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# Yeast Cell Death During the Drying and Rehydration Process

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## 1. Introduction

Dehydration and rehydration stress (DRS) is a serious problem affecting plants, animals and humans and much research has been devoted to the subject over the years. Attempts to enhance the desiccation tolerance of cells first focused on plant cells and seeds of agricultural significance, as the availability of water is one of the main parameters that limits plant productivity (Bartels et al., 2001). More recently, lyophilisation and other dehydration-based technologies have been explored by a number of groups for the purpose of cell and tissue preservation (Liang et al., 2002; Wolkers et al., 2002; Elliott et al., 2006). Furthermore, active dry yeast is commonly used in the food industry for the production of beer, wine and bread. *Saccharomyces cerevisiae*, in addition to being an excellent model for the study of eukaryotic cells, is an ideal starting point for deciphering DRS response mechanisms due to its anhydrobiotic qualities. The transformation of yeast cells from the state of vital activity to that of anhydrobiosis as a result of cell desiccation is followed by a period of suspended animation, and the subsequent recovery of metabolic functions. To understand what yeasts do, we must address controversial issues such as cell age, longevity, the structural and biochemical properties of anhydrous cytoplasm, and metabolic stasis (Beker & Rapoport, 1987). The resulting damage may be classified into damage of different macromolecules, structures, organelles, and defensive intracellular reactions. Membrane changes are especially interesting (Rapoport et al., 1994). Increased plasma permeability or rehydration has been proposed as the main cause of cell death during dehydration. In fact, the increase and decrease of osmotic pressure causes the leakage of nucleotides, ions and other soluble cell components into the surrounding medium (Attfield et al., 2000). The highly dynamic lipid bilayer of the plasma membrane is known to undergo phase transitions during dehydration (Laroche et al., 2005) and rehydration (Crowe et al., 1992). These phase transitions of some phospholipids in the membrane may be the cause of membrane rupture or changes in permeability (Laroche et al., 2003). Other authors suggest that the formation of endovesicles during dehydration leads to plasma membrane lysis during osmotic expansion when the cells are rehydrated (Mille et al., 2003). Yeast cells can recover faster or slower

depending on the culture conditions (Anand & Brown, 1968; Rodríguez-Porrata et al., 2011) and/or rehydration conditions (Poirier et al., 1999; Rodríguez-Porrata et al., 2008). Despite the accumulated knowledge about the structural changes and the mechanical damage to cells during DRS, little is known about the molecular mechanisms involved in yeast cell death under these stressful conditions. In recent years, it has become clear that yeast can succumb to cell death, exhibiting typical apoptotic markers (Ludovico et al., 2001; Madeo et al., 1997, Madeo et al., 1999). Moreover, the yeast genome codes for many proteins of the basic molecular machinery responsible for cell death, including orthologues of caspases (Madeo et al., 2002), AIF (Wissing et al., 2004), and yeast EndoG (NUC1) (Büttner, et al., 2007). In addition, programmed death in yeast has been linked to complex apoptotic scenarios such as mitochondrial fragmentation (Fannjiang et al., 2004), cytochrome *c* release (Ludovico et al., 2002), and aging (Fabrizio et al., 2004; Herker et al., 2004; Laun et al., 2001). Notably, histone H2B phosphorylation, which is considered to be a universal prerequisite for apoptosis execution (Cheung et al., 2003), was shown to be necessary for cell-death induction upon oxidative stress in yeast (Ahn et al., 2005). Recently, yeast apoptosis research has begun to resolve the complex interplay of mitochondrial cell death mediators. It is becoming increasingly clear that the connection between mitochondrial respiration and apoptosis is intricate, as suppression of respiration can either be beneficial or detrimental to the cell, depending greatly on the apoptotic scenario (Eisenberg et al., 2007). It is not likely by chance that nature has coupled pro-apoptotic potential to many molecules that have a genuine function in the respiratory chain of healthy cells, such as cytochrome *c*, AIF (apoptosis-inducing factor) or AMID (apoptosis-inducing factor-homologous mitochondrion-associated inducer of death). By simply changing the location from mitochondria, the daytime place of action, to the cytosol, cell death is executed in a redundant, highly effective manner. As a result, the permeabilisation of the mitochondrial outer membrane is probably the point of no return in cell death execution and thus an excellent target for clinical manipulations of apoptosis (Galluzzi et al., 2006) and perhaps even necrosis (Golstein & Kroemer, 2006).

Here, we identified a group of mitochondrial knockout mutants ( $\Delta Aif1$ ,  $\Delta Cpr3$ ,  $\Delta Nuc1$ , and  $\Delta Qcr7$ ) as hyper-tolerant to dehydration stress. Yeast cells were analysed for apoptotic hallmarks. DHE staining revealed that during dehydration and rehydration, the wild type showed enhanced ROS production compared to the mutants. Additionally, Annexin V/PI double staining indicated that, after the imposition of stress, the wild type culture also contains an elevated percentage of necrotic and late apoptotic/secondary necrotic cells. Further tests using the strains  $\Delta oxa1$ ,  $\Delta mgm1$ , and  $\Delta yac1$  suggested that cell death during dehydration stress is neither caspase nor respiratory dependent.

## 2. Materials and methods

### 2.1 Strains and growth conditions

Table 1 summarises the yeast strains used in this study. The single null mutant collection of strains and the reference strain, all in the BY4742 genetic background, were purchased from EUROSCARF (Frankfurt, Germany). Yeast strains were grown in shake flasks (150 rpm) in SC media containing 0.17% yeast nitrogen base supplied by Difco, 2% glucose, 0.5%  $(\text{NH}_4)_2\text{SO}_4$  and 25 mg l<sup>-1</sup> uracil, 84 mg l<sup>-1</sup> leucine, and 42 mg l<sup>-1</sup> lysine, and histidine. The

desiccation-rehydration process and the determination of yeast viability were performed as described in Rodríguez et al. (2011).

## 2.2 Determination of yeast viability

Yeast cells were cultivated in SC medium until the stationary phase and then some of the culture was transferred to a 12-well plate in the presence of trehalose at 10% W/V of the final concentration. Half of the cell suspension was transferred to another 12-well plate for drying. The cells in the second plate were air-dried at 28°C for 24 hours. They were then rehydrated with sterile water at 37°C for 30 minutes. To calculate cell survival, the cell cultures were diluted and cell concentration was determined with a CASY cell counter and aliquots containing 500 cells, which were spread onto YPD agar medium using a Whitley Automatic Spiral Plater furnished by AES Laboratoire (France). The number of colonies was determined after two days at 28°C. The CFU were quantified using a Lemnatec Microbiology-Colony-Counter and processed with ProtoCOL SR/HR counting system software version 1.27, supplied by Symbiosis (Cambridge, UK). After the colonies were counted, the percentage of viability was determined by means of a simple calculation.

## 2.3 Tests for apoptotic markers

Dihydroethidium (DHE) staining was performed with approximately  $5 \cdot 10^6$  cells per experiment, which were washed with PBS (pH 7.0) and resuspended with 250 ml of 2.5 mg ml<sup>-1</sup> DHE/PBS. After 5 min dark incubation at 25°C, the cells were washed with 250 ml PBS prior to both microscopic and flowcytometric evaluation. Each double Annexin V fluorescein and propidium iodide (PI) staining was carried out with  $2 \cdot 10^7$  yeast cells washed with 500 ml sorbitol buffer (1.2 M sorbitol, 0.5 mM MgCl<sub>2</sub>, 35 mM potassium phosphate, pH 6.8). The resuspended cells in 330 ml sorbitol buffer were incubated with 2.5 µl Lyticase and 15 µl beta-glucuronidase/arylsulfatase (Roche) for 1 h at 28°C. After this treatment the cells became spheroblasts, so the centrifugation and resuspension steps were very brief. The cells were harvested, washed again in 500 ml sorbitol buffer, and suspended in incubation buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl<sub>2</sub> at pH7.4) containing 0.6 M sorbitol. Then, 3 µl Annexin V acquired from Roche and 3 µl PI (100 µg ml<sup>-1</sup> in H<sub>2</sub>O) were added and the cells were dark incubated for 20 min at 25°C. After adding 500 µl incubation buffer containing 0.6 M sorbitol the cells were ready to be analysed with the flowcytometer using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a >560 nm filter for PI detection. TUNEL staining was performed as previously described in the literature (Büttner *et al.*, 2007). To determine frequencies of morphological phenotypes revealed by TUNEL, DHE- and Annexin V/PI double staining, at least 300 cells of three independent experiments were evaluated using flowcytometry and BD FACSDiva software.

## 2.4 Microscopy

Cultures of the strains were grown to the stationary phase in SC medium. The cells were washed with 1 x PBS buffer (pH 7.4) and fixed with 70% ethanol for 10 min at R.T. Images were taken using an Olympus model BH-2RFCA fluorescence microscope, an Olympus model c35AD-4 digital camera, and Metamorph® software provided by Soft Imaging System GmbH.

## 2.5 Statistical analyses

The results were statistically analysed by one-way ANOVA and the Scheffé test from the statistical software package SPSS 15.1. Statistical significance was set at  $P < 0.05$ .

## 3. Results

### 3.1 Drying and rehydration stress compromises yeast survival

To study the molecular response to drying and rehydration stress (DRS) in yeast, we analysed the viability of the complete EUROSCARF collection of *Saccharomyces cerevisiae* upon DRS. This collection comprises a total of 4794 mutants, each deleted in one of the non-essential genes. While viability in the wild-type reference strain BY4742 was approximately 40%, we detected a group of around 100 deletion mutants with viability of less than 10%. Figure 1 shows the functional distribution of the corresponding genes ranked according to their relative abundance. Pathways involving protein synthesis and the biogenesis of cellular components occurred more frequently, while pathways connected to cell fate, cellular rescue and environment interaction were the least abundant. Furthermore, we detected a group of 12 deletion mutants with viability values higher than those of the reference strain.

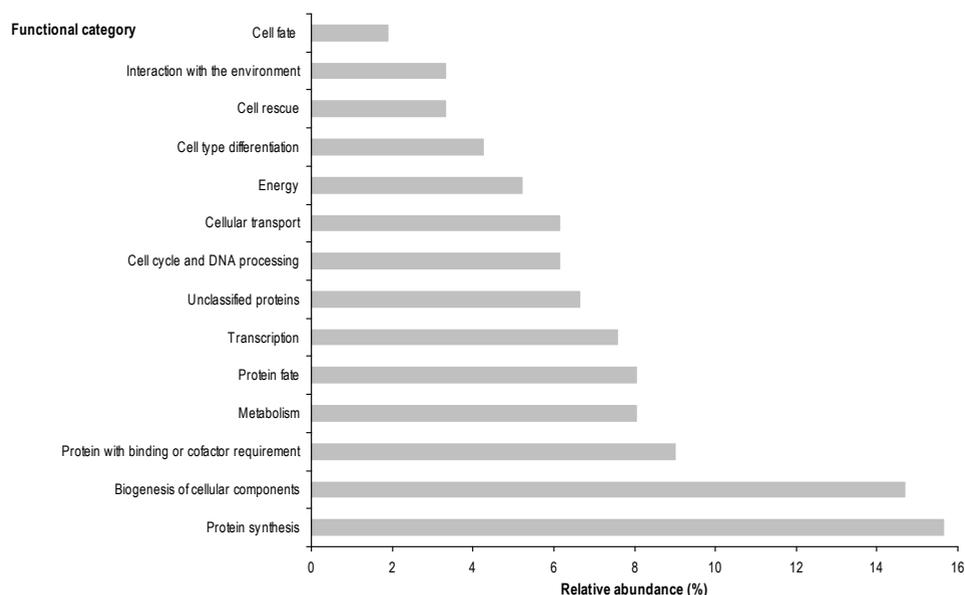


Fig. 1. Distribution of functional classes of 112 EUROSCARF deletion mutants showing less than 10% viability after drying and rehydration stress

### 3.2 Several mutants can rescue cell death upon rehydration stress

Among the 12 mutants that showed enhanced cell viability after dehydration stress, some are directly connected to programmed cell death (PCD). Figure 2 represents the fold

increase in viability of some mutants deleted in genes closely linked to PCD. Interestingly, the knockouts with viabilities higher than the reference strain BY4742 lack the mitochondrial genes *AIF1*, *CPR3* and *NUC1* (2-fold increase in viability) and *QCR7* (3-fold increase). *CPR3* encodes for a yeast homologue of cyclophilin D (Dolinski et al., 1997), which is a peptidyl-prolyl isomerase located within the mitochondrial matrix and which is a component of mPTP, along with adenine nucleotide translocator (ANT) and the voltage-dependent anion transporter (VDAC). Cyclophilin D is thought to facilitate a calcium-triggered conformational change in the ANT mitochondrial permeability transition associated with mitochondrial swelling, outer membrane rupture, and the release of apoptotic mediators (Halestrap, 2005). Cyclophilin D has previously been implicated in both necrosis and apoptosis programmes (Halestrap, 2005; Schneider, 2005). *Cpr3p* has been reported to be central to the PCD process induced by Cu in *S. cerevisiae* (Liang & Zhou, 2007). MPTP has been shown to be a key component of necrotic cell death caused by calcium overload and oxidative stress and does not usually play much of a role in apoptosis (Crompton et al., 1988). The release of proteins from the compartment between the two mitochondrial membranes, of which cytochrome C is the major player, triggers apoptosis in many cells through the caspase pathway. However, many researchers argue that this is unlikely because it would disrupt the production of ATP, which is required in the apoptosis process. This is supported by results obtained with cyclophilin D knockout mice in which the extensive apoptosis involved in development was not affected by the loss of cyclophilin D (Nakagawa et al., 2005). *AIF1* encodes for Aif1p, a homologue of the mammalian Apoptosis-Inducing Factor (AIF). Aif1p is a flavoprotein with NADH oxidase activity and contains a mitochondrial localisation sequence in the NH<sub>2</sub> terminus and a nuclear localisation sequence in the COOH terminus, as well as a putative DNA binding domain composed of positively charged amino acids (Wu et al., 2002). Upon the induction of apoptosis, Aif1p translocates to the nucleus, where it leads to chromatin condensation and DNA degradation (Susin et al., 1999; Madeo et al., 1999). It has recently been shown that chronological aging is a physiological trigger for apoptosis in yeast (Herker et al., 2004). The release of Aif1p from mitochondria may be subordinated to earlier caspase activation, supporting the notion that caspases and Aif1p may be engaged in cooperative or redundant pathways and they are activated by the same apoptotic stimulus (Arnoult et al., 2003; Madeo et al., 2002). *NUC1* encodes for the major mitochondrial nuclease and has RNase and DNA endo- and exonucleolytic activities. Nuc1p plays a role in mitochondrial recombination, apoptosis and the maintenance of polyploidy. Nuc1p and mammalian mitochondrial nuclease EndoG share well preserved residues in the catalytically active site, suggesting that both belong to the large family of DNA/RNA nonspecific bba-Me-finger nucleases (Schafer et al., 2004). Upon the induction of apoptosis, the translocation of mammalian EndoG to the nucleus coincides with large-scale DNA fragmentation (Li et al., 2001; Parrish et al., 2001). EndoG may have a genuine vital function in addition to its pro-apoptotic function, which plays a role in cell proliferation and replication, as has in fact been suggested by other authors (Huang et al., 2006). The last mutated strain showing enhanced viability after the dehydration and rehydration process lacks *QCR7*, which is associated with Qcr8p and cytochrome *b*, both constituents of one of the sub-complexes of the mitochondrial inner membrane electron transport chain of the yeast cytochrome *bc*<sub>1</sub>. The existence of this sub-complex has been proposed on the basis of the finding that the deletion of any one of these three genes leads to the disappearance of the other two subunits, and because the Qcr7p N-terminal was shown to stabilise the central core of the cytochrome *bc*<sub>1</sub> complex (Zara et al.,

2004; Lee et al., 2001). Furthermore, the  $\Delta qcr7$  strain does not continue the PDC process induced by Cu in *S. cerevisiae* (Liang & Zhou, 2007).

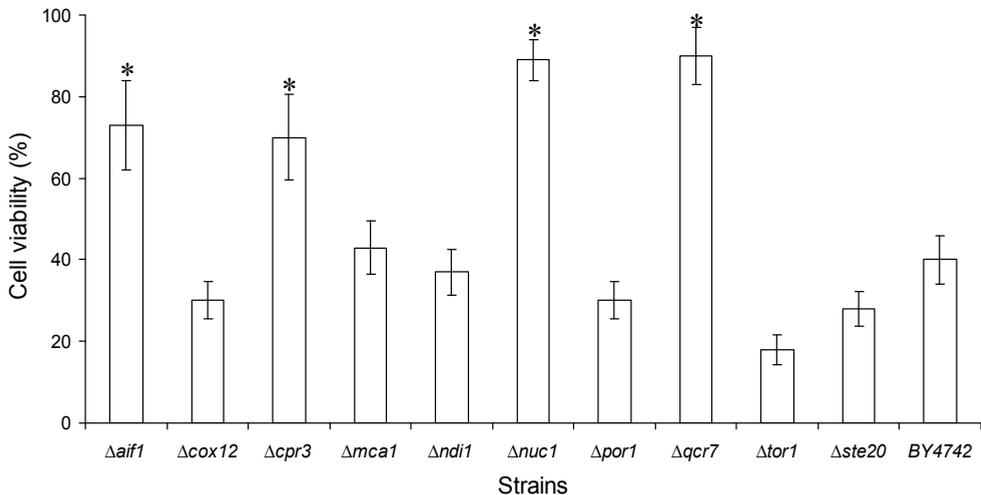


Fig. 2. Effect of deletion of genes linked to cell death mechanisms in cell viability after stress imposition. Values shown are means of  $n=3$  independent samples  $\pm$ SD. \*Significant differences ( $p \leq 0.05$ ) to BY4742 strain

Among the mutants evaluated and those deleted in genes closely linked to PCD, only four showed enhanced cell viability after the imposition of stress. We wanted to ascertain whether the higher viability rate for these four strains under dehydration stress could be due to differences in the apoptotic hallmark profile.

### 3.3 Dehydration survival is associated with diminished apoptosis and necrosis

We characterised the mode of cell death accompanying DRS by performing various assays using flowcytometry to quantify apoptotic and necrotic markers. The conversion of dihydroethidium (DHE) to fluorescent ethidium was used to visualise the accumulation of reactive oxygen species (ROS). DNA fragmentation was detected using TUNEL staining. Furthermore, Annexin V/propidium iodide (PI) co-staining was used to quantify the externalisation of phosphatidylserine, an early apoptotic event, and membrane permeabilisation, which is indicative for necrotic death. This staining allows a distinction to be made between early apoptotic (Annexin V positive, PI negative), late apoptotic (Annexin V positive, PI positive), and necrotic (Annexin V negative, PI positive) death. Figure 3 shows the results obtained for the apoptotic hallmarks of the BY4742,  $\Delta aif1$ ,  $\Delta cpr3$ ,  $\Delta nuc1$ , and  $\Delta qcr7$  strains before dehydration (BD) and after rehydration (AR), respectively. All strains began with comparable ROS levels before dehydration (Fig. 3-B). However, after rehydration, the  $\Delta aif1$ ,  $\Delta cpr3$ ,  $\Delta nuc1$  and  $\Delta qcr7$  strains show an approximate 20% reduction in ROS accumulation compared to the BY4742 strain. These reduced ROS levels in the mutated strains during stress imposition are accompanied by an increase in both early apoptotic and late apoptotic cell populations (Fig. 3 C-D), even when before the imposition of stress the

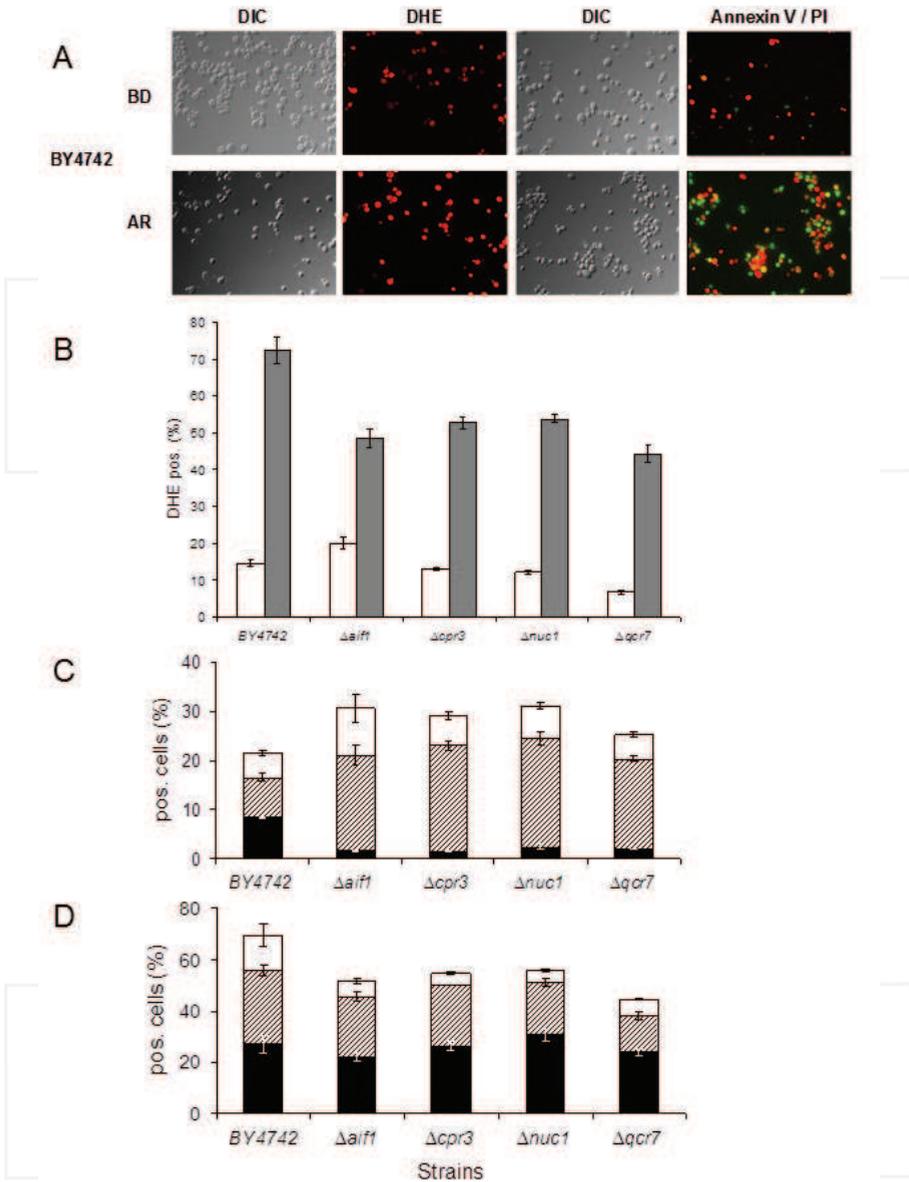


Fig. 3. Deletion of mitochondrial cell death genes prevents necrotic cell death. (A) These pictures are a representative example of fluorescence microscopy of DHE-, and Annexin V/PI-costaining of before dehydration (BD) and after rehydration (AR) of the same BY4742 cell samples. (B) Level of ROS-accumulating cells using DHE-staining before drying (white bars) and after rehydration (grey bars). Quantification of stained cells before (C) and after (D) stress imposition: V-/PI+ □, V+/PI+ ▨ and V+/PI- ■. In each experiment, 3 · 10<sup>4</sup> cells were evaluated using flowcytometry. DIC, differential interference contrast; DHE pos., DHE-positive cells

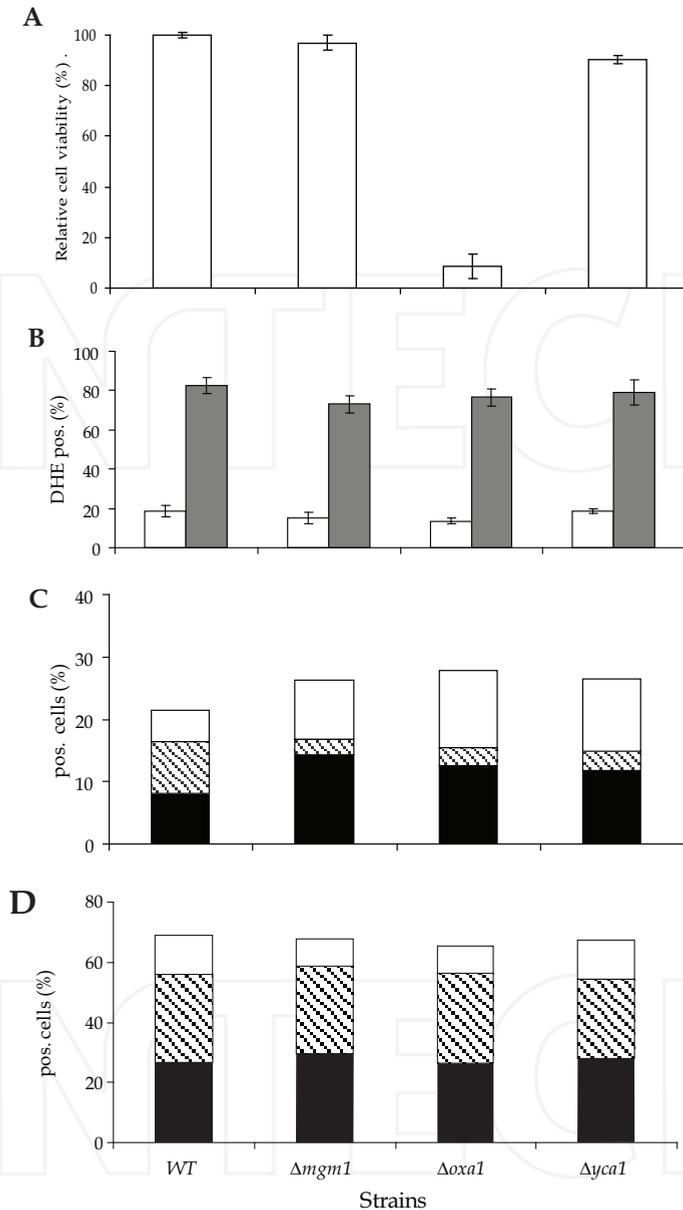


Fig. 4. Dehydration stress characterisation of respiratory deficient strains. (A) The scale of relative viability (%) indicates the percentage of experimental values for the different strains relative to the highest viability for *S. cerevisiae*. (B) ROS accumulating cells before dehydration (white bars) and after rehydration (grey bars). V-/PI<sup>+</sup> □, V+/PI<sup>+</sup> ▨, and V+/PI<sup>-</sup> ■ stained cells before (C) and after (D) stress imposition. Values shown are means of  $n=3$  independent samples  $\pm$ SD

mutants showed 8% less early apoptotic and 10% more late apoptotic cells than BY4742. However, after stress imposition cell necrosis and late apoptotic populations were reduced in  $\Delta$  strains by 17% and 10%, respectively. This result suggests that the aforementioned improved viability upon the absence of these genes corresponds to a prevention of necrosis and apoptosis under dehydration stress.

### 3.4 Respiration deficiency is not responsible for cell death prevention

Among the deleted mitochondrial strains, which provide resistance upon DRS, *QCR7* is essential to respiratory activity. We therefore evaluated two further respiratory deficient strains in order to rule out an effect due to loss of respiratory capacity. We chose the  $\Delta$ *mgm1* and  $\Delta$ *oxa1* mutants, which lack respiratory growth and have significantly different viability values after dehydration stress (40% and 5% respectively) (Fig. 4-A). Neither  $\Delta$ *mgm1* nor  $\Delta$ *oxa1* showed reduced ROS levels compared to the wild type (Fig. 4-B).

The analysis of apoptotic and necrotic markers further showed no significant differences in DNA fragmentation, phosphatidylserine externalisation or loss of cell integrity after rehydration (Fig. 4 C-D). Thus, we might suggest that the observed reduction in the apoptotic and necrotic populations for the  $\Delta$ *aif1*,  $\Delta$ *cpr3*,  $\Delta$ *nuc1* and  $\Delta$ *qcr7* strains after DRS is independent of respiration capacity.

### 3.5 Reduction of apoptotic and necrotic cell populations is caspase-independent

We tested the  $\Delta$ *yca1* strain to determine whether cell death was associated with the caspase pathway in our stress evaluation. The *S. cerevisiae* *YCA1* gene encodes for a metacaspase that is involved in yeast apoptosis in response to multiple stimuli, such as cell aging, oxidative stress, etc. (Herker et al., 2004; Madeo et al., 2002). The  $\Delta$ *yca1* strain does not show improved survival or reduced ROS levels compared to BY4742 after stress imposition (Fig. 4-A and -B). Furthermore, the analysis of apoptotic and necrotic markers also revealed no significant differences to the reference strain in DNA fragmentation, phosphatidylserine externalisation or loss of cell integrity after rehydration (Fig. 4-C and -D). This result supports the suggestion that the reduction in the apoptotic and necrotic populations observed during dehydration stress does not involve the metacaspase pathway.

Strain	Genotype	Source
BY4742	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0</i>	EUROSCARF
$\Delta$ <i>aif1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 aif1::kanMX4</i>	EUROSCARF
$\Delta$ <i>cox12</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 cox12::kanMX4</i>	EUROSCARF
$\Delta$ <i>cpr3</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 cpr3::kanMX4</i>	EUROSCARF
$\Delta$ <i>mca1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 mca1::kanMX4</i>	EUROSCARF
$\Delta$ <i>ndi1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 ndi1::kanMX4</i>	EUROSCARF
$\Delta$ <i>nuc1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 nuc1::kanMX4</i>	EUROSCARF
$\Delta$ <i>por1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 por1::kanMX4</i>	EUROSCARF
$\Delta$ <i>qcr7</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 qcr7::kanMX4</i>	EUROSCARF
$\Delta$ <i>tor1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 tor1::kanMX4</i>	EUROSCARF
$\Delta$ <i>ste20</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 ste20::kanMX4</i>	EUROSCARF
$\Delta$ <i>yca1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 yca1::kanMX4</i>	EUROSCARF
$\Delta$ <i>oxa1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 oxa1::kanMX4</i>	EUROSCARF
$\Delta$ <i>mgm1</i>	MATa <i>his3<math>\Delta</math>1 leu2<math>\Delta</math>0 lys2<math>\Delta</math>0 ura3<math>\Delta</math>0 mgm1::kanMX4</i>	EUROSCARF

Table 1. Yeasts strains used in this study

#### 4. Discussion and conclusions

Dehydration and rehydration stress (DRS) is of great interest in the food industry. For example, the rehydration of active dry wine yeast probably represents one of the most critical phases in the entire winemaking process. Only proper rehydration can ensure the viability of healthy cells which lead to efficient fermentation. Dehydration causes a rapid efflux of water through the cell membrane, resulting in the collapse of the cytoskeleton. This dry state has a deleterious effect on yeast cell physiology by altering the structure and function of the vacuole, and the integrity and functionality of nuclear and cell membranes (Walker and van Dijck, 2006). The liquid-crystalline phase transition experienced by both dry membranes and lipid bi-layers during rehydration leads to changes in their permeability (Crowe et al., 1992, 1998). In fact, dehydrated yeast has been shown to lose up to 30% of soluble cell compounds when rehydrated, thus proving the loss of cell membrane functionality (Beker et al., 1984; Rapoport et al., 1994; Rodríguez-Porrata et al., 2008). The yeast *S. cerevisiae* is one of the few organisms capable of resisting these complex changes, which may allow it to overcome the multifaceted stress of the desiccation-rehydration process. However, in this study we found that only approximately 40% of BY4742 cells are able to generate a colony in rich medium after DRS. We systematically analysed the viability of the complete EUROSCARF collection of *S. cerevisiae* upon DRS and detected a series of knockouts displaying increased viability compared to the reference strain. Interestingly, among them were the *AIF1*, *CPR3* and *NUC1* deleted mutants, all genes directly involved in yeast apoptosis and necrosis, and coding for mitochondrial proteins (Büttner et al., 2007; Madeo et al., 1999; Halestrap, 2005). Of note, the lack of an additional mitochondrial protein, Qcr7p (the subunit 7 of the ubiquinol cytochrome-*bc*<sub>1</sub> reductase complex), also provided resistance upon DRS. Beyond their importance in energy metabolism, mitochondria have emerged as crucial organelles in PCD control (Kroemer, 2002). Like mammals, yeast also bears mitochondrially dictated cell death pathways (Eisenberg et al., 2007). For instance, Aif1p and Nuc1p are caspase-independent pro-death mitochondrial factors that upon various stresses translocate from mitochondria to the nucleus to facilitate degradation of nuclear DNA (Wissing et al., 2004; Liang et al., 2008). Therefore, the lethal function of Aif1p has been shown to depend on the yeast homologue of cyclophilin A (Cande et al., 2004; Herker, 2004). However, until now there has been no direct mention of a link between Aif1p and Cpr3p. Our experiments suggest that Aif1p and Crp3p as well as Nuc1p are activated upon DRS. In addition, we show that they perform their lethal activity in a caspase-independent manner since metacaspase deficiency did not prevent DRS-mediated cell death. Importantly, mitochondria is a major source for reactive oxygen species (ROS), which play a central role in mediating yeast cell death (Mazzoni et al., 2003; Weinberger et al., 2003). The bulk of mitochondrial ROS generation occurs as a by-product of respiration in the electron transport chain (ETC), where Q-cytochrome c oxidoreductase (complex III) acts as a source of ROS (Cadenas et al., 2000; Turrens, 2003; Dröse, et al., 2008). In keeping with this, the deletion of *QCR7*, which derives in disassembly from complex III, reveals lower levels of ROS before and more markedly after the imposition of DRS. This effect, however, does not seem to rely solely on respiratory disruption, as other respiration deficient mutants did not show any rescuing effect. The molecular significance of Qcr7p in cell death will need to be further clarified in future studies. Interestingly, our results show that DRS mediates a type of death which combines both apoptosis and necrosis. The enhanced viability of the different deletion mutants is thereby accompanied by a reduction in both apoptotic and

necrotic markers. In fact, death mediated by mammalian AIF, cyclophilin D and endonuclease G has been described as including both types of death depending on the scenario (Madeo et al., 1999; Halestrap, 2005; Schneider, 2005; Büttner et al., 2006). It is thus possible that DRS activates both types of death in yeast which the proteins we describe are able to execute in parallel or in series.

In conclusion, based on our results we suggest that under DRS cell death is closely linked to molecular pathways that induce death by apoptosis and necrosis in a caspase- and respiratory-independent way, with DRS being dependent, at least partially, on mitochondrial death. The study of yeast genes involved in PCD under these stress conditions provides the opportunity to gain new insight into the mechanistic pathways behind DRS in high eukaryotic cells and the resulting pathologies in a legitimate PCD model organism. Additionally, it allows new cell death based strategies to be established in order to address the difficulties arising from DRS in any industry using dry yeast.

## 5. Acknowledgements

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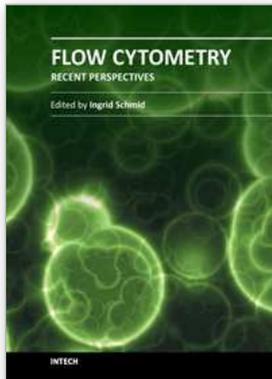
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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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