

1 **Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis**  
2 **in *Saccharomyces cerevisiae***

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1 **Abstract**

2

3 During the last years, several reports described an apoptosis-like programmed cell death  
4 process in yeast in response to different environmental aggressions. Here, evidence is  
5 presented that hyperosmotic stress induces in *Saccharomyces cerevisiae* a cell death  
6 process accompanied by morphological and biochemical indicators of apoptotic  
7 programmed cell death, namely chromatin condensation along the nuclear envelope,  
8 mitochondrial swelling and reduction of cristae number, production of reactive oxygen  
9 species and DNA strand breaks, with maintenance of plasma membrane integrity.  
10 Disruption of *AIF1* had no effect on cell survival, but lack of Yca1p drastically reduced  
11 metacaspase activation and decreased cell death indicating that this death process was  
12 associated to activation of this protease. Supporting the involvement of mitochondria  
13 and cytochrome *c* in caspase activation, the mutant strains *cyc1Δ cyc7Δ* and *cyc3Δ*, both  
14 lacking mature cytochrome *c*, displayed a decrease in caspase activation associated to  
15 increased cell survival when exposed to hyperosmotic stress. These findings indicate  
16 that hyperosmotic stress triggers *S. cerevisiae* into an apoptosis-like programmed cell  
17 death that is mediated by a caspase-dependent mitochondrial pathway partially  
18 dependent on cytochrome *c*.

# 1 **Introduction**

2

3 Apoptosis, one of the most common expressions of programmed cell death (PCD), has  
4 been observed in *Saccharomyces cerevisiae* aged cells (Herker *et al.*, 2004; Laun *et al.*,  
5 2001), after heterologous expression of human pro-apoptotic genes, such as Bax (Ligr *et*  
6 *al.*, 1998) or after mild treatment with stress agents. Yeast cells die and show typical  
7 markers of apoptosis after exposure to low doses of hydrogen peroxide (Madeo *et al.*,  
8 1999), acetic acid (Ludovico *et al.*, 2001), UV radiation (Del Carratore *et al.*, 2002),  $\alpha$   
9 mating-type pheromone (Severin and Hyman, 2002), salt (Huh *et al.*, 2002), aspirin  
10 (Balzan *et al.*, 2004) and by low sugar concentrations in the absence of additional  
11 nutrients (Granot *et al.*, 2003). Initial screens of the *S. cerevisiae* complete genome  
12 sequence have revealed few homologs of mammalian apoptotic regulators. Though  
13 recently, orthologs of key regulators such as the metacaspase (*YCA1/MCA1*) (Madeo *et*  
14 *al.*, 2002), the HtrA2-like protein (Fahrenkrog *et al.*, 2004), or the yeast apoptosis  
15 inducing factor (Aif1p) (Wissing *et al.*, 2004) have been observed in yeast. Fannjiang *et*  
16 *al.* (2004) also reported that Dnm1p, the *S. cerevisiae* homolog of the human  
17 mitochondrial fission protein Drp1p, was involved in yeast apoptosis.

18 In addition to the existence of Aif1p, HtrA2-like protein, Yca1p and Dnm1p, evidence  
19 has been provided for cytochrome *c* (cyt *c*)-associated mitochondrial involvement in  
20 yeast apoptosis. Yamaki *et al.* (2001) showed that cell death in the *asf1/cia1* deficient  
21 mutant, accompanied with predominant apoptotic features, was associated to decrease in  
22 mitochondrial membrane potential, dysfunction of mitochondrial ATPase complex and  
23 release of cyt *c*. Ludovico *et al.* (2002) observed in yeast cells undergoing apoptosis  
24 induced by acetic acid, cyt *c* release and a mitochondrial dysfunction pattern identical to  
25 that described in *S. cerevisiae* cells expressing Bax (Manon *et al.*, 1997) including

1 major alterations in the respiratory chain namely, decrease in the amount of cyt *c* and  
2 reduction of the cytochrome *c* oxidase (COX) activity.

3 To date, there are no reports of apoptosis in yeasts induced by hyperosmotic stress.  
4 However, it has been recognised that in mammalian cells, hyperosmotic stress induces  
5 apoptosis and is involved in several pathological states such as ischemia, septic shock  
6 and diabetic coma (Wright and Rees, 1998; Galvez *et al.*, 2001). Moreover, Chan *et al.*  
7 (1999) reported that during hyperosmotic shock-induced apoptosis in several cell types,  
8 p21<sup>Cdc42/Rac</sup>-activated kinase (PAK2) is cleaved and activated via a caspase-dependent  
9 mechanism, and suggested the involvement of oxidative stress in the induction of this  
10 process.

11 Here we report that hyperosmotic stress is also able to trigger *S. cerevisiae* into a PCD  
12 process associated with characteristic apoptotic markers namely, chromatin  
13 condensation along the nuclear envelope, reactive oxygen species (ROS) production,  
14 DNA strand breaks and metacaspase activation. Further evidence is provided supporting  
15 the involvement of mitochondria and a role for the yeast metacaspase Yca1p in the  
16 hyperosmotic induced-apoptosis.

17

## 18 **Results**

19

### 20 ***S. cerevisiae* dies in response to hyperosmotic stress**

21 Exposure of *S. cerevisiae* cells, strain PYCC3507, to hyperosmotic stress caused by  
22 high glucose or sorbitol concentrations resulted in cell death (Fig. 1A). Moreover, in  
23 medium with 70% (w/w) glucose cell death was shown to be temperature- and growth  
24 phase-dependent (Table 1). The results obtained showed that, at 28 or 35°C, stationary  
25 cells (that possess fully active respiring mitochondria and display a higher

Table 1

1 mitochondrial mass) exhibited higher death rates in comparison to exponential cells.  
2 Additionally, cell death was enhanced at higher temperatures (Table 1) and by aeration  
3 at 28°C, achieved by mechanical shaking (data not shown). Subsequently, PYCC3507  
4 stationary phase cells exposed to 60% (w/w) glucose or 60% (w/w) sorbitol in yeast  
5 morphology broth (YMB) at 28°C with aeration were used to characterize cell death  
6 mode under hyperosmotic conditions.

7 Huh *et al.* (2002) reported that ionic imbalance caused by high concentrations of NaCl  
8 induced a lysigenous apoptosis in *S. cerevisiae*. The possibility of cell death imparted  
9 by incubation under hyperosmotic conditions being due to cellular lysis was analysed  
10 by determining the percentage of cells with ultrastructural lytic alterations. The values  
11 obtained (5.9% for treated cells and 0.7% for the control) discard that possibility (Fig.  
12 2E). Propidium iodide (PI) staining (see below) supported the same interpretation. All  
13 these data pointed to a metabolic dependence of the death process and suggested that an  
14 active rather than an accidental process was underlying *S. cerevisiae* cell death under  
15 hyperosmotic stress.

16

### 17 **Characteristic markers of apoptosis accompany *S. cerevisiae* hyperosmotic stress-** 18 **induced cell death**

19 Preservation of plasma membrane integrity is one of the characteristic markers of  
20 apoptotic death. PI exclusion is most frequently used to assess that parameter. Dead or  
21 dying cells with compromised membrane incorporate this dye that stains nucleic acids  
22 (Haugland, 2000). Loss of *S. cerevisiae* proliferative capacity in response to  
23 hyperosmotic stress caused by 60% (w/w) glucose was not accompanied by significant  
24 loss of plasma membrane integrity. After 12 and 24 hours incubation, about 10 and 30%  
25 respectively, lost their membrane integrity suggesting that most cells were dying by

Figure 1

1 apoptosis rather than by necrosis. (Fig. 1B; third column). This increase in the  
2 percentage of cell with damaged membranes likely results from secondary necrosis, the  
3 terminal stage of yeast apoptosis (reviewed in Ludovico *et al.*, 2005). To assess whether  
4 the cell death induced by high glucose concentration is apoptotic, several apoptotic  
5 markers were investigated. Nuclear alterations along treatment of PYCC3507 *S.*  
6 *cerevisiae* cells with 60% (w/w) glucose were monitored by staining with 4,6-diamido-  
7 2-phenyl-indole (DAPI). Cell staining with this dye allowed visualizing well-defined  
8 nucleus in control cells (0 hours treatment, Fig. 2A). In contrast, treated cells displayed,  
9 very early after exposure to 60% (w/w) glucose, loose nuclei with kidney or ring shaped  
10 condensed chromatin characteristic of apoptotic cells (Fig. 2A). Fig. 1B shows that most  
11 cells with characteristic apoptotic nuclear alterations revealed by DAPI staining have  
12 preserved membrane integrity as shown by PI staining. This was confirmed by DAPI/PI  
13 double staining (data not shown). Electron microscopy analysis further confirmed the  
14 occurrence of nuclear alterations by showing chromatin condensation along the nuclear  
15 envelope in treated cells whereas nuclei of untreated cells were homogeneous in shape  
16 and density (Fig. 2B). Treated cells also exhibited mitochondrial ultrastructural changes  
17 namely, swelling and reduction of cristae number (Fig. 2C). In addition, TUNEL-  
18 positive cells displaying a nuclear green fluorescence were detected along exposure to  
19 60% (w/w) glucose (Fig. 2D) indicating the occurrence of DNA strand breaks. The  
20 percentage of cells displaying TUNEL-positive staining increased along time reaching a  
21 maximum at 12 hours (Fig. 1B; second column). On the other hand, TUNEL-positive  
22 cells were rare in control cells (Fig. 1B and 2A).

23 Comparison of the results regarding TUNEL and PI staining (Fig. 1B) revealed a great  
24 discrepancy. After 12 hours incubation, about 50% of the cells were TUNEL positive  
25 but only about 10% lost their membrane integrity. Although it is not possible to assess

1 at the individual level whether TUNEL-positive cells still maintain their membrane  
2 integrity, this comparison suggested that the majority of the cells with DNA strand  
3 breaks had preserved membrane integrity.

4 Since it is known that high glucose concentration causes several metabolic changes in  
5 the yeast cell it was addressed whether this was the cause of apoptosis induction rather  
6 than the reduced value of water activity. For this purpose the response of *S. cerevisiae*  
7 to 60% (w/w) sorbitol was analysed. Incubation of cells in YMB with 60% (w/w)  
8 sorbitol (corresponding to a water activity similar to the one obtained in YMB with 60%  
9 (w/w) glucose) also results in cell death. The PI staining kinetics was similar to the one  
10 obtained in YMB with 60% (w/w) glucose (data not shown). Moreover, under these  
11 conditions, cell death was accompanied by caspase activation as detected by flow  
12 cytometry (data not shown) and by fluorescence microscopy (Fig. 2F).

13 In summary, the data presented above support the interpretation that hyperosmotic  
14 stress trigger *S. cerevisiae* into a PCD with an apoptotic phenotype sharing common  
15 features to mammalian apoptosis.

16

### 17 **ROS are produced during apoptosis induced by hyperosmotic stress**

18 Production of ROS in *S. cerevisiae* cells dying due to exposure to 60% (w/w) glucose  
19 was monitored with dihydroethidium (DE) or 2',7'-dichlorodihydrofluorescein diacetate  
20 ( $H_2DCFDA$ ). DE passively diffuses into the cell and, in the presence of the superoxide  
21 anion, is oxidized to ethidium that intercalates within nucleic acids, staining the cell  
22 with a bright red fluorescence.  $H_2DCFDA$  also enters the cell and once inside a live cell  
23 is hydrolysed by intracellular esterases to  $H_2DCF$ . This compound is oxidised by ROS  
24 and leads to emission of green fluorescence (Haugland, 2000).

Figure 3

1 Most yeast cells from untreated samples did not show any fluorescence after staining  
2 with either DE or H2DCFDA (Fig. 3 A and B). On the other hand, some cells from  
3 yeast suspensions exposed to 60% (w/w) glucose for 3 hours, displayed a red  
4 fluorescence after staining with DE (Fig. 3A) or a green fluorescence after staining with  
5 H2DCFDA (Fig. 3B). The observed staining patterns with the two selected fluorophores  
6 indicate that ROS production is an early event of the hyperosmotic stress-induced  
7 apoptosis of *S. cerevisiae*.

8

### 9 **Mitochondria participate in the hyperosmotic stress-induced apoptosis**

10 Indication of mitochondrial involvement in apoptosis induced by hyperosmotic stress  
11 was given by the observation of mitochondrial ultrastructural alterations and significant  
12 ROS production, described above. Further confirmation of this hypothesis was achieved  
13 by the study of two *S. cerevisiae* BY4741 mutant strains, lacking nuclear encoded  
14 mitochondrial proteins, namely the *cyc1Δ cyc7Δ* strain, deleted in the genes encoding  
15 isoform 1 and 2 of cyt *c*, and the *cyc3Δ* strain lacking the cytochrome *c* heme lyase,  
16 essential for the covalent binding of the heme group to isoform 1 and 2 of  
17 apocytochrome *c* (Reilly and Sherman, 1965; Dumont *et al.*, 1987) These two strains  
18 share the lack of mature cyt *c*. The kinetics of cell death induced by incubation of wild-  
19 type strain BY4741 in SC (synthetic complete medium) with 60% (w/w) glucose (Fig.  
20 4) is similar to that described above for strain PYCC3507 in YMB broth with 60%  
21 (w/w) glucose or sorbitol (Fig. 1A). The two BY4741 mutant strains were found to be  
22 more resistant to death induced by hyperosmotic stress, comparatively to the wild-type  
23 strain (Fig. 4). However, in *cyc1Δ cyc7Δ* and *cyc3Δ* strains cell death was not  
24 completely abolished. Both mutants, likewise the wild-type strain, displayed DNA  
25 strand breaks, detected by TUNEL (data not shown), as well as caspase activation (Fig.

Figure 4



1 5B). On the other hand the *aif1*Δ mutant strain did not differ from the wild type strain  
2 regarding cell survival in response to hyperosmotic stress (Fig. 4).

3

4 **Apoptosis induced by hyperosmotic stress is mediated by a partially cyt *c*-**  
5 **dependent Yca1p activation**

Figure 5

6 To examine whether the yeast metacaspase Yca1p has a role in hyperosmotic stress-  
7 induced cell death, cell survival of a strain lacking Yca1p was firstly assessed. The  
8 results (Fig. 5A) showed that absence of the metacaspase increased cell viability  
9 consistent with the occurrence of a metacaspase-dependent active cell death process. In  
10 order to further confirm metacaspase involvement in this apoptotic process, cells were  
11 incubated with the FITC-labelled pan-caspase inhibitor VAD-fmk that binds to the  
12 active site of caspases, allowing flow cytometric determination of cells with active  
13 enzymes (Madeo *et al.*, 2002). Treatment for 2 or 6 hours led to an increase in the  
14 number of cells with active metacaspase in the wild-type strain (Fig. 5B) with about  
15 50% of cells with active metacaspase after 6 hours. Consistently, only slight  
16 metacaspase activation was observed in *YCA1* disrupted cells (Fig. 5B). Comparison of  
17 the levels of caspase activation in the wild type strain after 2 hours and *YCA1* null strain  
18 after 6 hours incubation in presence of 60% (w/w) glucose, showed that, for similar  
19 values of cell survival, metacaspase activation is three times higher in the wild type  
20 strain. Moreover, exposure of the wild-type strain to 60% (w/w) glucose in the presence  
21 of zVAD-fmk caused a drastic decrease in the percentage of cells with active caspase  
22 (data not shown). In order to ascertain the role of cyt *c* in metacaspase activation  
23 induced by hyperosmotic stress, strains *cyc1*Δ *cyc7*Δ and *cyc3*Δ were also monitored for  
24 this protease. Metacaspase activation was detected in these two mutant strains under

1 hyperosmotic stress, although at a much lower rate than the one observed with the wild  
2 type strain (Fig. 5B).

3

4

## 5 **Discussion**

6

7 An increasing number of reports show that different molecules, at a given concentration  
8 range, are able to trigger *S. cerevisiae* into a PCD process sharing features common to  
9 those of mammalian apoptosis (for a review see Madeo *et al.*, 2004 and Ludovico *et al.*,  
10 2005). Here we show that cell death induced by hyperosmotic stress in a *S. cerevisiae*  
11 osmosensitive strain is not a lytic process and is temperature-, aeration- and growth  
12 phase-dependent, indicating that cells were dying by a metabolic process. That death  
13 induced by hyperosmotic stress in the *S. cerevisiae* strain PYCC3507 is apoptotic is  
14 concluded from the occurrence of chromatin condensation, DNA strand breaks, ROS  
15 production, metacaspase activation and preservation of plasma membrane integrity.  
16 Some of these apoptotic markers were also observed in *S. cerevisiae* BY4741, a strain  
17 with a different genetic background. Yeast and plant cells exposed to hyperosmotic  
18 NaCl concentrations die by an apoptotic process (Huh *et al.*, 2002). However, this  
19 apoptotic process is a consequence of an ion disequilibrium rather than of a  
20 hyperosmotic stress. Hence, the present paper is the first report on yeast apoptosis  
21 induced by hyperosmotic stress.

22 Evidences regarding mitochondria involvement in yeast apoptosis in response to  
23 different stimuli have been obtained (Ludovico *et al.*, 2002; Severin and Hyman, 2002;  
24 Wissing *et al.*, 2004). Here we present several results indicating that mitochondrial  
25 function maybe required for cell death induced by hyperosmotic stress. Absence of

1 holocytochrome *c* in *cyc1Δ cyc7Δ* and *cyc3Δ* mutant strains enhances cell survival. This  
2 can have two possible explanations. First, this could be due to the lack of cyt *c*, second  
3 to retrograde response due to respiratory deficiency. However, under the apoptotic  
4 inducing conditions used, namely, high glucose concentrations (Liao *et al.*, 1991) and  
5 presence of glutamate in the medium (Liu and Butow, 1999), retrograde response is  
6 repressed. These evidences indicate that the retrograde pathway is not involved and that  
7 mature cyt *c* is important for the execution of the death program induced by  
8 hyperosmotic stress. Additionally, we found that this death process is accompanied by  
9 ROS production and mitochondrial ultrastructural alterations. These alterations were  
10 also detected in apoptosis induced by low doses of acetic acid in *S. cerevisiae* and  
11 *Zygosaccharomyces bailii* (Ludovico *et al.*, 2002; Ludovico *et al.*, 2003). Likewise  
12 swelling and reduction in cristae number, have been found in several instances of  
13 mammalian apoptotic cell death (reviewed by Vieira *et al.*, 2000). Brown (1975) had  
14 already reported that high glucose concentrations provoked structural and functional  
15 mitochondrial alterations in a sensitive *S. cerevisiae* strain including decrease in cristae  
16 number, respiration rate, NADH oxidase activity and cyt *c* content. Taking into account  
17 these results it is conceivable that the mitochondrial ultrastructural alterations seen in  
18 cell death induced by hyperosmotic stress represent the same mitochondrial molecular  
19 events described by Brown (1975). Moreover, because of the absence of mitochondrial  
20 respiration in the *cyc1Δ cyc7Δ* and *cyc3Δ* strains, it could be argued that the decreased  
21 ability of those strains to develop the hyperosmotic-induced apoptosis would be due to  
22 the failure in mitochondrial ATP generation. Nevertheless, this is not the case because  
23 cells of wild-type strain were able to commit apoptosis even when mitochondrial ATP  
24 synthesis was inhibited by oligomycin (data not shown). A similar response was  
25 observed for acetic acid-induced apoptosis (Ludovico *et al.*, 2002).

1 Several reports describe Yca1p involvement in yeast apoptosis in response to different  
2 stimuli (Madeo *et al.*, 2002; Herker *et al.*, 2004; Wadskog *et al.*, 2004; Wissing *et al.*,  
3 2004). Here we show that deletion of *YCA1* reduced cell death in response to  
4 hyperosmotic stress. Moreover, cell death was accompanied by metacaspase activation  
5 and the deletion of Yca1p consistently resulted in a drastic decrease of metacaspase  
6 activation. Recently, Wysocki and Kron (2004) reported that FITC-VAD-fmk, the  
7 substrate we used for detection of metacaspase activation, binds nonspecifically to dead  
8 cells. These authors claimed that staining with fluorochrome-conjugated caspase  
9 inhibitors is subjected to artifacts. Yet this does not appear to happen under our  
10 conditions. Firstly, our results show the presence of caspase positive/PI negative cells  
11 (Fig. 1F). Secondly the results presented above show that the percentage of cells with  
12 active metacaspase is not strictly correlated with the percentage of dead cells (e.g. after  
13 2 hours treatment, the wild type strain displayed approximately the same cell survival as  
14 the *yca1Δ* strain at 6 hours, but metacaspase activation was three times higher). All  
15 these evidences suggest that unspecific labelling by FITC-VAD-fmk is not occurring  
16 and further reinforce the involvement of Yca1p in hyperosmotic stress-induced  
17 apoptosis.

18 Participation of cyt *c* and Yca1p in different scenarios of yeast apoptosis has been  
19 reported before. However, a link between these two proteins had never been shown. The  
20 results presented above showing that, under hyperosmotic stress, there was a decrease in  
21 the percentage of cells with active metacaspase in the mutant strains *cyc1Δ cyc7Δ* and  
22 *cyc3Δ* in comparison with the wild type strain, point to a causal relationship between  
23 cyt *c* and metacaspase activation. Hindrance of ROS production often results in lack of  
24 activation of mammalian effector caspases and could be a reason for the observed  
25 decrease in metacaspase activation in *cyc1Δ cyc7Δ* and *cyc3Δ* strains (Baker *et al.*,

1 2000). However, this may not be the case in our study since *cyt c* is not essential for  
2 ROS production (Severin and Hyman, 2002; Pozniakovsky *et al.*, 2005), which can  
3 occur in the absence of an intact mitochondrial respiratory chain (Heeren *et al.*, 2004).  
4 Our result with *cyc1Δ cyc7Δ* and *cyc3Δ* strains is the first indication in favour of the  
5 interpretation that *cyt c*, at least in yeast apoptosis induced by hyperosmotic stress, is  
6 important for metacaspase activation and probably acts upstream of this protease in the  
7 apoptotic process. However, the observation that the two *cyt c* mutant strains tested still  
8 present caspase activation, although delayed in comparison to the wild type strain,  
9 indicates that *cyt c* is not indispensable for the activation of this protease but appears  
10 critical for a rapid onset of the activation process.

11 Although our results show that metacaspase Yca1p and *cyt c* are key factors in the  
12 apoptotic process, it should be stressed that their absence only reduces but not abolishes  
13 the apoptotic death. Since *AIF1* has been shown to be involved in apoptosis induced by  
14 H<sub>2</sub>O<sub>2</sub> and chronological aging (Wissing *et al.*, 2004) its involvement in apoptosis  
15 induced by hyperosmotic stress was also studied. However, lack of Aif1p had no effect  
16 on cell survival indicating the existence of alternative cell death pathways.

17 As a whole, these results indicate that, under hyperosmotic stress, *S. cerevisiae* cells  
18 triggers a PCD with an apoptotic phenotype that is partially mediated by a metacaspase-  
19 and mitochondria-dependent pathway. Furthermore, several of the nuclear and  
20 mitochondrial changes that accompany apoptosis induced by hyperosmotic stress in *S.*  
21 *cerevisiae*, appear to be common to those described in *S. cerevisiae* undergoing  
22 apoptosis following Bax expression (Manon *et al.*, 1997; Ligr *et al.*, 1998) and in *Z.*  
23 *bailii* cells under apoptosis induced by acetic acid (Ludovico *et al.*, 2003) suggesting  
24 that a common mitochondrial apoptotic pathway can be activated in yeast in response to  
25 different signals.

1 Several implications in the biotechnological field can be anticipated from this study. A  
2 significant number of yeast species has been described as usual contaminants of high  
3 sugar foods and these species are usually osmotolerant. Understanding the mechanism  
4 that triggers apoptotic cell death under hyperosmotic stress in osmosensitive strains and  
5 how osmotolerant yeast are able to prevent the activation of this cell death program will  
6 allow the development of new preservation strategies for conservation of high sugar  
7 foods. Moreover, the elucidation of the cytotoxic effects induced by high glucose  
8 concentrations can contribute to the optimisation of the industrial fermentative yeast  
9 performance under hyperosmotic stress.

10 It has been recognised that several pathological states such as ischemia, septic shock  
11 and diabetic coma can be associated to hyperosmotic changes (Wright and Rees, 1998;  
12 Galvez *et al.*, 2001), and that hyperosmotic stress induces apoptosis in human cell lines  
13 (Matthews *et al.*, 1997; Edwards *et al.*, 1998; Chan *et al.*, 1999; Hoover *et al.*, 2000;  
14 Mockridge *et al.*, 2000; Morales *et al.*, 2000). As in the case with *S. cerevisiae*,  
15 apoptosis induced by hyperosmotic stress in mammalian cells is caspase-dependent and  
16 seems to involve oxidative stress (Chan *et al.*, 1999). Thus, the genetically tractable  
17 yeast appears as a promising model to unravel apoptotic mechanisms occurring in  
18 human pathological conditions associated to hyperosmotic stress.

19

## 20 **Experimental procedures**

21

### 22 **Yeast strains, plasmids and growth conditions**

23 The yeast strains used in this study are listed in Table 2. The *cyc7Δ* Y10280 strain  
24 (EUROSCARF) was transformed with a DNA fragment carrying the HphMX4  
25 (hygromycin) resistance gene from the vector pAG32 (Goldstein and McCusker, 1999).

Table 2

1 Chromosomal DNA was prepared from the resulting hygromycin resistant and G418  
2 sensitive strain and used as template for PCR using primers GTT ATA GCG CCC CTT  
3 ATT GAA TTA T and TTC CTC TAC TGC TAC TAA GAA CGG A, amplifying a  
4 fragment containing the *cyc7::hphMX4* gene. The *cyc1Δ* strain Y06846  
5 (EUROSCARF) was transformed with the PCR product and the correct integration into  
6 the *CYC7* locus was confirmed with colony PCR. The resulting *cyc1Δ cyc7Δ* strain was  
7 unable to grow on media with glycerol as carbon source.

8 *S. cerevisiae* PYCC3507 strain was grown for 2 days in slants of yeast morphology agar  
9 (YMA; yeast extract, 3 g/l, malt extract, 3 g/l, proteose peptone, 5 g/l and agar, 20 g/l)  
10 and was inoculated in 250 ml flasks containing 100 ml of YMB (yeast extract, 3 g/l,  
11 malt extract, 3 g/l and proteose peptone, 5 g/l) supplemented with 1% (w/w) glucose.  
12 The cultures were incubated in a mechanical shaker (150 rpm) at 28°C.

13 The BY4741-based strains were pre-grown for 24 hours in glass tubes containing SC  
14 medium consisting of 0.17% yeast nitrogen base (Difco), 0.5% ammonium sulfate, 80  
15 mg/L of all amino acids (except 20 mg/L adenine, 400 mg/L leucine and 8 mg/L para-  
16 aminobenzidine) and supplemented with 2% (w/w) glucose as carbon source (SCD).  
17 After 24 hours cells were harvested and transferred to 50 ml flasks containing 10 ml of  
18 SCD. The cultures were incubated on a mechanical shaker (200 rpm) at 30°C.

19

## 20 **Cell death assays**

21 Exponential and stationary cells of *S. cerevisiae* PYCC3507 strain grown in YMB with  
22 1% (w/w) glucose were centrifuged and suspended to a final concentration of  $5 \times 10^7$   
23 cells/ml in 50 ml flasks containing 30 ml of YMB supplemented with 70% (w/w)  
24 glucose and incubated at 28°C without agitation. Loss of cell viability was assessed by  
25 c.f.u. counts after 3 days incubation at 30°C on YMA plates supplemented with 18%

1 (w/w) glucose. No further colonies appeared after this incubation period. Death rates  
2 were also assessed at 4 and 35°C and the effect of aeration (150 rpm) on cell death was  
3 tested at 28°C.

4 Further characterization of cell death induced by high glucose concentrations was  
5 performed with early stationary cells. The cells were harvested and inoculated to a final  
6 concentration of  $2 \times 10^7$  cells/ml in 100 ml flasks containing 25 ml of YMB  
7 supplemented with 60% (w/w) glucose or 60% (w/w) sorbitol. The treatment was  
8 carried out for 24 hours at 28°C with mechanical shaking (150 rpm).

9 To study the role of *cyt c*, *Yca1p* and *Aif1p*, BY4741-based strains were used. Early  
10 stationary cells, grown in SCD medium, were inoculated to a final concentration of  
11  $2 \times 10^7$  cells/ml in 50 ml flasks containing 5 ml of SC medium with 60% (w/w) glucose.  
12 The treatment was carried out for 6 hours at 30°C with mechanical shaking (200 rpm).  
13 Loss of cell viability was assessed as described above.

14

#### 15 **Transmission electron microscopy analysis**

16 To analyse nuclear and lytic ultrastructural alterations, cells from different treatments  
17 were harvested, suspended in 2.5% (v/v) glutaraldehyde in 40mM  
18 phosphate/magnesium buffer, pH 6.5 and fixed overnight at 4°C. After cell wall  
19 digestion with lyticase of the pre-fixed yeast cells (Ludovico *et al.*, 2001), protoplasts  
20 were washed and fixed with aqueous 2% (w/v) osmium tetroxide (2 hours) followed by  
21 postfixation (30 minutes) with 1% (w/v) aqueous uranyl acetate (Silva *et al.*, 1987).  
22 Percentage of lytic cells was determined by analysing 152 control cells and 167 cells  
23 exposed for 12 hours to 60% (w/w) glucose.

24 To analyse mitochondrial ultrastructure, cells were prefixed with glutaraldehyde as  
25 above, fixed with aqueous 2% (w/v) potassium permanganate (1 hour), and postfixed



1 with uranyl acetate as above. Dehydration was performed in ethanol. After 100%  
2 ethanol washes, the samples were transferred to 100% propylene oxide, and infiltrated  
3 with 50% (v/v) propylene oxide and 50% (v/v) Epon (TAAB Laboratories) for 30  
4 minutes and with 100% Epon overnight. Cells were transferred to gelatin capsules with  
5 100% Epon and incubated at 60°C for 48 hours before cutting thin sections and staining  
6 with uranyl acetate and lead citrate (Silva *et al.*, 1987). Micrographs were taken with a  
7 Zeiss EM 10C electron microscope.

8

### 9 **DAPI and PI staining**

10 Nuclear staining protocol with DAPI (Sigma) was adapted from Madeo *et al.* (1997).  
11 Yeast cells were collected, resuspended in 3.7% formaldehyde for 30 minutes, washed  
12 three times with PBS and incubated with 2 µg/ml of DAPI. After 10 minutes at room  
13 temperature cells were washed with PBS and mounted on a slide with a drop of anti-  
14 fading agent Vectashield (Vector laboratories, Inc.).

15 PI staining was used to monitor cell membrane integrity as previously described  
16 (Ludovico *et al.*, 2003). Along exposure to 60% (w/w) glucose in YMB, 300 µl of yeast  
17 samples were taken and incubated with 1.5 µl of a stock solution (1mg/ml) of PI  
18 (Sigma) for 10 minutes at room temperature. Cell suspensions in YMB with 60% (w/w)  
19 glucose boiled for 5 minutes were used as positive control whereas cells not subjected  
20 to hyperosmotic glucose (YMB with 1% (w/w) glucose) were used as a negative  
21 control.

22 The samples were observed under a Leitz Laborlux S epifluorescence microscope  
23 equipped with a 50 W mercury lamp and appropriate filter setting. The digital images  
24 were acquired with a 3CCD colour video camera (SONY, DXC-9100P), a frame

1 grabber (IMAGRAPH, IMASCAN/Chroma-P) and software for image archival and  
2 management (AxioVision 3.0, Carl Zeiss Vision GmbH)

3

#### 4 **Assessment of ROS production**

5 ROS production was detected with DE (Molecular Probes) or H<sub>2</sub>DCFDA (Molecular  
6 Probes), essentially as described by Madeo *et al.* (1999). Before treatment with glucose  
7 (60%, w/w), cells were preloaded with 10 µg/ml of DE or 40 µg/ml of H<sub>2</sub>DCFDA for 45  
8 minutes at 30°C. After preloading with the probe cells were transferred to YMB with  
9 60% (w/w) glucose to induce hyperosmotic stress and ROS production was monitored  
10 by epifluorescence microscopy. Cells not subjected to hyperosmotic stress were used as  
11 a negative control. Microscope and image acquisitions were performed as described for  
12 DAPI and PI staining.

13

#### 14 **TUNEL assay**

15 DNA strand breaks were detected by the "In Situ Cell Death Detection Kit, Fluorescein"  
16 (Roche Applied Science) using a protocol previously described (Ludovico *et al.*, 2001)  
17 with slight modifications. Yeast cells were fixed with 3.7% (v/v) formaldehyde, the cell  
18 wall was digested with lyticase as referred above, and cells were applied to poly-lysine-  
19 coated slides. The slides were rinsed with PBS, incubated in permeabilization solution  
20 (0.1%, v/v, Triton X-100 and 0.1%, w/v, sodium citrate) for 2 min in ice, rinsed twice  
21 with PBS and incubated with 10 µl TUNEL reaction mixture (terminal  
22 deoxynucleotidyl transferase 200 U/ml, FITC-labeled dUTP 10 mM, 25 mM Tris-HCl,  
23 200 mM sodium cacodylate and 5 mM cobalt chloride) for 60 minutes at 37°C. Finally  
24 the slides were rinsed three times with PBS and a coverslip was mounted with a drop of

1 anti-fading agent Vectashield (Vector Laboratories, Inc). Microscope and image  
2 acquisitions were performed as described for DAPI and PI staining.

3

#### 4 **Detection of metacaspase activity**

5 Detection of active metacaspase was performed using the "CaspACE, FITC-VAD-fmk  
6 In Situ Marker" (Promega) and a protocol adapted from Madeo *et al.* (2002). Briefly,  
7  $1 \times 10^6$  cells were washed in PBS, resuspended in 100  $\mu$ l staining solution containing 50  
8  $\mu$ M of FITC-VAD-FMK and incubated for 20 minutes at 30°C. After incubation cells  
9 were washed once and resuspended in PBS. Cells were subsequently incubated with 2  
10  $\mu$ g/ml of PI for 10 min at room temperature for double staining with PI.

11 Flow cytometric analysis was performed in an Epics<sup>®</sup> XL-MCL<sup>™</sup> (Beckman Coulter)  
12 flow cytometer equipped with an argon-ion laser emitting a 488-nm beam at 15mW.  
13 Green fluorescence was collected through 488 blocking filter, a 550 nm long-pass  
14 dichroic and a 525 nm band-pass. Twenty thousand cells were analysed per sample at  
15 low flow rate. Data were analysed by WinMDI 2.8 software. Microscope and image  
16 acquisitions were performed as described for DAPI and PI staining.

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2

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1 **Figure legends**

2

3 **Figure 1.** Cell survival and apoptotic markers in the *S. cerevisiae* PYCC3057 strain  
4 during hyperosmotic stress. (A) Cell survival evaluated by c.f.u. (100% corresponds to  
5 the number of cells at time zero) in medium with 60% (w/w) glucose (empty circles) or  
6 with 60% (w/w) sorbitol (full circles). Values are mean  $\pm$  SEM of a representative  
7 experiment of three independent experiments. (B) Percentage of cells displaying  
8 chromatin condensation (DAPI +), DNA strand breaks (TUNEL +) and loss of  
9 membrane integrity (PI +) as detected by DAPI staining, TUNEL assay and PI staining,  
10 respectively, after exposure to 60% (w/w) glucose. To determine the percentage of  
11 positive cells, at least 300 cells were evaluated for each parameter. Data are from a  
12 representative experiment of three independent experiments.

13

14 **Figure 2.** Nuclear and mitochondrial apoptotic alterations and metacaspase activation in  
15 *S. cerevisiae* PYCC3507 cells under hyperosmotic stress. (A) DAPI staining images of  
16 control cells (0 hours) and cells treated during 2 and 10 hours; bar 10  $\mu$ m. (B) Electron  
17 microscopy images of control cells (0 hours) and cells treated during 12 hours, with  
18 fixation with glutaraldehyde-OsO<sub>4</sub>-uranyl acetate. N–nucleus. Arrows mark chromatin  
19 condensation; bar 0.3  $\mu$ m. (C) Electron microscopy images of control cells (0 hours)  
20 and cells treated during 12 hours, with fixation with glutaraldehyde-KMnO<sub>4</sub>-uranyl  
21 acetate. Arrowheads mark swollen mitochondria; bar 0.3  $\mu$ m. (D) TUNEL images of  
22 control and of cells treated for 8 or 12 hours. Left panel: phase contrast microscopy;  
23 right panel: fluorescence microscopy of the same cells; bar 10  $\mu$ m. (E) Electron  
24 microscopy image showing ultrastructural lytic alterations, with fixation with  
25 glutaraldehyde-OsO<sub>4</sub>-uranyl acetate; bar 0.3  $\mu$ m. (F) Metacaspase activation image in

1 cells treated with 60% (w/w) sorbitol during one hour. Left panel: FITC filter; right  
2 panel: PI filter; bar 10  $\mu$ m.

3

4 **Figure 3.** ROS production in *S. cerevisiae* PYCC3507 cells during hyperosmotic stress.

5 ROS production detected by dihydroethidium (A) or H<sub>2</sub>DCFDA (B) in control cells and

6 cells treated for 3 hours. In A and B the upper panels show phase contrast microscopy;

7 the lower panels show fluorescence microscopy of the same cells. Bar 10  $\mu$ m.

8

9 **Figure 4.** Involvement of cyt *c* but not of Aif1p in hyperosmotic induced cell death.

10 Cell survival evaluated by c.f.u. (100% corresponds to the number of cells at time zero)

11 of the wild-type BY4741 strain and of three mutant strains lacking cyt *c* (*cyc1 $\Delta$  cyc7 $\Delta$* ),

12 mature cyt *c* (*cyc3 $\Delta$* ) or the yeast Aif1p (*aif1 $\Delta$* ). Values are mean  $\pm$  SEM of five

13 independent experiments. \* denotes values significantly different from the control

14 ( $P < 0.05$ ; unpaired Student's *t*-test).

15

16 **Figure 5.** Metacaspase involvement in death induced by hyperosmotic stress. (A) Cell

17 survival evaluated by c.f.u. (100% corresponds to the number of cells at time zero) of

18 the wild-type BY4741 strain and of a strain deleted in the yeast metacaspase (*yca1 $\Delta$* ).

19 Values are mean  $\pm$  SEM of five independent experiments and are significantly different

20 ( $*P < 0.05$ ,  $**P < 0.001$ ; unpaired Student's *t*-test) from those obtained with the control.

21 (B) Percentage of cells with active metacaspase of the wild-type BY4741 strain and

22 three mutant strains (*cyc1 $\Delta$  cyc7 $\Delta$* , *cyc3 $\Delta$*  and *yca1 $\Delta$* ) during hyperosmotic stress.

23 Control cells (0 hours) and treated cells (2 and 6 hours) labelled for active metacaspase

24 with FITC-VAD-fmk and analysed by flow cytometry. Data represent one of three

25 independent experiments.

**Table 1**

Specific death rates ( $\mu_d$ ) of exponential and stationary cells of *S. cerevisiae* PYCC3507 in YMB 70% (w/w) glucose at different temperatures. The values are mean  $\pm$  SD of two independent experiments.

Temperature (°C)	$\mu_d$ (days <sup>-1</sup> ) <sup>a</sup>	
	Exponential cells	Stationary cells
4	0.407 $\pm$ 0.0044	0.226 $\pm$ 0.0053
28	0.458 $\pm$ 0.0028	0.566 $\pm$ 0.0324
35	0.648 $\pm$ 0.0023	0.813 $\pm$ 0.0191

<sup>a</sup> The values of  $\mu_d$  were estimated from the slope of the linear part of the semilogarithmic plot of the number of colony forming units as a function of incubation time.

**Table 2**

Yeast strains used in this study.

Strain	Genotype	Source
BY4741	MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Euroscarf
<i>cyc1Δ cyc7Δ</i>	MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> <i>cyc1::kanMX4 cyc7::hphMX4</i>	This study
<i>cyc3Δ</i>	MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> <i>cyc3::kanMX4</i>	Euroscarf
<i>yca1Δ</i>	MATa <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> <i>yca1::kanMX4</i>	Euroscarf
<i>aif1Δ</i>	MATa <i>his3Δ1 leu20 met15Δ0 ura3Δ0</i> <i>aif1::kanMX4</i>	Euroscarf
PYCC3507		PYCC <sup>a</sup>

<sup>a</sup> Portuguese Yeast Culture Collection