a less efficient manner (Borner and Monney, 1999). Notable is the role of reactive oxygen species as mediators of—in many cases—caspase-independent apoptosis in mammalian cells (Xiang *et al.*, 1996; Carmody and Cotter, 2000). Recently, a mammalian apoptosis-inducing factor, AIF, was identified that has closely related orthologues in all phyla (Susin *et al.*, 1999; Lorenzo *et al.*, 1999). Consequent to its translocation from mitochondria to the nucleus, this factor triggers a cell death process with all of the cytological hallmarks of apoptosis but without the activation of caspases. Thus, the target compartment of AIF and the major place of localization of Stm1 is the same—the nucleus—and both share similar function—induction of caspase-independent cell death resembling apoptosis. The tempting hypothesis to be tested is that AIF, Stm1, and their respective orthologues participate in the same caspase-independent pathway in yeast and mammals.

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R., Moore, D., Seidmann, J.G., Smith, J.A., and Struhl, K. (1989). Current Protocols in Molecular Biology, New York: Wiley.

Bishop, D.K. (1994). RecA homologs Dmc1 and Rad51 interact to form multiple nuclear complexes prior to meiotic chromosome synapsis. Cell *79*, 1081–1092.

Borner, C., and Monney, L. (1999). Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ. *6*, 497–507.

Bourns, B.D., Alexander, M.K., Smith, A.M., and Zakian, V.A. (1998). Sir proteins, Rif proteins, and Cdc13p bind *Saccharomyces* telomeres in vivo. Mol. Cell. Biol. *18*, 5600–5608.

Byers, B., and Goetsch, L. (1991). Preparation of yeast cells for thin-section electron microscopy. Methods Enzymol. *194,* 602–608.

Carmody, R.J., and Cotter, T.G. (2000). Oxidative stress induces caspase-independent retinal apoptosis in vitro. Cell Death Differ. *7*, 282–291.

Cerbón, J., and Calderón, V. (1991). Changes of the compositional asymmetry of phospholipids associated to the increment in the membrane surface potential. Biochim. Biophys. Acta *1067*, 139–144.

Collins, J.A., Schandi, C.A., Young, K.K., Vesely, J., and Willingham, M.C. (1997). Major DNA fragmentation is a late event in apoptosis. J. Histochem. Cytochem. *45*, 923–934.

Collinson, L.P., and Dawes, I.W. (1992). Inducibility of the response of yeast cells to peroxide stress. J. Gen. Microbiol. *138*, 329–335.

Costanzo, M.C., Hogan, J.D., Cusick, M.E., Davis, B.P., Fancher, A.M., Hodges, P.E., Kondu, P., Lengieza, C., Lew-Smith, J.E., Lingner, C., Roberg-Perez, K.J., Tillberg, M., Brooks, J.E., and Garrels, J.I. (2000). The yeast proteome database (YPD) and *Caenorhabditis elegans* proteome database (WormPD): comprehensive resources for the organization and comparison of model organism protein information. Nucleic Acids Res. *28*, 73–76.

Dai, R.M., Chen, E., Longo, D.L., Gorbea, C.M., and Li, C.C.H. (1998). Involvement of valosin-containing protein, an ATPase co-purified with IκBα and 26S proteasome, in ubiquitin-proteasome mediated degradation of IκBα. J. Biol. Chem. *273*, 3562–3573.

Drexler, H.C. (1997). Activation of the cell death program by inhibition of proteasome function. Proc. Natl. Acad. Sci. USA *94*, 855–860.

Drexler, H.C.A. (1998). Programmed cell death and the proteasome. Apoptosis *3*, 1–7.

Engelberg-Kulka, H., and Glaser, G. (1999). Addiction modules and programmed cell death and antideath in bacterial cultures. Annu. Rev. Microbiol. *53*, 43–70.

Espinet, C., Angeles de Tore, M., Aldea, M., and Herrero, E. (1995). An efficient method to isolate yeast genes causing overexpression-mediated growth-arrest. Yeast *11*, 25–32.

Frantz, J.D., and Gilbert, W. (1995). A yeast gene product, G4p2, with a specific affinity for quadruplex nucleic acids. J. Biol. Chem. *270*, 9413–9419.

Fröhlich, K.-U., and Madeo, F. (2000). Apoptosis in yeast: a monocellular organism exhibits altruistic behavior. FEBS Lett. *473*, 6–9.

Ghislain, M., Dohmen, R.J., Levy, F., and Varshavsky, A. (1996). Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. EMBO J. *15*, 4884–4899.

Gietz, R.D., Schiestl, R.H., Willems, A.R., and Woods, R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast *11*, 355–360.

Güldener, U., Heck, S., Fiedler, T., Beinhauer, J., and Hegemann, J.H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. *24*, 2519–2524.

Hampsey, M. (1997). A review of phenotypes in *Saccharomyces cerevisiae*. Yeast *13*, 1099–1133.

Heinemeyer, W., Gruhler, A., Möhrle, V., Mahé, Y., and Wolf, D.H. (1993). *PRE2*, highly homologous to the human major histocompatibility complex-linked *RING10* gene, codes for a yeast proteasome subunit necessary for chymotryptic activity and degradation of ubiquitinated proteins. J. Biol. Chem. *268*, 5115–5120.

Heinemeyer, W., Kleinschmidt, J.A., Saidowsky, J., Escher, C., and Wolf, D.H. (1991). Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. EMBO J. *10*, 555–562.

Hilt, W., Enenkel, C., Gruhler, A., Singer, T., and Wolf, D.H. (1993). The *PRE4* gene codes for a subunit of the yeast proteasome necessary for peptidylglutamyl-peptide-hydrolyzing activity: mutations link the proteasome to stress- and ubiquitin-dependent proteolysis. J. Biol. Chem. *268*, 3479–3486.

Hilt, W., and Wolf, D.H. (1996). Proteasomes: destruction as a program. Trends Biochem. Sci. *21*, 96–102.

Hilt, W., and Wolf, D.H. (1999). Protein degradation and proteinases in yeast. In: Posttranslational Processing: A Practical Approach, ed. S.J. Higgins and B.D. Hames, Oxford, U.K.: Oxford University Press, 263–302.

Kerr, J.F.R., 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.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell *96*, 635–644.

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.

Liu, Z., Frantz, J.D., Gilbert, W., and Tye, B.K. (1993). Identification and characterization of a nuclease activity specific for G4 tetra-stranded DNA. Proc. Natl. Acad. Sci. USA *90*, 3157–3161.

Lorenzo, H.K., Susin, S.A., Penninger, J., and Kroemer, G. (1999). Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ. *6*, 516–524.

Luo, W., Liang, T.C., Li, J.M., Hsieh, J.T., and Lin, S.H. (1996). A universal tag for recombinant proteins. Arch. Biochem. Biophys. *329*, 215–220.

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

Martin, S.J., Reutelingsperger, C.P.M., McGahon, A.J., Rader, J., van Schie, C.A.A., LaFace, D.M., and Green, D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and AB1. J. Exp. Med. *182*, 1545–1557.

Meyer, H.H., Shorter, J.G., Seemann, J., Pappin, D., and Warren, G. (2000). A complex of mammalian Ufd1 and Npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. EMBO J. *19*, 2181–2192.

Nakai, K., and Kanehisa, M. (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics *4*, 897–911.

Nelson, L.D., Musso, M., and van Dyke, M.W. (2000). The yeast *STM1* gene encodes a purine motif triple helical DNA-binding protein. J. Biol. Chem. *275*, 5573–5581.

Rich, T., Watson, C.J., and Wyllie, A. (1999). Apoptosis: the germs of death. Nat. Cell Biol. *1*, E69–E71.

Rout, M.P., Aitchison, J.D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B.T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. J. Cell Biol. *148*, 635–651.

Shirogane, T., Fukada, T., Muller, J.M., Shima, D.T., Hibi, M., and Hirano, T. (1999). Synergistic roles for Pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis. Immunity *11*, 709–719.

Sikorski, R.S., and Boeke, J.D. (1991). In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. Methods Enzymol. *194,* 302–318.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M., and Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. Nature *397*, 441–446.

Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M., Johnston, M., Fields, S., and Rothberg, J.M. (2000). A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature *403*, 623–627.

Webb, S.J., Harrison, D.J., and Wyllie, A.H. (1999). Apoptosis: an overview of the process and its relevance in disease. In: Advances in Pharmacology, ed. S.H. Kaufmann, San Diego, CA: Academic Press, 1–34.

Wu, D., Chen, P.J., Chen, S., Hu, Y., Nunez, G., and Ellis, R.E. (1999). *C. elegans* MAC-1, an essential member of the AAA family of ATPases, can bind CED-4 and prevent cell death. Development *126*, 2021–2031.

Xiang, J., Chao, D.T., and Korsmeyer, S.J. (1996). BAX-induced cell death may not require interleukin 1β-converting enzyme-like proteases. Proc. Natl. Acad. Sci. USA *93*, 14559–14563.