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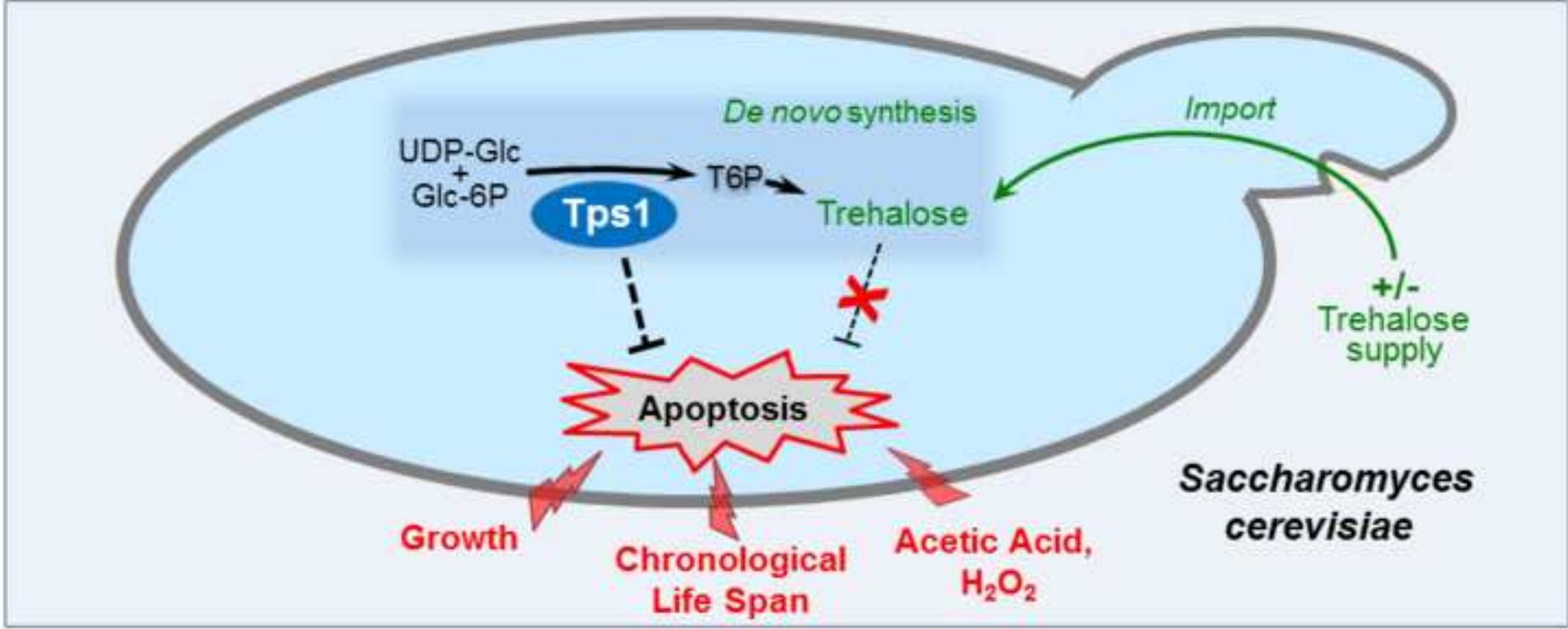
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- The trehalose is not required for yeast longevity and the inhibition of apoptosis
- The Tps1 protein itself protects cells from commitment to apoptosis during growth
- The lack of Tps1p shortens CLS and sensitizes to H₂O₂ and AA-triggered apoptosis
- The pro-survival function of Tps1p probably relies on the maintain of ATP charge

1 **A new function for the yeast Trehalose-6P Synthase (Tps1) Protein, as key pro-**
2 **survival factor during growth, chronological ageing, and apoptotic stress.**

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19 **ABSTRACT**

20
21 Looking back to our recent work that challenged the paradigm of trehalose in stress resistance
22 in yeast, our objective was to revisit the role of this disaccharide in chronological life span
23 (CLS), and in the control of apoptosis. Using a catalytically dead variant of the trehalose-6-
24 phosphate synthase (Tps1) protein, (the first enzyme in the trehalose biosynthetic pathway),
25 and by manipulating intracellular trehalose independently of this pathway, we demonstrated
26 that trehalose has no role in CLS or in the inhibition of acetic acid or H₂O₂-triggered cell
27 death. We showed instead that, in the absence of any apoptotic stimulus, the Tps1 protein
28 itself was necessary in preventing massive, spontaneous commitment of yeast cells to
29 apoptosis during growth. Without Tps1p, the life span was shortened and cells were sensitized
30 to acetic acid (AA) and H₂O₂, whereas the overexpression of the inactive variant of Tps1p
31 almost abolished AA-triggered apoptosis. Genetic interaction analysis of *TPS1* and genes
32 such as *YCA1*, *NUC1* and *AIF1* indicated that these key executioners of cell death partially
33 relayed *tps1Δ*-triggered signalling. Our results suggested that the pro-survival role of Tps1p
34 could be connected with its ability to preserve ATP levels in yeast cells.

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36 **Keywords:** Anti-apoptotic; Chronological Life Span; Oxidative stress; Acetic Acid; ATP.

1. INTRODUCTION

Apoptosis is described as an evolutionary conserved cell suicide program in response to intracellular or environmental stimuli (Galuzzi, 2014; Falcone & Mazzoni, 2016). The existence of apoptosis in yeast was first reported in 1997, using a *Saccharomyces cerevisiae* strain carrying a mutation on the *CDC48* gene (Madeo et al., 1997). Since that time, studies have assessed the existence of apoptosis in response to various stresses, for example, exposure to acetic acid or to drugs, and also during different physiological scenarios such as chronological aging (CLS) (Fabrizio and Longo, 2003; Ruckenstuhl and Carmona-Gutierrez, 2010; Swinnen et al., 2014). It was indeed shown that chronologically aged yeast cultures die, undergoing an altruistic death program that exhibits typical markers of apoptosis (Allen et al., 2006; Fabrizio et al., 2004; Herker et al., 2004). This active process may allow the most aged or damaged cells to provide nutrients and resources for quiescent cells waiting for growth resumption under more favourable conditions (Allen et al., 2006; Herker et al., 2004). Several orthologues of mammalian apoptotic proteins have been discovered in yeast revealing conserved pathways, which have highlighted the yeast *S. cerevisiae* as a powerful model for apoptosis study in general (Büttner et al., 2006; Carmona-Gutierrez et al., 2010; Greenwood and Ludovico, 2010; Liang et al., 2008; Longo and Fabrizio, 2002; Ludovico et al., 2005; Madeo et al., 2009; 2004; Mazzoni and Falcone, 2008).

The process of cell death and its control remains an intricate molecular puzzle, whose complexity and phenotypic markers are still under debate far beyond the yeast model (Galuzzi et al., 2015; Ludovico et al., 2005; Wanichthanarak et al., 2013). Many subtypes of genetically-controlled cell death processes could be proposed, based on the nature, extent and kinetics of the occurrence of biochemical and morphologic features. As in higher eukaryotic cells, these features include, amongst others, DNA cleavage and chromatin condensation,

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63 phosphatidyl serine (PS) externalization on the outer leaflet of the plasma membrane and also
64 alterations in mitochondrial structure and functions (Braun and Westermann, 2011;
65 Guaragnella et al., 2012; Ludovico et al., 2001a; Mirisola et al., 2014; Wloch-Salamon and
66 Bem, 2013). These mitochondrial dysfunctions are, for example, extensive fragmentation,
67 reactive oxygen species (ROS) accumulation and alteration of membrane potential. Another
68 hallmark of apoptosis is the release of proteins from this compartment, such as the
69 cytochrome c (Ludovico et al., 2002), the NADH dehydrogenase Ndi1p (Cui et al., 2012; Li,
70 2006), as well as the so-called cell death ‘executioners’ such as Aif1p and Nuc1p. The
71 cytochrome c was the first mitochondrial protein shown to have an apoptotic function
72 different from its role as an electron carrier in the respiratory chain (Ludovico et al., 2002).
73 The apoptosis-inducing factor (AIF) is a highly conserved protein from yeast to human, which
74 translocates to the nucleus where it participates in chromatinolysis in response to apoptotic
75 stimuli (Wissing et al., 2004). Nuc1p is the yeast homologue of the metazoan endonuclease G
76 (EndoG), a mitochondrial protein with DNase/RNase activity also involved in apoptotic DNA
77 degradation after its mitochondrio-nuclear translocation (Büttner et al., 2007a). Another key
78 executioner of cell death is the type I metacaspase encoded by *YCAI/MCAI* (Madeo et al.,
79 2002; Tsiatsiani et al., 2011; Uren et al., 2000), of which the only substrate described so far is
80 the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase, which is cleaved under
81 peroxide-induced apoptosis (Silva et al., 2011).

82 The link between apoptosis and metabolism, that relied notably on antioxidant detoxifying
83 activities and redox homeostasis (Ayer et al., 2014), is being reinforced by studies that unveil
84 functions of ‘metabolic’ genes in promoting cell death, or conversely exerting pro-survival
85 functions (*e.g.* *ACHI* (Orlandi et al., 2012), *GPHI* (Favre et al., 2008), *GUP1* (Tulha et al.,
86 2012), *HXK2* (Amigoni et al., 2013), *ISCI* (Almeida et al., 2008), *PGKI* (Mazzoni et al.,
87 2009) or *YDC1* (Aerts et al., 2008)). The accumulation of toxic metabolic intermediates over

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88 time, especially in mitochondria in the absence of specific clearance mechanism, could be one
89 of the causes of the loss of mitochondrial integrity and commitment to cell death (Breitenbach
90 et al., 2014). Other reports strengthened the importance of neutral amino acids which may
91 sustain the vacuolar–mitochondrial cross-talk (Hughes and Gottschling, 2012), and the
92 nucleocytosolic pool of acetyl-CoA that may link histone acetylation and epigenetic changes
93 in chromatin, with autophagy and longevity (Eisenberg et al., 2014; Mirzaei and Longo,
94 2014).

95 Other evidence for the link between apoptosis and carbon metabolism rely on trehalose,
96 which may be essential in defining yeast longevity (Kyryakov et al., 2012; Ocampo et al.,
97 2012; Samokhvalov et al., 2004a) and in the inhibition of apoptosis (Kyryakov et al., 2012;
98 Lu et al., 2011). This non-reducing, highly stable disaccharide is especially known as a potent
99 molecular chaperone able to protect cells against a variety of environmental and nutritional
100 stresses (Elbein et al., 2003). It is also known as a reserve carbohydrate present in a large
101 variety of organisms such as fungi, insects and plants (Elbein et al., 2003). In the yeast
102 *S. cerevisiae*, trehalose is synthesized in a two-step reaction. The trehalose-6P synthase
103 (Tps1p) first produces trehalose-6-phosphate (T6P) from glucose-6P and UDP-Glucose. The
104 T6P is then dephosphorylated by a specific trehalose-6P phosphatase (Tps2p), to yield to
105 trehalose. Strains deleted for *TPS1* cannot synthesize trehalose and they exhibit a plethora of
106 phenotypes (François and Parrou, 2001). The most outstanding one is the inability of a *tps1Δ*
107 mutant to grow on rapidly assimilated carbon sources (*e.g.* glucose or fructose), galactose
108 being therefore the usual, permissive carbon source used to cultivate this mutant strain. This
109 inability to grow on glucose or fructose is explained by the rapid drop of ATP and the
110 hyperaccumulation of phosphorylated sugars upon addition of such carbon sources. A
111 consequence of this metabolic defect is the loss of the mutant strain's viability (Blazquez et
112 al., 1993; Gancedo and Flores, 2004; González et al., 1992; Walther et al., 2013). These

113 results led to the suggestion that Tps1p and/or the trehalose pathway control the entrance of
114 glucose into glycolysis (Thevelein and Hohmann, 1995), but in spite of several models
115 proposed so far (Gancedo and Flores, 2004; Hohmann et al., 1996; van Heerden et al., 2014),
116 the molecular mechanisms underlying this phenotype remain unsolved.

117 In recent work we revisited the paradigm of trehalose as a stress-protectant in yeast and we
118 showed that the Tps1 protein itself, not trehalose, was the main determinant for stress
119 resistance (Gibney et al., 2015; Petitjean et al., 2015). The objective of the present study is to
120 identify which of the three elements; *i.e.* the Tps1 protein *per se*, T6P or trehalose, actually
121 help maintain yeast longevity and block commitment to apoptotic cell death in a wild type
122 strain. To this end, we used the same strategy as in Petitjean et al. (Petitjean et al., 2015). It
123 consisted of the expression of a catalytically inactive variant of Tps1p, encoded by *tps1-156*,
124 which was unable to synthesize trehalose. In parallel, we manipulated trehalose levels by the
125 use of the laboratory CEN.PK strain (Van Dijken et al., 2000), which enabled the uptake of
126 the exogenous disaccharide by the constitutively expressed Agt1 transporter (Plourde-Owobi
127 et al., 1999). Our results proved that trehalose does not protect cells from entering apoptosis, a
128 role that is fully endorsed by the Tps1 protein itself, independently of its known catalytic
129 function. We also showed that a basal level of the Tps1 protein is necessary, and sufficient, to
130 protect cells from premature cell death during growth on any carbon source, to maintain
131 chronological life span and to constrain the apoptotic response to different stimuli. Altogether,
132 these results led to the proposition that the Tps1 protein is a major pro-survival factor in the
133 yeast *Saccharomyces cerevisiae*.

134

2. MATERIAL and METHODS

2.1. Strains constructions and validation

The *S. cerevisiae* strains used in this study were derived from the CEN.PK113-7D, a prototrophic *MAL* constitutive strain (Van Dijken et al., 2000). Strains *tps1*Δ (JF1476) and *tps2*Δ (JF248) were previously described in Guillou et al. (2004) and Petitjean et al. (2015), respectively. Construction of the *tps1-156* allele leading to the catalytically dead Tps1^{D156G} variant of the Tps1 protein, has been described in our recent paper (Petitjean et al., 2015).

All other knockout mutants presented in this work were constructed using the Yeast Knockout strain Collection from Open Biosystems (#YSC1053), available in the BY strain background. Using recommended primers for locus amplification and validation, each knockout of interest was PCR amplified and both the control (*TPS1*) and *tps1*Δ strains were transformed by these PCR products, leading either to single or to double mutants with *tps1*Δ, in the CEN.PK strain background. Overexpression of *TPS1* and *tps1-156* alleles was carried out using the pGP564 2μ plasmid (Jones et al., 2008), with coding sequences under the native *TPS1* promoter (pMP26 and pMP27, respectively). As in Petitjean et al. (2015), the strain that overexpresses the *tps1-156* allele was validated, to demonstrate that this allele fails to make trehalose even when expressed at high-copy (data not shown). First, Western blotting against Tps1 validated the overexpression of the protein. Second, this strain exhibited no measurable trehalose-6P synthase activity *in vitro*. Third, *in vivo*, trehalose-6P was below the detection level upon glucose addition to trehalose grown cells, even in the *tps2*Δ background. As a final proof for trehalose-6P synthase deficiency *in vivo*, we validated the inability of cells to produce trehalose under heat shock to 42 °C.

2.2. Culture conditions

Unless otherwise stated, yeast cells were cultivated in YN synthetic medium (yeast nitrogen base without amino acids and ammonium at 1.7 g/L (Difco)), supplemented with ammonium

160 sulfate at 5 g/L, complete drop-out when required (MP-Biomedicals CSM) and 2% (w/v)
161 galactose (YN Gal medium) or 2% galactose plus 1% trehalose (YN GalTre medium). The
162 medium was buffered at pH 4.8 by the addition of 14.3 g of succinic acid liter⁻¹ and 6 g of
163 NaOH liter⁻¹. This buffered pH allowed us to maintain optimal growth, (exponential until A_{600}
164 3-4, as judged from exponential regression analysis of the growth curves, (see a representative
165 experiment in Fig S1, and Parrou et al. (1999)), even when the cultures were conducted in
166 shake flasks (0.5-liter Erlenmeyer flasks with a working volume of 100 mL, at 30 °C and
167 200 rpm on a rotary shaker).

2.3. Yeast Chronological Life Span assay

169 Yeast Chronological Life Span (CLS) was assayed following a standard procedure (Longo
170 and Fabrizio, 2012). Overnight cultures from a single colony were diluted (1:200) into 10 mL
171 fresh YN Gal medium, grown to exponential phase and further diluted to A_{600} 0.5–0.7 in
172 100 mL YN Gal or YN GalTre medium. These cultures were then incubated at 30 °C on a
173 rotary shaker (200 rpm) for several weeks, taking as reference time the moment when the
174 culture reached A_{600} 1.0 (exponential growth).

2.4. Stress exposure

176 Stress exposure was carried out on exponentially growing cells (A_{600} 0.5–0.7) cultivated on
177 YN Gal or YN GalTre medium at 30 °C, after inoculation with a cell density of A_{600} 0.025.
178 For the oxidative stress, the culture was directly treated by addition of hydrogen peroxide
179 0.5 M (H₂O₂, sigma, 7722-84-1) to a final concentration of 1 mM, 5 mM or 10 mM. Acetic
180 acid exposure was carried out according to previously described procedures (Giannattasio et
181 al., 2005; Ludovico et al., 2001a). Exponentially growing cells were transferred in a YN Gal
182 adjusted to pH 3,0 with HCl, and containing or not (control) acetic acid 40 mM, 80 mM and
183 120 mM (adapted from Ludovico et al. (2001b)). Incubation in the presence of the chemicals
184 was performed at 30 °C, up to 4 h.

185 **2.5. Other analytical procedures**

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2 186 Cell sampling and metabolites extraction were carried out as previously described in Walther
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5 187 et al. (2010). The ATP content was determined enzymatically by the production of NADH as
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7 188 follows. The reaction was carried out at 30 °C in Tris-HCl buffer with NAD⁺ 0.4 mM,
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10 189 glucose 1 mM, 4.5 U/ml Glucose-6-phosphate dehydrogenase and 1.5 U/ml hexokinase.
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12 190 NADH production was then assessed at 340 nm (spectrophotometer Agilent G1103A).
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14 191 Trehalose measurement was done according to Parrou and François (1997).
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17 **2.6. General settings for flow cytometric analysis**

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20 193 Flow cytometric analyses were carried out on the Attune[®] Acoustic Focusing Cytometer (Life
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23 194 Technologies, Inc.). Cytometer acquisition was run at 100 µL/min to ensure high specificity,
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25 195 on a total number of 100,000 events/run. For all the assays described below, the source of
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27 196 excitation was the 488-nm laser (blue). Voltage calibration of the cytometer was always
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30 197 performed by using the unstained, control sample of the WT strain and further checked on the
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32 198 *tps1Δ* strain. Routinely applied voltages were 2,000 mV for the forward scatter (FSC) and
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35 199 2,600 mV for the side scatter (SSC). To get auto-fluorescence signal below the 10³-threshold,
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37 200 calibration settings of the emission filters were 1,200 mV for the 525-nm filter (BL-1),
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40 201 1,900 mV for the 575-nm filter (BL-2) and 2,100 mV for the 670-nm filter (BL-3). Besides
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42 202 the *TPS1* and *tps1Δ* control strains, unstained samples from strains of interest were analyzed
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45 203 to ensure that these calibration settings for the side scatter-forward scatter plot and for the
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47 204 auto-fluorescence could apply.
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50 **2.7. Cell viability**

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53 206 The relative percentage of living and non-viable cells in the population was determined using
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55 207 the Guava[®] ViaCount[®] reagent (4000-0040, purchased from Millipore). Flow cytometric
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58 208 analysis was performed using the 525-nm emission filter (BL-1) for the nuclear dye that stains
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60 209 only nucleated cells, and the 670-nm filter (BL-3) for the viability dye that brightly stains
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210 dying cells. Full procedure for sample handling and data analysis has been thoroughly
211 described in Petitjean et al. (2015).

212 **2.8. Mitochondrial membrane potential**

213 Mitochondrial membrane potential was measured using the fluorescent, potential-sensitive
214 dye Rhodamine 123 (Rh123, Molecular Probes #R-302), following an assay adapted from
215 Ludovico et al. (2001a). Briefly, cells were diluted at a concentration of 10^6 cells/ml in PBS
216 (pH 7,0) and independently stained with Rh123 50 nM for 10 min at room temperature, in the
217 dark, and with $1\mu\text{g/ml}$ of Propidium Iodide (PI, #V13241, Life Technologies). Analysis was
218 performed using the 525-nm emission filter (BL-1), concluding for hyperpolarization of
219 mitochondria when the cells presented a fluorescence signal above the 10^4 -treshold, while
220 cells with depolarized mitochondria led to a signal below 10^2 AU (AU, Arbitrary Units of
221 fluorescence), indistinguishable from the autofluorescence. To determine the mitochondrial
222 potential only on viable and apoptotic cells, PI-positive cells were excluded from the
223 measurement (Ludovico et al., 2001a). Hyperpolarization and depolarization profiles were
224 confirmed on WT cells treated by nigericin and FCCP, respectively, according to Ludovico et
225 al., (2001a) (Fig. S2).

226 **2.9. DNA fragmentation**

227 DNA fragmentation was assayed using the APO-BrdU™ TUNEL assay (#A35125, Life
228 technologies) according to (Madeo, 1999) recommendations. For fixation, cells were
229 suspended at 2×10^6 cells/ml in 500 μL PBS. This cell suspension was mixed with 5 mL of
230 3.7% formaldehyde and incubated on ice for 15 min. After centrifugation (500 g, 5 min), cells
231 were washed two times with PBS, suspended in 5 ml PBS and then added to 5 ml ice-cold
232 ethanol 70% for overnight incubation at $-20\text{ }^\circ\text{C}$. For strand breaks labelling, ethanol-fixed
233 cells were rinsed in the provided wash-buffer and incubated in extemporaneously prepared
234 DNA-labelling solution, at $37\text{ }^\circ\text{C}$ with mild shaking on a steering wheel. After 4 hours, cells

235 were washed and incubated for 30 min at room temperature in the antibody staining solution,
236 then finally stained for another 30 min in the PI staining buffer. Samples were directly
237 analyzed by flow cytometry using the 525-nm (BL-1) and 575-nm (BL-2) emission filters.
238 Negative cells to staining overlaid cells auto-fluorescence (signal between 10^2 and 10^3 AU),
239 while positive cells positioned between 10^4 and 10^5 AU on the BL-1 axis. For each
240 independent experiment, the positive and negative controls provided in the kit were performed
241 and analyzed in parallel.

2.10. Caspase activity

243 Caspase quantification was performed according to Madeo et al. (2002) using the CaspACE™
244 assay (#7461, Promega). A total of 5×10^6 yeast cells were harvested, washed once in PBS,
245 and incubated for 20 min at 30 °C with 200 µl of staining solution containing the FITC-VAD-
246 FMK substrate. Cells were then washed in PBS and suspended in 200 µl PBS for flow
247 cytometry analysis using the 525-nm emission filter (BL-1). Quantification was performed on
248 the FSC vs. BL-1 dot blot, where positive cells for caspase activity positioned between 10^3
249 and 10^5 AU on the BL-1 axis.

2.11. ROS accumulation

251 ROS detection *in situ* was performed using the the CellROX® Green Reagent (Life
252 technologies, #C10444) according to manufacturer recommendations. Briefly, cells were
253 concentrated at 2×10^7 cells/ml and the CellROX® Reagent was added at 5 µM final
254 concentration. After 30 minutes at 37 °C, cells were washed three times with PBS. Sample
255 analysis by flow cytometry was performed using the 525-nm emission filter (BL-1).
256 Quantification was performed on the FSC vs. BL-1 dot blot, where fluorescence signal above
257 the 10^3 -threshold on the BL-1 axis indicated positive cells for ROS accumulation.

258 **2.12. Membrane modifications**

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2 259 Externalization of phosphatidyl serine on the outer leaflet of the plasma membrane was
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5 260 assayed using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 & Propidium
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7 261 Iodide (#V13241, Life Technologies). For samples preparation, cells were washed in cold
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10 262 PBS and diluted in the 1X annexin-binding buffer at a final concentration of 2×10^6 cells/ml.
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12 263 For each 100 μ l aliquot, 10 μ l of conjugated Annexin V solution and 1 μ l of 100 μ g/ml PI
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14 264 solution were added, and cells were incubated for 15 minutes at room temperature. After this
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17 265 incubation period, 400 μ l of 1X annexin-binding buffer was added and samples were analyzed
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19 266 by flow cytometry using the 525-nm (Annexin V) and 575-nm (PI) emission filters. When
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22 267 analyzing the 525-nm vs. 575-nm scatter plot, fluorescence below 10^3 AU for both axes
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24 268 indicated viable cells. Cells with fluorescence signal beyond 10^3 AU on the Annexin V axis
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27 269 with low PI signal were considered as primary apoptotic cells. Conversely, cells positioning
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29 270 beyond 10^3 AU on the PI axis with low annexin V signal were considered as necrotic. Finally,
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32 271 cells positioning beyond 10^3 AU on both axes were considered as secondary apoptotic cells.
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34 35 272 **2.13. Visualization of mitochondria by confocal microscopy**

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37 273 To visualize mitochondrial network organization, *TPS1* and *tps1* Δ cells were transformed with
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40 274 a pYX222-mtDsRed plasmid (Campbell Gourlay's plasmid, kind gift from Dr Paula
41
42 275 Ludovico, Life and Health Sciences Research Institute (ICVS), Braga, Portugal). Confocal
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45 276 microscopy was performed with a confocal disk system (Revolution Nipkow; Andor
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47 277 Technology) installed on an inverted microscope (IX-81; Olympus) featuring a CSU22
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50 278 confocal spinning disk unit (Yokogawa Corporation of America) and an EM charge-coupled
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52 279 device (CCD) camera (DU 888; Andor Technology). This system was controlled using the
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55 280 mode "Revolution FAST" of Andor Revolution IQ1 software (Andor Technology). Images
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57 281 were acquired using a 100 \times objective lens (Plan-Apochromat, 1.4 NA, oil immersion;
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59 282 Olympus). Single laser lines used for excitation were Diode Pumped Solid State Lasers
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283 (DPSSL) exciting DsRed at 561 nm (50 mW; Cobolt Jive). A bandpass emission filter (FF01-
284 512/630-25, Semrock) allowed collection of the red fluorescence. 250-nm z steps were used.
285 Exposure time was 2 ms.

286 **2.14. Statistical analysis**

287 Statistical analyses, that is, One-Way and Two-Way ANOVA tests, were conducted by using
288 the PRISM software, and p-value below 0.01 as statistically significant level. When it
289 appeared necessary to sustain ambiguous conclusions, p-value results have been shown, as
290 star(s) that sit on top of brackets, on Excel graphs. The Hierarchical Ascendant Classification
291 (HAC) of single and double mutants was carried out using the STATGRAPHICS Centurion
292 16 software. This analysis was conducted according to the Ward method, with default
293 parameters (standardization of the data and squared Euclidean distances), by using the whole
294 set of quantitative data available for these strains (*i.e.* viability and all apoptotic markers data).

295

296 **3. RESULTS**

297 **3.1. The deletion of *TPS1* impacts cell growth and viability.**

298 Among the numerous phenotypes of *TPS1* loss-of-function, we particularly observed that
299 *tps1Δ* cells growing on any permissive carbon sources had a very low cell viability (Petitjean
300 et al., 2015). We also noticed that the growth rate on galactose of this mutant was not only
301 slower than that of the wild type, but highly variable between experiments, and that this
302 growth rate correlated with cell viability of the population (Fig. S1.B). Based on the general
303 growth kinetics principle that at any time during growth cells can either divide, or die
304 (Fig. S1.C1), we found that viable *tps1Δ* cells grow and divide as fast as WT cells, with a
305 mean intrinsic growth rate of about 0.34 h⁻¹ (Fig. S1.C4). We therefore concluded that this
306 apparent low growth rate of the *tps1Δ* mutant resulted from massive and premature cell death
307 within the population. We have already shown that this lethality could not be prevented by
308 preloading *tps1Δ* cells with trehalose (Petitjean et al., 2015). Conversely, this cell death was
309 not observed in *tps1-156* cells expressing the catalytically inactive Tps1^{D156G} variant,
310 indicating a crucial role of the Tps1 protein *per se* in cell viability during growth.

311 **3.2. The Tps1 protein protects growing cells from spontaneous apoptotic cell**
312 **death.**

313 This unexpected commitment to cell death of a *tps1* null mutant prompted us to characterize
314 this death process by analyzing specific morphological and biochemical markers (Carmona-
315 Gutierrez et al., 2010). As is shown in Figure 1, almost 40% of *tps1Δ* cells in the exponential
316 phase of growth on galactose presented phosphatidyl serine externalization (~5% for the WT
317 strain) and 20% of them exhibited caspase activity and DNA fragmentation (less than 5% for
318 the WT). Chromatin condensation was also investigated by DAPI staining and microscopy,
319 but no significant difference was observed between WT and *tps1Δ* nuclei. In contrast, the
320 *tps1Δ* mutant showed a clear mitochondrial DNA fragmentation and altered mitochondrial

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321 function, as 80% of these cells presented hyperpolarization of mitochondria (see also Fig. S2)
322 and more than 40% exhibited increased ROS levels (less than 10% for the WT). We finally
323 looked at the structure of the mitochondrial network by using the mtDsRed mitochondrial
324 marker. While the WT showed a clear tubular structure, we observed a total disruption of the
325 mitochondrial network in the *tps1Δ* mutant, with unambiguous thread-grain transition
326 (Fig. 1B). Considering these characteristics, we concluded that the *tps1Δ* mutant strain
327 committed to an apoptotic cell death process.

328 Very interestingly, these apoptotic traits were abolished in the *tps1-156* strain that expresses a
329 catalytically dead variant of Tps1p. As for the WT strain however, the basal, yet statistically
330 significant 10% loss of viability during growth on galactose, which correlated with 5-10%
331 positive cells for apoptotic markers, was still observed in this *tps1-156* strain. Further, this
332 basal loss of viability was not overridden by the overexpression of Tps1p, either from *tps1-156*
333 or *TPS1* alleles on a 2μ plasmid. These apoptotic markers were similarly observed in cells
334 cultivated on other carbon sources (data not shown). These included galactose supplemented
335 with trehalose, also a mix of glycerol-lactate-ethanol used as a pure respiratory carbon source
336 medium, or trehalose alone, a condition that perfectly mimics glucose-limited oxidative
337 growth in chemostat (Jules et al., 2005, 2004). Taken together, these results showed that the
338 apoptotic cell death occurring in the *tps1Δ* mutant during growth was due to the lack of the
339 Tps1 protein itself, not to trehalose that had no role in protecting cells against this process.

340 **3.3. The Tps1 protein, not trehalose, warrants a normal chronological lifespan.**

341 It has been emphasized that trehalose plays an essential role in the extension of yeast
342 chronological life span (Kyryakov et al., 2012; Palabiyik and Jafari Ghods, 2015;
343 Samokhvalov et al., 2004a, 2004b; Trevisol et al., 2011). The above results prompted us to
344 revisit this conclusion by determining viability and evolution of apoptotic markers during
345 long-term growth of the *tps1-156* strain, which expresses the Tps1 protein but is unable to

346 synthesize trehalose. As can be seen in Figure 2A, the viability of the *tps1Δ* mutant dropped
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2 347 by *approx.* 1,000-fold during a 4-weeks' incubation, whereas that of WT and *tps1-156* strains
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4 348 remained high with a 30% survival rate at the end of the assay. By cultivating these strains on
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6 349 YN GalTre medium, (which leads to the huge accumulation of trehalose in these cells), cell
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8 350 death kinetics were not altered in any of the three strains (only *tps1Δ* is shown). However, by
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10 351 overexpressing *TPS1*, or by deleting *TPS2*, survival rates were slightly lowered after 4 weeks
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12 352 (15% for these two strains *versus* 30% for the WT strain). This slight drop of viability
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14 353 occurred independently of the presence of trehalose in the culture medium (data not shown).
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16 354 These results suggested that T6P, whose levels were significantly higher in these two mutants
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18 355 as compared to the WT (data not shown but see Walther et al. (2013)), had a negative effect
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20 356 on yeast CLS. From these data, we also concluded that the Tps1 protein, and not trehalose, the
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22 357 end product of Tps1p metabolic pathway in yeast, is needed for normal survival during
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24 358 chronological ageing.

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32 359 Figure 2 also reports the evolution of apoptotic markers during ageing. The most singular
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34 360 phenotype for the *tps1Δ* strain, whose mitochondria were hyper-polarized during the
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36 361 exponential growth ($A_{600} \sim 1$), was the massive de-energization of mitochondria, which started
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38 362 even before the diauxic shift (data not shown) and persisted all along the assay as seen for
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40 363 days 9 and 22. Overall, it could be seen that the percentage of positive cells for ROS
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42 364 accumulation, AnV staining and caspase activity, steadily increased during the assay in all
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44 365 strains (Fig. 2B-E), with no statistically significant difference between *tps1-156* and
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46 366 overexpressing strains as compared to the WT strain. Also, we were not able to measure any
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48 367 significant difference for these strains in the presence of trehalose (YN Gal *versus* YN GalTre
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50 368 culture media, data not shown). These data further discarded any role of trehalose in
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52 369 preventing commitment to apoptosis.

3.4. The Tps1 protein constrains both acetic acid and H₂O₂-triggered apoptotic responses

The unpredicted apoptotic state of *tps1*Δ cells growing in the absence of any stimulus, led us to investigate the behavior of this strain when challenged to various exogenous apoptotic stimuli (Fig. 3). To visualize the sole effect of stress exposure, we considered the variable, initial apoptotic states of the different strains in the exponential phase. We then calculated the changes in the percentage of viable cells and positive cells for apoptotic markers, that is, the difference between the values measured after exposure to the stress of interest for 4 hours, and the values measured during the exponential growth. As is shown in Figure 3A, the deletion of *TPS1* greatly sensitized yeast cells to the exposure to 40–120 mM acetic acid (AA) and to 1–10 mM peroxide (H₂O₂), while the WT and *tps1-156* strains showed similar dose-response patterns to these chemicals. Furthermore, we found that preloading the cells with trehalose had no protective effect against these apoptotic stimuli (not shown). These results indicated that the presence of the sole Tps1 protein was sufficient to guarantee WT phenotypes. It is worth noting that, as compared to other yeast strains reported in published data, the lower sensitivity to chemicals of the WT strain used in this study may be explained by the use of the CEN.PK strain background, which was already found to be more resistant than the BY strain to heat shock, oxidative stress and dehydration (see Fig. S3 and Petitjean et al. (2015)).

A singularity of *tps1*Δ in response to H₂O₂ treatment was the significant increase in the proportion of cells presenting AnV/PI co-staining and PI-only staining (*approx.* 60% and 10%, respectively, under 10 mM H₂O₂, Fig. 3D). This indicated that exposure to this chemical strongly damaged cells that committed to secondary apoptosis, and necrosis for a few of them. With regard to acetic acid exposure; the lower dose of 40 mM AA led to a 40% drop of viability for the mutant, as compared to 8% for the WT, which was accompanied by a marked rise in positive cells for ROS accumulation and PS externalization. However, the *tps1*Δ strain

395 exhibited moderate changes in the number of positive cells with alterations of mitochondrial
396 polarization, caspase activity and DNA fragmentation, even when challenged with increasing
397 AA concentration. This counter-intuitive result could be explained by the strong apoptotic
398 features of the *tps1Δ* strain that are observed already in exponential phase cells, such as
399 hyperpolarized mitochondria, which may preclude further extensive changes of these
400 parameters under stress.

401 Remarkably, increasing the amount of the Tps1^{D156G} protein by overexpressing the *tps1-156*
402 allele very nearly removed the sensitization of yeast cells to AA exposure. It was illustrated
403 by a marginal drop of viability and almost no increase in positive cells for the different
404 apoptotic markers, even at the higher AA concentration. This effect was not observed in yeast
405 cells overexpressing the wild type *TPS1* allele, indicating that, as for CLS assay, the Tps1p-
406 dependant hyperaccumulation of T6P is somewhat toxic and antagonistic to the positive effect
407 of the sole Tps1 protein that should have been expected. Curiously, contrary to AA exposure,
408 the overexpression of the *tps1-156* allele did not provide any benefits to *tps1Δ* cells exposed
409 to peroxide. This suggested that the molecular targets of Tps1p, that help constraining these
410 various apoptotic responses, are multiple and partly specific to the pathway that is triggered.

3.5. Molecular machinery involved in *tps1Δ*-triggered cell death execution during growth and under exposure to extrinsic stimuli

413 The commitment to apoptosis of *tps1Δ* led us to investigate the genetic interaction between
414 *TPS1* and a few genes coding for effectors of apoptosis, namely *AIF1*, *NUC1*, *YCA1* and
415 *NDH1*. The whole set of quantitative data on viability and apoptotic markers, (which were
416 determined in each single and double mutant strain during exponential growth), allowed us to
417 conduct a cluster analysis (HAC) that classified the strains into two categories (Fig. 4). One
418 class contained all the strains bearing the *tps1Δ* mutation. The second one gathered the WT
419 and *tps1-156* strains together with all single mutants. This classification was not surprising,

420 since, in agreement with literature, none of the single disruptants in the WT's cluster
421 exhibited apoptotic features during growth. A notable exception was the *nuc1Δ* strain that
422 classified in this WT's cluster, and did not exhibit any apoptotic feature, while it has been
423 shown to exhibit apoptotic cell death during the exponential growth on a glucose synthetic
424 medium (Büttner et al., 2007b).

425 The cluster of all double disruptants with *tps1Δ* indicated that none of these deletions enabled
426 to fully recover WT profile. However, more detailed analysis of the different patterns showed
427 that three out of the four mutations brought partial rescue of *tps1Δ* phenotypes, that is, *aif1Δ*,
428 *nuc1Δ* and *yca1Δ*. Deletion of *NUC1* and *AIF1* in the *tps1Δ* background similarly improved
429 viability and diminished the percentage of positive cells for most of the parameters (Fig. 4A-
430 E). Nevertheless, *NUC1* loss-of-function suppressed *tps1Δ*-induced DNA fragmentation,
431 while no statistical difference could be observed between *tps1Δ aif1Δ* and *tps1Δ* cells. The
432 yeast caspase Yca1p also appeared as a mediator of some of the *tps1Δ*-related apoptotic traits,
433 as the deletion of *YCA1* utterly eliminated caspase activity, suppressed membrane
434 modifications, and reduced positive cells for ROS accumulation from 40 to 25% of cell
435 population. However, *YCA1* deletion was without effect on the viability and percentage of
436 cells with mitochondria hyperpolarization, providing another example of cell death triggering
437 that depends only partially on caspase action (Madeo et al., 2009).

438 All single and double disruptants grown to exponential phase were then exposed to AA
439 120 mM and H₂O₂ 10 mM, respectively (Fig. 5). As in Figure 3, we considered in calculations
440 the initial apoptotic states of the different mutants in the exponential phase, to visualize the
441 sole effect of stress exposure. When analyzing consequences of gene deletions in the *tps1Δ*
442 background that strongly sensitized cells to stress, *yca1Δ* was the only mutation able to
443 suppress AA-triggered apoptosis. This double *yca1Δ tps1Δ* disruptant exhibited features of

444 the single *yca1Δ* mutant and unambiguously supported a caspase-dependent pathway for AA-
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2 445 induced cell death. With regard to the peroxide exposure assay, both *NUC1* and *AIF1*
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5 446 deletions significantly limited the sensitivity of *tps1Δ* cells. They led to a drop of viability and
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7 447 an increase in the number of cells with AnV/PI co-staining comparable to those observed in
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10 448 the WT strain. However, neither *nuc1Δ* nor *aif1Δ* in this *tps1Δ* background was able to
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12 449 prevent peroxide-induced DNA fragmentation, contrary to what was observed in the *TPS1*
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15 450 context, where these single disruptants blocked the rise in positive cells for DNA
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17 451 fragmentation. In contrast to *NUC1* and *AIF1* deletions, neither *YCA1* nor *NDI1* deletion
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20 452 helped *tps1Δ* strains to better survive this harmful, peroxide-triggered oxidative stress.
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23 453 **3.6. The occurrence of apoptosis in *tps1Δ* cells correlated with a rapid drop of** 24 25 454 **ATP levels**

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27 455 It is well known that a *tps1Δ* mutant cannot maintain its ATP levels, notably in response to
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30 456 glucose exposure (Walther et al., 2013) and when under heat stress (Petitjean et al., 2015).
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32 457 Likewise, we observed that exposure of *tps1Δ* cells to the lower doses of AA and peroxide
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34 458 caused an irreversible drop of ATP levels, in less than 3 hours (Fig. 6A and 6B). A continuous
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37 459 drop of the ATP charge was also noticed during growth of the *tps1Δ* mutant strain (Fig. 6C),
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39 460 (see the decrease of ATP levels from A_{600} 1.0 (early exponential) to A_{600} 3.0 (late
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42 461 exponential)), with full depletion of this crucial metabolite even before the onset of the
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44 462 diauxic shift. Conversely, this ATP charge remained high in the WT and *tps1-156* strains.
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464 **4. DISCUSSION**

465 **Trehalose does not prevent the loss of viability and commitment to apoptosis.**

466 The disaccharide trehalose has been reported as an essential component in yeast longevity
467 (Kyryakov et al., 2012; Samokhvalov et al., 2004a) and in the inhibition of apoptosis (Hu et
468 al., 2014; Kyryakov et al., 2012; Lu et al., 2011). By way of example, it has been suggested as
469 a negative regulator of peroxide-induced apoptosis, through the inhibition of Ca²⁺ signaling
470 pathway and caspase activity (Lu et al., 2011). We revisited these conclusions in the present
471 work, as we recently showed that the trehalose-6-phosphate synthase (Tps1 protein) was the
472 main determinant for stress resistance in yeast (Gibney et al., 2015; Petitjean et al., 2015),
473 contrary to long-standing claims on the essential role of trehalose in stress resistance (Elbein
474 et al., 2003). By using the same experimental strategy (Petitjean et al., 2015), especially the
475 expression of a catalytically inactive variant of Tps1p, we proved that it is the lack of Tps1
476 protein, and not that of trehalose, which significantly reduces chronological lifespan and
477 hypersensitizes yeast cells to external apoptotic stimuli (*e.g.* acetic acid and peroxide).
478 Interestingly, we also found that the Tps1 protein, and not trehalose, was able to prevent
479 spontaneous apoptotic cell death during exponential growth. This useless role of trehalose was
480 consistent with the absence of intracellular trehalose during the exponential growth of WT
481 cells (Parrou et al., 1999). These results highlighted the potential pitfalls of both correlation-
482 based studies and the use of gene deletions (*e.g.* *tps1*Δ), strengthening the idea that correlation
483 is not causality. In light of these and previous results (Wilson et al., 2007; Petitjean et al.,
484 2015), affirmations of trehalose function that have been raised based on *TPS1* deletion in any
485 fungal species, must be carefully revisited.

486 **The lack of the Tps1 protein leads to stochastic commitment to cell death and**
487 **variable population growth rate.**

488 Our preliminary observations suggest that living *tps1* Δ cells divide almost as fast as the WT
489 cells, the low growth rate of the population being explained by the significant fraction of cells
490 that commit to cell death. Interestingly, the high culture-to-culture growth rate variation that
491 we noticed for *tps1* Δ cells, was also observed in perfectly controlled bioreactors (our
492 unpublished data), which minimizes the possible influence of extrinsic factors. It fits instead
493 with published reports that heterogeneity in growth rate, even for genetically identical cells,
494 can be propagated over several generations (Kelly and Rahn, 1932; Wheals and Lord, 1992).
495 A very recent work by Cerulus et al. (2016) quantified this phenomenon and demonstrated
496 that clonal populations that are growing in the exponential phase could show considerable
497 heterogeneity and epigenetic inheritance of single-cell doubling times, possibly leading to
498 heterogeneity of population growth rate. Whether, in the absence of the Tps1 protein, both
499 stochastic commitment to cell death and heterogeneous doubling times between the remaining
500 living-cells explain such culture-to-culture growth rate variation, should deserve further
501 investigations.

502 **The lack of Tps1p affects apoptosis through cell death executioners' dependent**
503 **and independent pathways.**

504 Negative regulators of apoptosis have been described in yeast (Owsianowski et al., 2008).
505 Only in a few cases, *e.g.* *CDC48*, *ASF1*, *LSM4* or *UBP10* (Bettiga et al., 2004; Madeo et al.,
506 1997; Mazzoni et al., 2003; Yamaki et al., 2001), loss-of-function of the gene committed cells
507 to apoptosis during exponential growth. The pro-survival role of Tps1p, remarkably illustrated
508 by the spontaneous commitment to apoptosis of exponentially growing *tps1* Δ cells, indicated
509 that the very small amount of Tps1p present during growth (Ghaemmaghami et al., 2003;
510 Parrou et al., 1999) is sufficient to guarantee this function in WT cells. Further, this small
511 intracellular level of Tps1 was not only sufficient, but also optimal if remembering that the

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512 overexpression of *TPS1*, through T6P overaccumulation, appeared somewhat antagonistic to
513 the positive effect of the Tps1 protein itself. However, by increasing the amount of the
514 Tps1^{D156G} variant, AA-triggered apoptosis was very nearly abolished. Our data thus evidenced
515 that in a WT strain, Tps1p could interfere with AA-triggered cell death signaling. The TOR
516 pathway is an appealing target, as it was recently reported as an important regulatory node
517 during AA-induced apoptosis (Almeida et al., 2009).

518 Among downstream players of the apoptotic pathways that were analyzed for their putative
519 involvement in *tps1Δ*-triggered cell death, the deletion of *AIF1*, *NUC1* and *YCA1* partially
520 rescued *tps1Δ* phenotypes during the exponential growth. The deletion of *YCA1* suppressed
521 caspase activity and membrane modifications. However, the fact that viability and other
522 apoptotic markers remained altered as in the single *tps1Δ* strain during growth, provided
523 another example of cell death process that depends only partially on caspase action (Madeo et
524 al., 2009). The deletion of *YCA1* was however able to fully suppress AA-triggered apoptosis,
525 even in the highly sensitive *tps1Δ* background, which unambiguously supported a caspase-
526 dependent pathway for AA-induced cell death.

527 The comparable effects of *AIF1* and *NUC1* deletions supported the idea that *tps1Δ*-triggered
528 cell death execution during the exponential growth relies, at least to some extent, upon these
529 key effectors. But the translocation of Aif1p from mitochondria to the nucleus was
530 questionable, as we did not observe any effect on chromatin fragmentation levels while
531 deleting *AIF1*, contrary to what was observed for *NUC1* deletion. Under peroxide-triggered
532 apoptosis, the viability of *tps1Δ* was only restored by the deletion of *AIF1* or *NUC1*.
533 However, these deletions did not block the significant rise in the number of cells exhibiting
534 DNA fragmentation, reinforcing the idea that Aif1p and Nuc1p mutually compensate for

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2 535 vitality under various conditions of environmental stress and endogenous-induced apoptosis
3 536 (Büttner et al., 2007b).
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6 537 Finally, we analyzed the putative role of Ndi1p in *tps1Δ*-triggered ROS accumulation, as
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8 538 dysfunctional mitochondria are most routinely cited as the major source of mitochondrial
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10 539 ROS (Cui et al., 2012; Li, 2006). Against all odds, this protein did not appear to mediate
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12 540 *tps1Δ*-triggered cell death, as the *tps1Δndi1Δ* double mutant perfectly clustered with the
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14 541 *tps1Δ* strain, maintaining high levels of ROS positive cells. This result raised the possibility
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16 542 that ROS production may arise from the ER-localized NADPH oxidase, Yno1p (Rinnerthaler
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18 543 et al., 2012), which accounted for the majority of detectable ROS in the context of reduced
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20 544 cytochrome c oxidase (COX) activity (Leadsham et al., 2013).
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26 545 **The clear-cut apoptotic phenotypes of the *tps1Δ* strain are independent of the**
27 **permissive carbon source.**
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30 547 The most outstanding phenotype of the *tps1Δ* mutant is its inability to grow on rapidly
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32 548 assimilated carbon sources (*e.g.* glucose or fructose), galactose being therefore the usual,
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34 549 permissive carbon source used to cultivate this mutant strain. Clear-cut apoptotic phenotypes
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36 550 of the *tps1Δ* strain were observed during early exponential growth, independently of the
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38 551 nature of the permissive carbon source used (*i.e.* respiratory, or respiro-fermentative as
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40 552 galactose (our unpublished data and (Fendt and Sauer, 2010; Ostergaard et al., 2000a, 2000b,
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42 553 2001; Syriopoulos et al., 2008)). This result contrasted with the *nuc1Δ* strain whose
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44 554 phenotypes were qualified as subtle; either protecting against, or sensitizing to, stress-
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46 555 triggered cell death when the strain was grown in glycerol or glucose respectively (Büttner et
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48 556 al., 2007b). In our study however, even if they grew on galactose and fermented, we did not
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50 557 observe any apoptotic markers for *nuc1Δ* cells that clustered with the WT strain, contrary to
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52 558 what might have been expected from these cells grown on glucose (Büttner et al., 2007b). A
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54 559 few, similar discrepancies could be noticed for some of the single mutants exposed to
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1 560 apoptotic stimuli (Fig. 5). We still don't know whether the carbon source is the only reason
2 561 for those variations, as different genetic backgrounds or any other, undetermined factor may
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4 562 also affect the occurrence of few of these specific apoptotic features. For that reason, we at
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7 563 least duplicated, and validated, all results in the BY background, for the WT, *tps1Δ* and *tps1-*
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10 564 *156* strains, under all relevant conditions of the study.

13 565 **The pro-survival role of the Tps1 protein probably relies on the maintenance** 14 566 **of ATP homeostasis, but not only**

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17 567 In previous works, we reported that a *tps1Δ* strain cannot maintain ATP levels under
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19 568 environmental changes (Petitjean et al., 2015; Walther et al., 2013). Interestingly, declining
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22 569 ATP levels and redox alteration were considered as potential causes of regulated cell death
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24 570 (Galluzzi et al., 2015; Pereira et al., 2010; Sousa et al., 2013). We can, therefore, argue that
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27 571 part of the pro-survival function of the Tps1 protein may rely on the capacity to preserve ATP
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29 572 levels under apoptotic stimuli; hence providing substrate to 'ATP hungry' proteins. By way of
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32 573 example, we may cite proteins as Cdc48p, which belong to the class of AAA+ proteins
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34 574 (ATPases associated with various cellular activities) and are components of the machinery
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36 575 necessary for maintaining cell integrity. Also, failure to stabilize ATP levels in the *tps1Δ*
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39 576 background may affect ATP-dependent processes, as actin cytoskeleton dynamics, whose
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41 577 regulation has been shown to influence the decision to commit to apoptosis (Gourlay and
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44 578 Ayscough, 2005a, 2005b; Gourlay et al., 2004, 2006; Leadsham et al., 2010; Smethurst et al.,
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46 579 2014).

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49 580 However, perturbation of the energy status of the cell may not be considered as the sole
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52 581 reason for apoptotic cell death of the *tps1Δ* strain, as early exponentially growing cells
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54 582 committed to apoptosis, while they showed almost wild type ATP levels. These data, together
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57 583 with the possible interaction of Tps1p with the TOR pathway during AA-induced apoptosis
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59 584 (see above), indicate that the function of Tps1p implicates specific targets that remain to be
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2 585 identified. We recently demonstrated that the Tps1 protein itself is crucial for cell integrity
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4 586 under stress exposure as heat shock (Petitjean et al., 2015), independently of apoptotic cell
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6 587 death processes (data not shown). These new results provide unbiased evidence for an
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8 588 alternative function of this protein as anti-apoptotic factor, and allowed us to further confirm
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10 589 the idea that Tps1p is a moonlighting protein (Flores and Gancedo, 2011; Gancedo and
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12 590 Flores, 2008).

15 591 **Conclusions**

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18 592 The search for pro-survival factors is relevant for improving stress resistance and preventing
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20 593 cell death of microbial cells under industrial processes. Here, the Tps1 protein and its
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22 594 interplay with apoptotic processes delineates novel targets for the selection and engineering of
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24 595 more robust industrial strains. Conversely, Almeida et al. (2008) reported the urgent need for
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26 596 a new generation of antifungal drugs that could enable the specific manipulation of yeast cell
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28 597 death, without causing side effects on human cells. Despite the high conservation of cell death
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30 598 processes between yeasts and mammals, our finding that yeast apoptotic cell death was
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32 599 greatly accelerated upon deletion of *TPS1* could fulfil this need, since the Tps1 protein is only
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34 600 present in bacteria, fungi and plants, but absent in mammals. Together with the key function
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36 601 of the Tps1 protein in providing tolerance to hostile environments (Petitjean et al., 2015), this
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38 602 architectural difference between apoptotic regulators of yeast and mammalian cells appeared
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40 603 as a possible Achilles' heel of yeast. Beyond *S. cerevisiae*, the Tps1 protein could therefore
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42 604 represent an appealing target for the selective destruction of closely related pathogens (*e.g.*
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44 605 *Candida albicans*) and evolutionarily more distant fungi (*e.g. A. fumigatus*).

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12
13 613 interests to declare.
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18 614 **Supplementary data**

19 615 Figures S1 - S3 and Tables S1 - S5
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Figure legends

Figure 1: The deletion of *TPS1* triggers a massive apoptotic cell death during exponential growth

(A) Flow cytometry analysis of the percentage of viable cells and of positive cells for apoptotic markers during exponential growth. Strains were grown in YN Gal medium to $A_{600} \sim 1.0$. In addition to the three reference strains of this work (WT, *tps1* Δ and *tps1-156*), the *tps1* Δ strain was also transformed with a 2 μ plasmid bearing the *tps1-156* and *TPS1* alleles, respectively (Strains #4 and #5). Stacked bars from the ‘Membrane modifications’ category gather together the percentages of positive cells for AnV staining (grey), AnV/PI co-staining (orange) and PI-only staining (green). The data are represented as means \pm 2 SEM of three to five biological replicates. Results of the One-way ANOVA test for WT vs. *tps1* Δ (significance level of 1%) are given in Table S1.

(B) Analysis of mitochondrial morphology by confocal microscopy. To visualize mitochondrial network organization, *TPS1* and *tps1* Δ cells were transformed with the pYX222-mtDsRed plasmid. Single laser lines used for excitation were Diode Pumped Solid State Lasers (DPSSL) exciting DsRed at 561 nm (50 mW; Cobolt Jive). A bandpass emission filter (FF01-512/630-25, Semrock) allowed collection of the red fluorescence. Pixel size was 65 nm. 250-nm z step were used for the acquisition.

Figure 2: Chronological lifespan in strains affected for trehalose metabolism

(A) Survival of strains grown in YN Gal medium and left for 4 weeks at 30 °C, with continuous shaking. *Day 0*, start of the experiment ($A_{600} \sim 1.0$). Cells were also grown in the presence of trehalose (+, YN GalTre medium), but only the *tps1* Δ strain is shown for the sake of clarity. The data are represented as means \pm 1 SEM of three independent replicates (*i.e.* different calendar periods).

908 (B–E) Percentage of positive cells for apoptotic markers after 9 and 22 days of chronological
909 aging (*Day 0* as reference). (B), Percent of positive cells for ROS accumulation; (C), Percent
910 of cells presenting hyper- or depolarization of mitochondria (grey and orange, respectively);
911 (D), Membrane modifications with percentage of positive cells for AnV staining (grey),
912 AnV/PI co-staining (orange) and PI-only staining (green); (E), Percent of cells presenting
913 caspase activity. The data are represented as means \pm 2 SEM of three independent replicates.
914 Results of the One-way ANOVA test for WT vs. *tps1* Δ (significance level of 1%) are given in
915 Table S2.

916 **Figure 3: Acetic acid and H₂O₂-triggered apoptosis in strains affected for trehalose** 917 **metabolism**

918 (A) “Drop of viability” after exposure to different concentrations of acetic acid (40 to
919 120 mM) and peroxide (1 to 10 mM). The “drop of viability” was calculated as the difference
920 between the viability measured after exposure to the stress of interest for 4 hours, and the
921 viability observed during exponential growth (A_{600} ~0.5-0.7). (B-E) “Changes in the number
922 of positive cells” for apoptotic markers (%), calculated as the difference between values
923 measured after exposure to the stress of interest for 4 hours, and the values measured during
924 exponential growth. (B), ROS accumulation; (C), Hyper- or depolarization of mitochondria
925 (grey and orange, respectively). Filled and dashed grey bars indicate respectively an increase
926 and a decrease in the number of hyperpolarized cells; (D), Membrane modifications with AnV
927 staining (grey), AnV/PI co-staining (orange) and PI-only staining (green); (E), Caspase
928 activity (green) and DNA fragmentation (purple). The data are represented as means \pm 2 SEM
929 of three biological replicates. For the sake of clarity, statistics were not plotted but exhaustive
930 results of the Two-way ANOVA test (significance level of 1%) could be found in Table S3.

931 **Figure 4: Genetic interaction between *TPS1* and genes encoding executioners of**
932 **apoptotic cell death in exponentially growing cells.**

933 Hierarchical Ascendant Classification (HAC) of single and double mutants between *tps1Δ* and
934 knockouts of known effectors of apoptosis. This Classification was performed according to
935 the Ward method, with default parameters (standardization of the data and squared Euclidean
936 distances), by using the whole set of quantitative data (*i.e.* viability and all apoptotic markers,
937 presented in panels A–E and measured from cells grown to $A_{600} \sim 1.0$ in YN Gal medium, with
938 the exception of DNA fragmentation results that were not included in the analysis as solely
939 evaluated for *nuc1Δ* and *aif1Δ* strains).

940 (A-E) Strains were ordered on the horizontal axis as obtained from the HAC. (A), Percent of
941 viable cells; (B), Percent of positive cells for ROS accumulation; (C), Percent of cells with
942 mitochondria hyper-polarization; (D), Membrane modifications with percent of positive cells
943 for AnV staining (grey), AnV/PI co-staining (orange) and PI-only staining (green); (E),
944 Positive cells presenting caspase activity (green) or showing DNA fragmentation (purple).
945 The data are represented as means \pm 2 SEM of three biological replicates. Results of the One-
946 way ANOVA test for *tps1Δ* vs. double mutants (significance level of 1%) are given in Table
947 S4.

948 **Figure 5: Molecular machinery of *tps1Δ*-triggered cell death execution under**
949 **acetic acid and peroxide exposure.**

950 Legend as in Figure 3. (A), “drop of viability” after exposure to acetic acid 120 mM and
951 peroxide 10 mM, for 4 hours. (B-E), Changes in the number of positive cells for apoptotic
952 markers. (B), ROS accumulation; (C), hyper- or depolarization of mitochondria (grey and
953 orange, respectively). Filled and dashed grey bars indicate respectively an increase and a
954 decrease in the number of hyperpolarized cells; (D), Membrane modifications (AnV staining
955 (grey), AnV/PI co-staining (orange) and PI-only staining (green)); (E), Caspase activity

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2 956 (green) and DNA fragmentation (purple). The data are represented as means \pm 2 SEM of three
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4 957 biological replicates (excepted for caspase and DNA fragmentation assays with only two
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6 958 biological replicates). For the sake of clarity, statistics were not plotted, but results of the
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8 959 Two-way ANOVA test for *tps1* Δ vs. double mutants (significance level of 1%) could be
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10 960 found in Table S5.

11 12 13 961 **Figure 6: The Tps1 protein guarantees the maintain of ATP levels**

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15 962 Levels of ATP during exposure of yeast cells to acetic acid (A), peroxide (B), or during
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17 963 growth (C). (A) and (B), ATP as a function of exposure time; (C), ATP as a function of A_{600} .
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20 964 Data are represented as mean \pm SD of 3 independent biological replicates.
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967 **Supplementary material for :**

1 968 **“A new function for the yeast Trehalose-6P Synthase (Tps1) Protein, as key pro-survival**
2
3
4 969 **factor during growth, chronological ageing, and apoptotic stress.”**

7 970

10 971 Supplementary Figure Legends

14 972 **Figure S1: Living *tps1* cells divide almost as fast as WT cells.**

16 973 (A) Representative plot of the evolution of A_{600} as a function of time, during the exponential
17
18 974 phase of growth. For each sample, we also measured the total cell number (N_t , *per ml* of
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21 975 culture), and the number of viable cells (N_v , *per ml*), which allowed us to calculate the
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23 976 viability as the ratio between viable cells (N_v) and the total number of cells (N_t). The
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26 977 exponential fitting curve enables to access the apparent specific growth rate of cell population
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28 978 (μ) during this exponential phase of growth. (B) Plot of the “*apparent*” specific growth rate
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31 979 (μ) as a function of the viability. Each point of the graph represents an independent culture,
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33 980 either from the WT (black) or of the *tps1* Δ strain (red). The viability value (x-axis) is the
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35
36 981 mean viability calculated from all samples analyzed during the exponential phase of growth.
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38 982 (C1-C4) indirect evaluation of the “*intrinsic*” growth rate (μ_i). (C1) At any time during
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41 983 growth, viable cells can either divide (intrinsic growth rate of dividing cells, μ_i), or die (death
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43 984 rate, μ_d). In a closed system (batch culture), and if considering a quasi-stationary state during
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45 985 this exponential phase (*i.e.* constant evolution rates μ_i & μ_d), the two differential equations (1)
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48 986 and (2) give the evolution of the viable cells, and of the total number of cells, respectively.
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50 987 The independent, integral forms of these equations (B2), enable to determine the two
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53 988 unknowns μ_i and μ_d ; (C4), same as in (B), with the “*intrinsic*” specific growth rate (μ_i) as a
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55 989 function of viability.

990 **Figure S2: Monitoring of Rh123-based mitochondrial membrane potential by flow**
991 **cytometry.**

992 Representative BL-1-RL-2 scatter plots of Rh123-stained cells (log scale, BL-1 (525 nm
993 filter), RL-2 (665 nm filter)). (A) Plots obtained from exponentially growing cells ($A_{600} \sim 1$) of
994 the WT and *tps1* Δ strains. (B) Control assays for depolarization or hyperpolarization of
995 mitochondria, after exposure of WT cells to FCCP 5 μ M and Nigericin 5 μ M, respectively.
996 Rh123-staining analysis was performed on BL-1, concluding for hyperpolarization when the
997 cells presented a fluorescence signal above the 10^4 -treshold, while cells with depolarized
998 mitochondria led to a signal bellow 10^2 AU (AU, Arbitrary Units of fluorescence).

999 **Figure S3: Comparative analysis of AA- and H₂O₂-triggered loss of viability, in**
1000 **CEN.PK and BY strain backgrounds.**

1001 “Drop of viability” after exposure to different concentrations of acetic acid (40 to 120 mM)
1002 and peroxide (1 to 10 mM). CEN.PK (upper graph) or BY strain background (lower graph).
1003 Legend as in Figure 3.

Figure 1

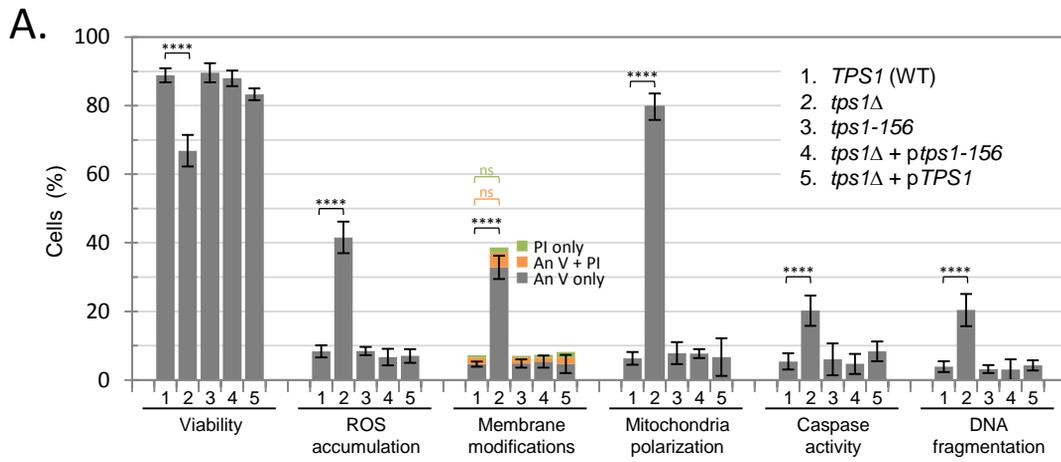
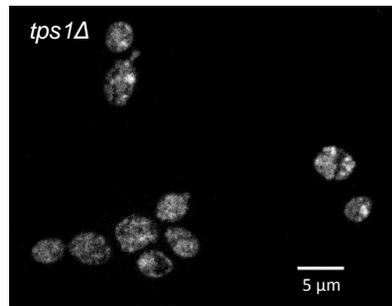
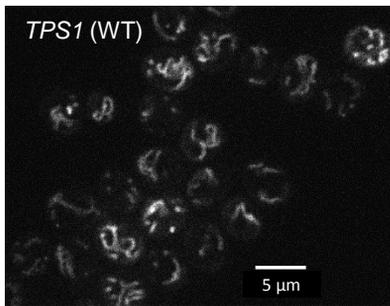
**B.**

Figure 2

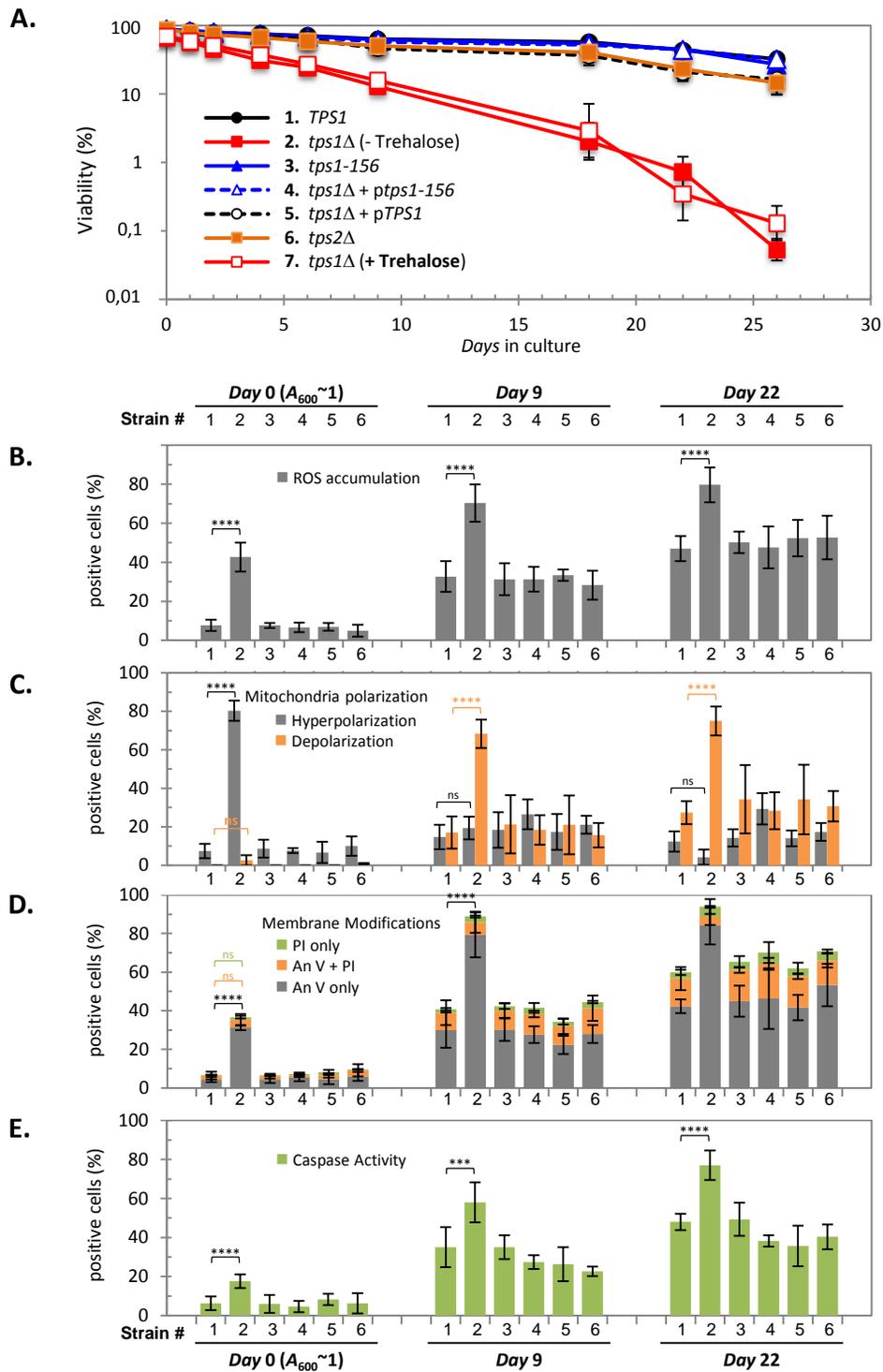


Figure 3

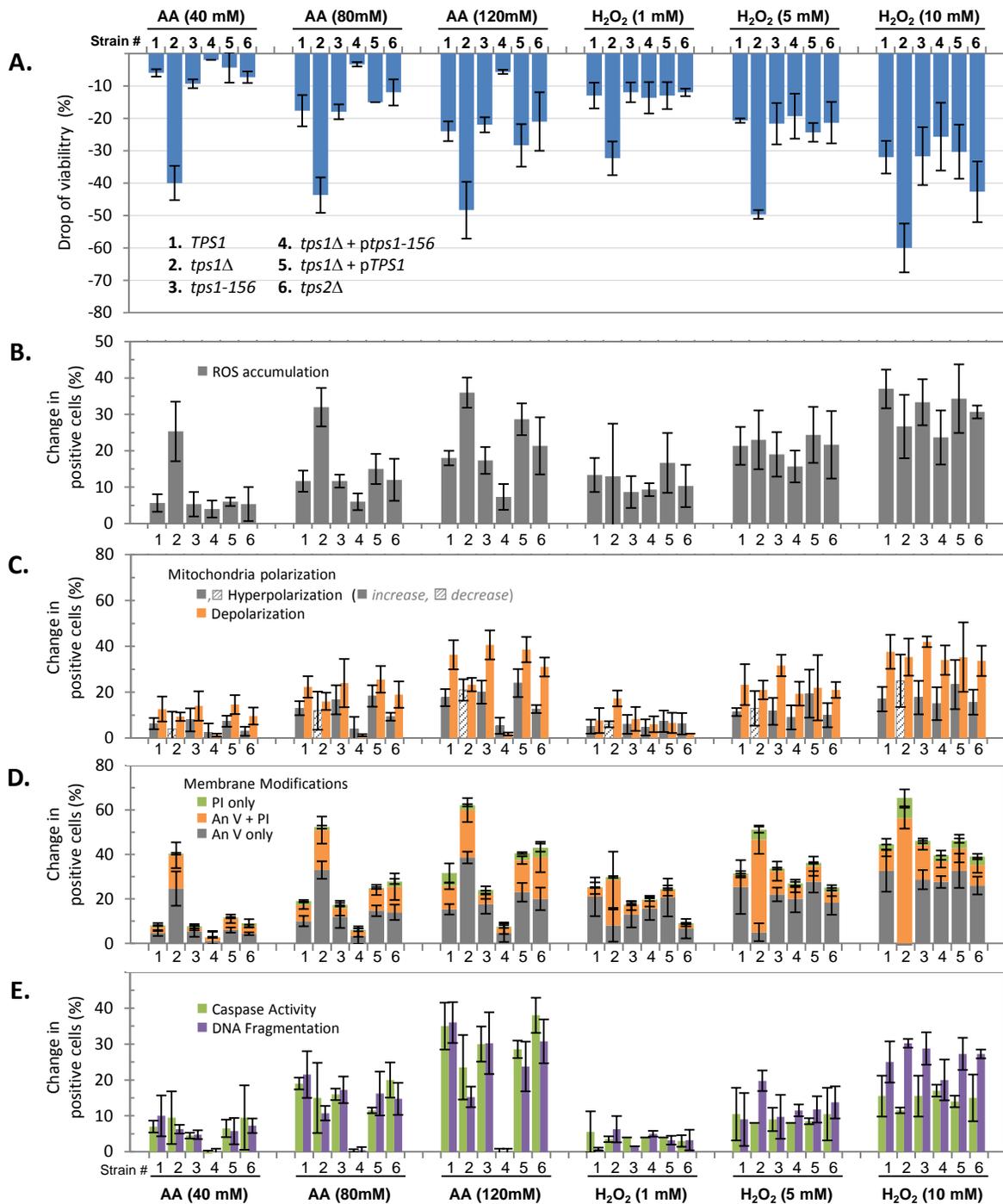


Figure 4

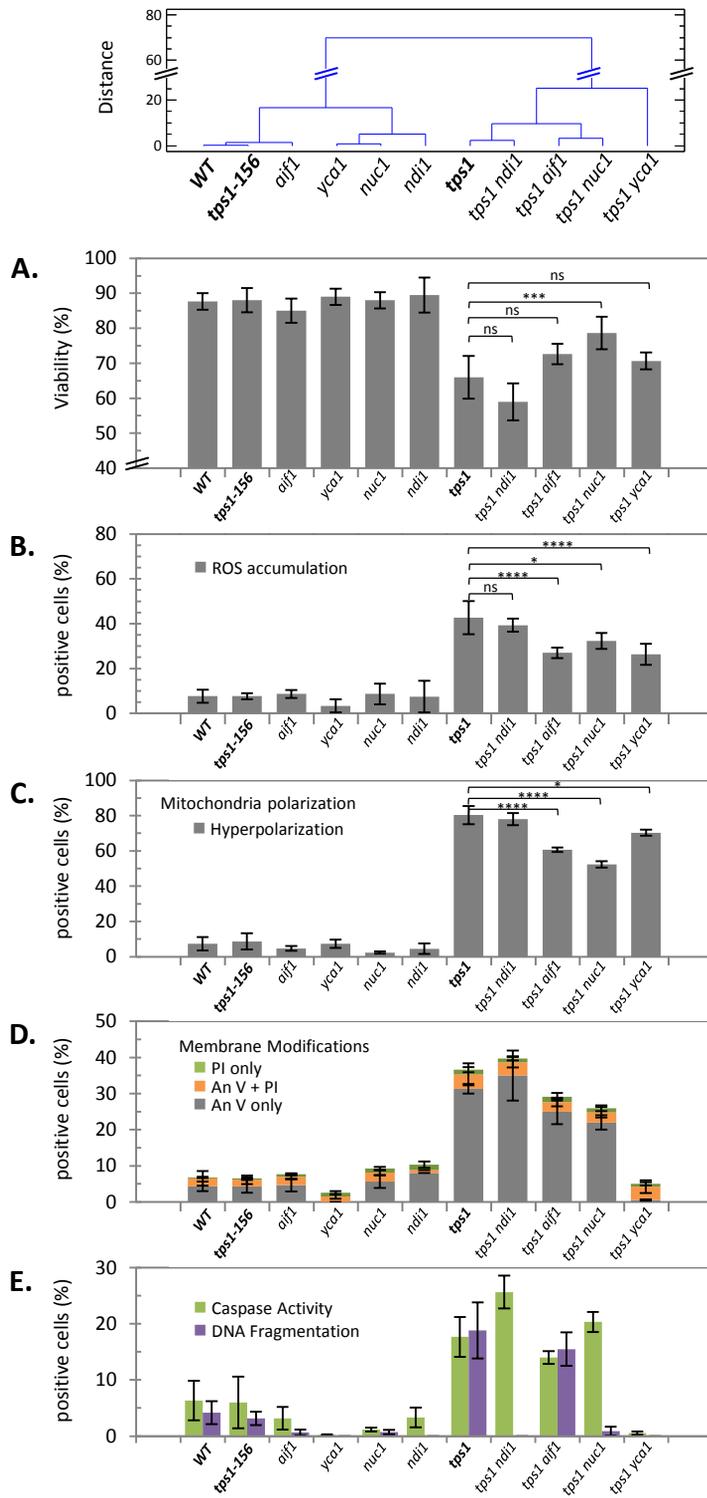


Figure 5

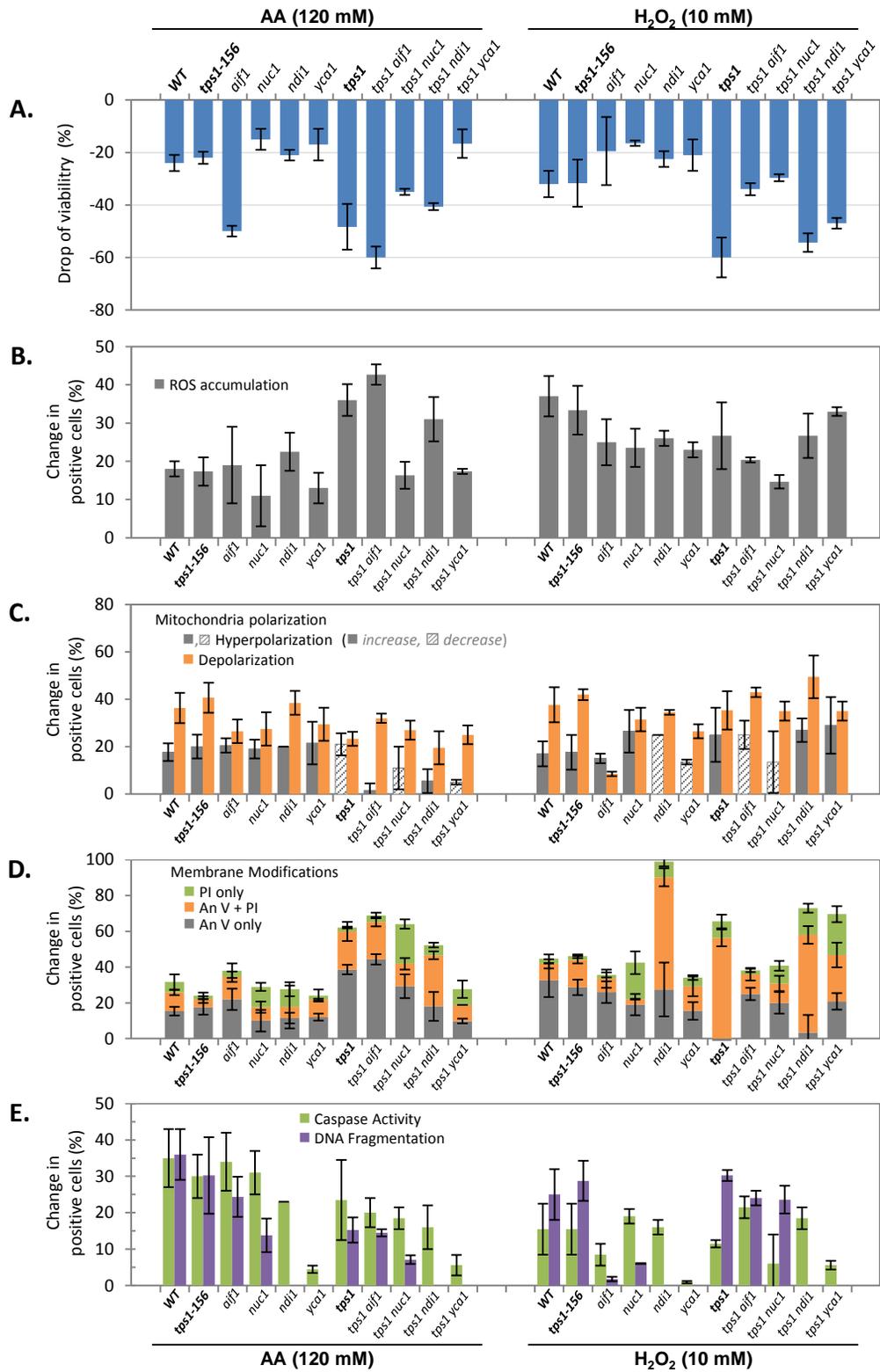
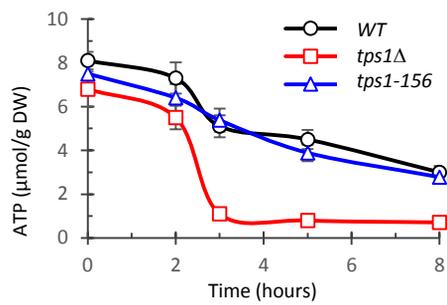
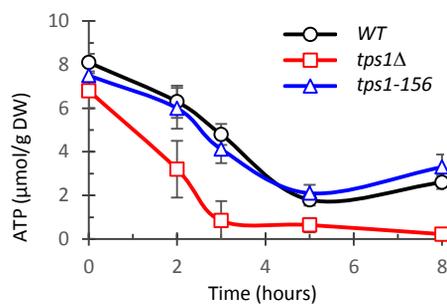


Figure 6

A. AA (40 mM)



B. H₂O₂ (1 mM)



C. Exponential phase of growth

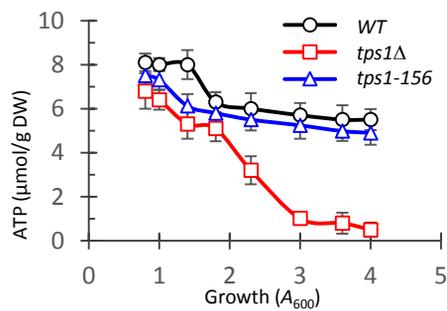
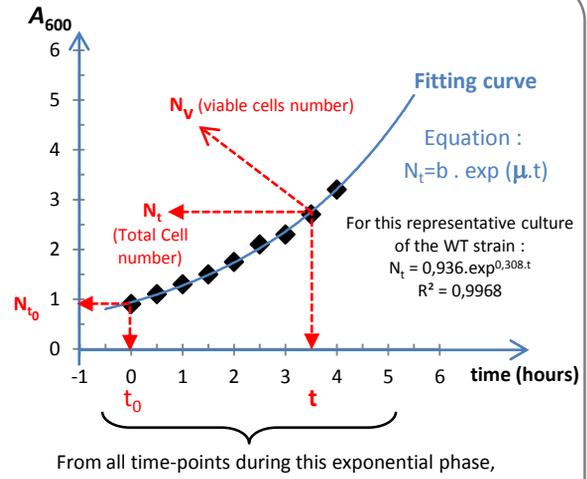


Figure S1

A. Representative growth curve, (evolution of A_{600} as a function of time), (or total cell number as a function of time), with :

- t_0 : arbitrary origin of time (first sample taken during early log phase)
- t : time of sampling
- N_t : **Total cell number**, (dead + viable cells), determined by cytometry.
- N_v : **Viable (i.e. dividing) cells number**, evaluated by cytometry (Guava® ViaCount® Viability kit)



Each of these experimental growth curves, enables :

Direct calculation of :

- ✓ μ , the « Apparent » specific growth rate of cell population, from the fitting exponential curve
- ✓ **Viability***, i.e. the mean viability value observed during this specific culture

Indirect estimation (Panel C1 to C4) of :

- ✓ μ_i , the « Intrinsic » specific growth rate of dividing cells

C.1. Viable cells can **DIVIDE**, but some of them **DIE**

In a closed system (batch culture), if considering a quasi-stationary state (i.e. constant evolution rates μ_i & μ_d),

Evolution of viable cells:

$$(1) \quad dN_v = N_v \mu_i \cdot dt - N_v \mu_d \cdot dt$$

Evolution of total number of cells ($dN_t = dN_v + dN_d$)

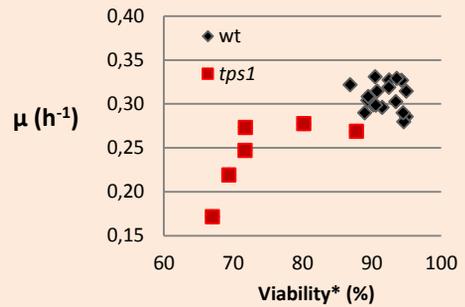
Considering (1) and $dN_d = N_v \mu_d \cdot dt$:

$$(2) \quad dN_t = N_v \mu_i \cdot dt$$

- N_t : **Total** cell number, (dead + viable cells), at t
- N_v : Number of **Viable (i.e. dividing)** cells, at t
- N_d : Number of **dead** cells, at t
- μ_i : **Growth rate of dividing cells (i.e. « intrinsic » growth rate)**
- μ_d : **Death rate**

B.

μ (h^{-1}), « Apparent » specific growth rate of cell population



One point = one culture, as the one shown in panel A.
* Mean viability value during the culture

C.2.

Integral forms of equations (1) and (2) :

$$(3) \quad N_v = N_{v0} \cdot \exp [(\mu_i - \mu_d) \cdot t]$$

$$(4) \quad N_t = N_{t0} + N_{v0} \cdot [\mu_i / (\mu_i - \mu_d)] \cdot (\exp [(\mu_i - \mu_d) \cdot t] - 1)$$

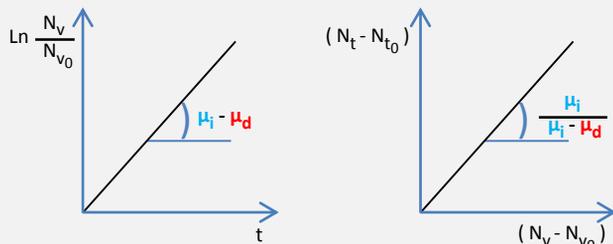
- N_{t0} and N_{v0} : total and viable cells, respectively, at t_0

C.3.

From these two, independant equations ((3) and (4)), we can solve the system and determine the two unknowns μ_i and μ_d by plotting :

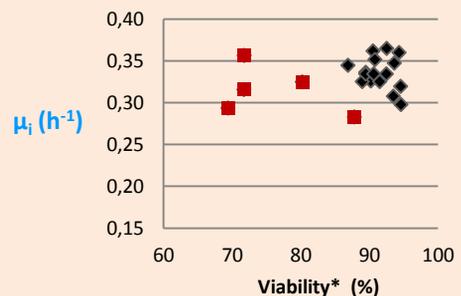
$$(5) \quad \ln (N_v / N_{v0}) = (\mu_i - \mu_d) \cdot t$$

$$(6) \quad (N_t - N_{t0}) = [\mu_i / (\mu_i - \mu_d)] \cdot (N_v - N_{v0})$$



C.4.

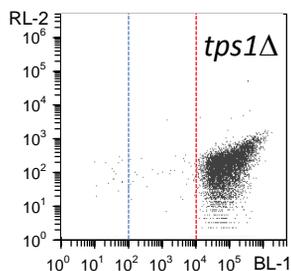
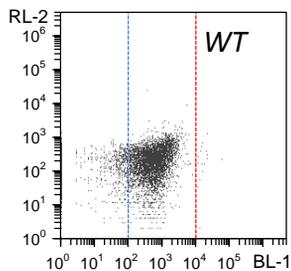
μ_i (h^{-1}), « Intrinsic » specific growth rate of dividing cells



* Mean viability value during the culture

Figure S2

A. Exponentially growing strains ($A_{600} \sim 1$)



B. Control assays for depolarization or hyperpolarization, on WT cells

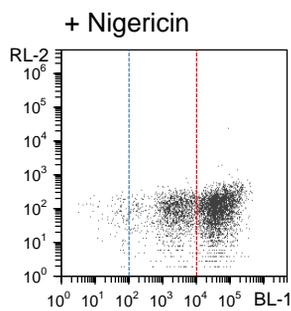
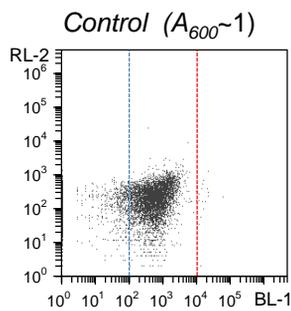
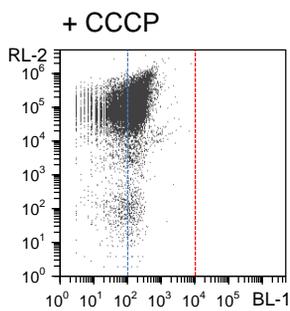
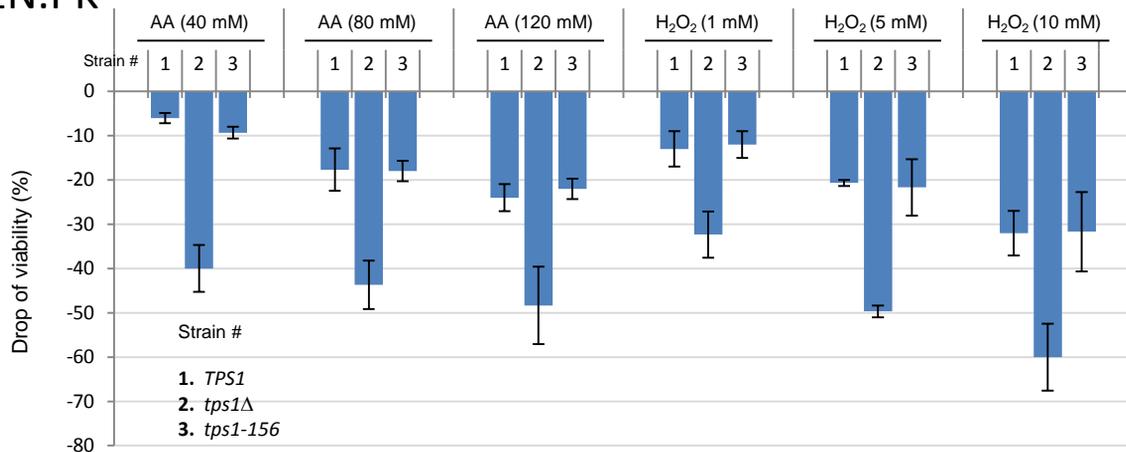
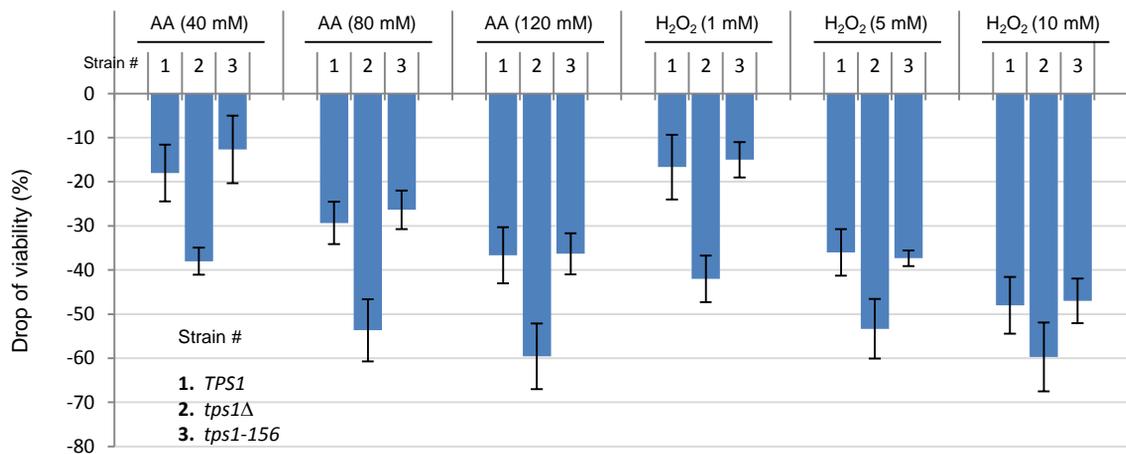


Figure S3

CEN.PK



BY



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