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# Yeast Tolerance to Various Stresses Relies on the Trehalose-6P Synthase (Tps1) Protein, Not on Trehalose\*

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**Background:** Decades of observations strengthened the idea that trehalose is a chemical chaperone.

**Results:** A catalytically inactive variant of the trehalose-6P synthase (Tps1) maintains cell survival and energy homeostasis under stress exposure.

**Conclusion:** The Tps1 protein itself, not trehalose, is crucial for cell integrity.

**Significance:** This work provides unbiased evidence for an alternative function of Tps1, a new “moonlighting” protein.

Trehalose is a stable disaccharide commonly found in nature, from bacteria to fungi and plants. For the model yeast *Saccharomyces cerevisiae*, claims that trehalose is a stress protectant were based indirectly either on correlation between accumulation of trehalose and high resistance to various stresses or on stress hypersensitivity of mutants deleted for *TPS1*, which encodes the first enzyme in trehalose biosynthetic pathway. Our goal was to investigate more directly which one, between trehalose and/or the Tps1 protein, may serve yeast cells to withstand exposure to stress. By employing an original strategy that combined the use of mutant strains expressing catalytically inactive variants of Tps1, with *MAL*<sup>+</sup> yeast strains able to accumulate trehalose from an exogenous supply, we bring for the first time unbiased proof that trehalose does not protect yeast cells from dying and that the stress-protecting role of trehalose in this eukaryotic model was largely overestimated. Conversely, we identified the Tps1 protein as a key player for yeast survival in response to temperature, oxidative, and desiccation stress. We also showed by robust RT-quantitative PCR and genetic interaction analysis that the role of Tps1 in thermotolerance is not dependent upon Hsf1-dependent transcription activity. Finally, our results revealed that the Tps1 protein is essential to maintain ATP levels during heat shock. Altogether, these findings supported the idea that Tps1 is endowed with a regulatory function in energy homeostasis, which is essential to withstand adverse conditions and maintain cellular integrity.

Trehalose ( $\alpha$ -D-glucopyranosyl-1,1- $\alpha$ -D-glucopyranoside) is a highly stable, nonreducing disaccharide found in many organisms including bacteria, archaea, plants, invertebrates, and fungi.

The chemical structure enables this sugar to form hydrogen bonds with polar residues in proteins and to substitute for water that normally comprises a “hydration shell” surrounding hydrophilic biomolecules (1). These properties likely account for the widespread use of trehalose as cryoprotectant of biological samples (2). Trehalose is highly abundant in anhydrobiotes, including the yeast *Saccharomyces cerevisiae* that can withstand long period of desiccation, which suggests a role for this sugar in desiccation survival, because of its proposed ability to stabilize membranes and proteins (3). In plants, trehalose is thought to play a role as osmoprotectant (4), and in flying insects, it serves as the “blood” sugar in hemolymph (5).

The physiological roles of trehalose have been investigated in the greatest detail in the yeast *S. cerevisiae* because of the ease of manipulating levels of this metabolite by genetic means and cultivation conditions (6). As a result of more than 40 years of research in this model eukaryote, two major functions of trehalose have emerged, namely energy reserve and stress protectant. The role of energy store has been ignored for a long time because, at variance to the true carbon reserve glycogen, which accumulates when glucose is still abundant, trehalose synthesis takes place only in stationary phase cultures when glucose is no longer present (7, 8). Also in the sporulation process, trehalose remains in mature spores, whereas glycogen is utilized during spore maturation (9). Two recent reports, however, provided evidence that accumulated trehalose can be the energy fuel to the yeast cell. Using a yeast mutant able to take up exogenous trehalose, we showed that the accumulated trehalose was readily consumed under carbon-starved conditions by a mechanism implicating the periplasmic acid trehalase (Ath1) (10). Also, Shi *et al.* (11) reported a rapid trehalose mobilization upon exit of yeast cells from quiescent state. The stress-protecting function, which is the second major function ascribed to trehalose, was mainly deduced from correlations between trehalose levels and cell survival under adverse environmental conditions, such as high thermotolerance of stationary phase cells coincidentally with high level of trehalose (12). Analysis of phenotypes associated with the deletion of the *TPS1* gene encoding the trehalose-6-phosphate synthase that catalyzes the first enzymatic step of trehalose synthesis further supported this proposition. Indeed, *tps1* $\Delta$  mutants showed hypersensitivity to stress of various

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# Role of *Tps1* in Yeast Stress Tolerance

**TABLE 1**

CEN.PK and BY background strains used in this study

| Name in the text                | Origin <sup>a</sup> | Genotype <sup>a</sup>   | Reference | Source         |
|---------------------------------|---------------------|---|-----------|----------------|
| <i>TPS1</i> <sup>c</sup>        | CEN.PK              | MATa <i>ura3-52</i>   | JF1094    | Ref. 25        |
| <i>tps1Δ</i>                    | CEN.PK              | MATa <i>ura3-52 tps1::hisG</i>  | JF1476    | Lab collection |
| <i>tps1-156</i>                 | CEN.PK              | MATa <i>ura3-52 tps1::tps1-156 KanMx4</i>   | JF118     | This study     |
| <i>tps2Δ</i>                    | CEN.PK              | MATα <i>tps2::KanMx4</i>  | JF248     | This study     |
| <i>tps1Δ tps2Δ</i>              | CEN.PK              | MATa <i>ura3-52 tps1::hisG tps2::KanMx4</i>   | JF87      | This study     |
| <i>tps1-156 tps2Δ</i>           | CEN.PK              | MATa <i>ura3-52 tps1::hisG tps2::KanMx4, YCpLac33 tps1-156</i>                                  | JF451     | This study     |
| <i>TPS1 hsf1Δ pTet-HSF1</i>     | CEN.PK              | MATa <i>ura3-52 trp1-289 leu2,3-112 hsf1::KanMx6, pTet-O<sub>7</sub> HSF1</i>                   | JF91      | This study     |
| <i>tps1Δ hsf1Δ pTet-HSF1</i>    | CEN.PK              | MATa <i>ura3-52 trp1-289 tps1::hisG hsf1::KanMx6, pTet-O<sub>7</sub> HSF1</i>                   | JF90      | This study     |
| <i>tps1-156 hsf1Δ pTet-HSF1</i> | CEN.PK              | MATa <i>ura3-52 trp1-289 tps1::hisG hsf1::KanMx6, pTet-O<sub>7</sub> HSF1 YCpLac33 tps1-156</i> | JF101     | This study     |
| <i>tps1Δ pHSP104</i>            | CEN.PK              | MATa <i>leu2,3-112 tps1::hisG, pGP564 HSP104</i>  | JF154     | This study     |
| <i>tps1Δ pHSP82</i>             | CEN.PK              | MATa <i>leu2,3-112 tps1::hisG, pGP564 HSP82</i>   | JF155     | This study     |
| <i>tps1Δ pHSC82</i>             | CEN.PK              | MATa <i>leu2,3-112 tps1::hisG, pGP564 HSC82</i>   | JF156     | This study     |
| <i>tps1Δ pSSA3</i>              | CEN.PK              | MATa <i>leu2,3-112 tps1::hisG, pGP564 SSA3</i>  | JF160     | This study     |
| <i>TPS1</i> <sup>d</sup>        | BY4741              | MATa  | JF1412    | Euroscarf      |
| <i>tps1Δ</i>                    | BY4741              | MATa <i>tps1::hisG</i>  | JF1927    | Lab collection |
| <i>tps1-156</i>                 | BY4741              | MATa <i>tps1::tps1-156 KanMx4</i>   | JF173     | This study     |

<sup>a</sup> Strain genetic background. All the CEN.PK113-7D-derived strains bear the *MAL2-8c* and *SUC2* alleles.

<sup>b</sup> The plasmids present in the strain are given in bold type.

<sup>c</sup> Referred to as strain *TPS1 TPS2* in Fig. 2A.

<sup>d</sup> This strain is the BY4741 reference strain. All BY strains bear the *his3-Δ1 leu2-Δ0 ura3-Δ0 met15-Δ0 ADE2 CAN1 LYS2 TRP1* alleles.

kinds, including heat, freezing shock, hydrostatic pressure, desiccation, and oxidative, ethanol and osmotic stresses (13–19). However, the loss of the *TPS1* gene is also accompanied by several other phenotypes, apparently unrelated to the lack of trehalose, such as the inability to grow on rapidly fermentable sugars, hyperaccumulation of glycogen, sporulation and respiratory defects (20–22). In the rice blast fungus *Magnaporthe oryzae*, Talbot's group (23, 24) succeeded in demonstrating that *Tps1*, in addition to trehalose-6P synthase enzymatic activity, possesses a regulatory function essential for plant infection by the fungus.

These observations led us to suggest that the yeast *Tps1* protein may have regulatory functions independent from its sole enzymatic role in the biosynthesis of trehalose. To elucidate whether the trehalose and/or the *Tps1* protein may help yeast to withstand stress exposure, we constructed and expressed catalytically inactive variants of *Tps1* unable to synthesize trehalose-6P and trehalose. Moreover, we used the *Mal*<sup>+</sup> CEN.PK strain (25) because it constitutively expresses the *Agt1*-mediated trehalose import (26), allowing manipulation of trehalose levels in the absence of *Tps1* or any stress. Combining these strategies, we demonstrated that the tolerance of yeast cells to different stresses was largely independent of trehalose but relied on the *Tps1* protein itself, independently of its annotated catalytic function.

## Experimental Procedures

**Construction of Yeast Strains and Plasmids**—The *S. cerevisiae* strains used in this study (Table 1) were derived from the CEN.PK113-7D, a prototrophic *MAL* constitutive strain (25), or from the BY4741, a *mal*<sup>−</sup> strain (EUROSCARF collection). Primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The *TPS1* alleles were obtained by site-directed mutagenesis. As an overview of the construction process, 139-bp sequences corresponding to 5' and 3' ends of *hisG* were added by recombinant PCR to the extremities of *TPS1*. This mosaic PCR product was cloned into a yeast shuttle plasmid (pMP1, YCpLac33 backbone) and marked with the kanMX4 selection marker, yielding to pMP2. This vector was then used as template for site-directed mutagenesis by inverse

PCR, yielding to different *tps1* alleles (*tps1-102* in pMP8, *tps1-111* in pMP7, and *tps1-156* in pMP6). As a final step, the kanMX-tagged *tps1* alleles were excised from the YCpLac33 backbone and integrated at the *tps1::hisG* locus of JF1476 strain, by homologous recombination via the flanking *hisG* sequences. Because the three alleles led to catalytically dead *Tps1*, we arbitrarily used in this work the *tps1-156* strain expressing the *Tps1*<sup>D156G</sup> variant.

The *HSF1* gene was cloned under the Tet-O<sub>7</sub> promoter in a pCM173 backbone (27) using the In-Fusion system (Clontech) with primers 65 and 66. *HSF1* gene was then deleted by KanMx6 cassette, using homologous recombination in cells transformed by pMP20, after amplification of the KanMx6 cassette on the pFA6a plasmid with primers 82 and 83. Strain genotypes were confirmed by PCR. Overexpression of HSPs was carried out using the 2μ plasmid from the yeast tilling collection (Open Biosystems, YSC4613 (28)) and subcloning of the gene of interest in the same pGP564 backbone vector.

**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/liter (Difco)), supplemented with ammonium sulfate at 5 g/liter, complete drop-out when required (MP-Biomedicals CSM) and 2% (w/v) galactose (YN Gal) or 2% galactose plus 1% trehalose (YN GalTre medium). Cultures were conducted in shake flasks (0.5-liter Erlenmeyer flasks with a working volume of 100 ml) at 30 °C and 200 rpm on a rotary shaker. Cell plating for viability analysis (cfu assay) was performed on YP Gal medium (10 g/liter yeast extract, 10 g/liter bacto peptone, 20 g/liter bacto agar, and 20 g/liter galactose). For modulation of *HSF1* transcript levels using the Tet-O<sub>7</sub> promoter, cells were cultivated according to Ref. 27 in the presence of 0.5 μg/ml tetracyclin (Sigma, stock solution of 5 mg/ml in 50% ethanol).

**Stress Tolerance Assays**—All stress experiments were carried out using exponentially growing cells (*A*<sub>600</sub> 0.5–0.7, corresponding to 1 × 10<sup>7</sup> cells/ml) cultivated on YN Gal or YN Gal-Tre medium at 30 °C, after inoculation of the culture with a cell density corresponding to *A*<sub>600</sub> 0.025. For mild heat shock (37 °C to 42 °C), cells were incubated in an oven after initial immersion

**TABLE 2**  
Primers used in this study

|    | Name                  | Sequence <sup>a</sup>  |
|----|-----------------------|--|
| 1  | hisG 1/24 (Sac1)      | <u>AGGAGCT</u> CATGTTAGACAACACCCGCTTACGC                                 |
| 2  | hisG 100/72 (TPS)     | CGTCTCTGGCTCTATCGTAAAGTACCAATTAATTTTATGCCGACGCGGGCCAG                    |
| 3  | hisG 803/834 (TPS)    | TCACCGTATAGGATATCGGATCCTCAGCAGCGAAACGTTGTTCTGGGAAACCATG                  |
| 4  | hisG 900/879 (Sph1)   | <u>TCCGATGCT</u> CACTCCATCATCTTCTCGATC                                   |
| 5  | TPS1 1021/1059 (hisG) | GCGGCATAAAAATTAATT <u>GGTACCT</u> TTACGATAGAGCCAGAGACGATGCCGACGAAGATGAAG |
| 6  | TPS1 3684/3656 (hisG) | ACAACGTTTCGCTGCTGAGGATCCGATATCCTATACGGTGAAAAAACTACTTGCGCAG               |
| 7  | SP-TPS1-T304G-A305T   | CGATGAAATCGTCGACTTACACGTCACCGGTTTCAGTAATTCCTATT                          |
| 8  | ASP-TPS1-T304G-A305T  | AATAGAATTACTGAACCCGTTGACGTGTAAGTCGACGATTTCCATCG                          |
| 9  | SP-TPS1-G332C         | ACAACGGGTCAGTAATTCATATCTATCGCCGTTATTCATT                                 |
| 10 | ASP-TPS1-G332C        | AATGGAATAACGGCGATAGAATAGAATTAATGACCCGTTGT                                |
| 11 | SP-TPS1-A467G         | CGATTTAACCTAGGTGCATGGTTACCATTTTGATGTTGGTTC                               |
| 12 | ASP-TPS1-A467G        | GAACCAACATCAAATGGTAACCATGCACCTAGGTTAAATCG                                |
| 65 | SP/HSF1-His-IF        | TCAATTCGGGGATCCATGAATAATGCTGCAAAATACAGGGACGACC                           |
| 66 | ASP/HSF1-His-IF       | TGCGGCCCTCCTGCAGCTAATGGTGTGATGATGATGTTTCTTAGC                            |
| 82 | SP/HSF1del-KanMX      | CCAAATAGCAGTACTGTTCCGGTAGATAAAGGCAAAGAGTTAGAGATGGGTAAGGAAAAGACTCA        |
| 83 | ASP/HSF1del-KanMX     | GAAAACTCATCGAGCATCATTAATGATTATATACGCTATTTAATGACCTGCCTGTGTACTA            |

<sup>a</sup> Restriction sites are underlined.**TABLE 3**  
Plasmids used in this study

| Identifying | Name                     | Description   | References |
|-------------|--------------------------|---|------------|
| pMP 6       | YcpLac33 <i>tps1-156</i> | YCpLac33; CEN origin; <i>TPS1</i> promoter                            | This study |
| pMP 20      | pTet- <i>HSF1</i>        | pCM173 backbone (27); CEN origin; Tet- <i>O</i> <sub>7</sub> promoter | This study |
| pMP 38      | p <i>HSP104</i>          | pGP564 backbone (28); 2 $\mu$ origin; native promoters                | This study |
| pMP 39      | p <i>HSC82</i>           | Same as pMP 38  |            |
| pMP 40      | p <i>HSP82</i>           | Same as pMP 38  |            |
| pMP 41      | p <i>SSA3</i>            | Same as pMP 38  |            |

in water bath set at the desired temperature for optimum heat transfer and temperature increase. High temperature heat shock (45 °C to 52 °C) was carried out in a water bath. For acquired thermotolerance, cells were preheated for 1 h at 37 or 42 °C and then transferred to a 50 °C water bath for 2 h. Tolerance to ethanol, oxidative, and osmotic stress was analyzed in the presence of 5% ethanol (v/v), 5 mM H<sub>2</sub>O<sub>2</sub>, and 1 M NaCl, respectively. A short term desiccation assay was carried out according to the vacuum method (18) and operated at 30 °C for 5 to 60 h. At the indicated times, aliquot were directly analyzed for viability by flow cytometry and trehalose content.

**Analysis of Viability by Flow Cytometry**—Flow cytometric analysis of the relative percentage of living and nonviable cells in the population was determined using the **Guava<sup>®</sup> ViaCount<sup>®</sup> reagent (4000-0040)**, purchased from Millipore) and the Attune<sup>®</sup> Acoustic Focusing Cytometer (Life Technologies, Inc.). Exponentially growing cells (approximately 2 × 10<sup>7</sup> cells) were harvested and sonicated to ensure single cell suspensions (40 Hz, 10 s) and then mixed to the Guava<sup>®</sup> reagent according to manufacturer indications and incubated for 5 min at room temperature. Cytometer acquisition was run at 100  $\mu$ l/min to ensure high specificity, on a total number of 100,000 events/run. Excitation was performed from the 488-nm laser (blue). Voltage calibration of the cytometer was always performed by using the unstained, control sample of the WT and further checked on the *tps1* $\Delta$  strain. Routinely applied voltages were 2,000 mV for the forward scatter and 2,600 mV for the side scatter. For optimal positioning of the whole cell population within the autofluorescence black gate (signal below 10<sup>3</sup> thresholds for both the **BL-1 (525 nm)** and **BL-3 (670 nm)** filters), we set BL-1 and BL-3 to 1,200 and 2,100 mV, respectively.

In addition to the control strains, unstained samples from all strains of interest, before and after stress exposure, were analyzed to ensure that these calibration settings for the side scatter-forward scatter plot or for the autofluorescence could apply.

Sample analysis was performed using a combination of two emission filters: the **525-nm filter (BL-1)** to analyze the nuclear dye, which stains only nucleated cells, and the **670-nm filter (BL-3)** to analyze the viability dye, which brightly stains dying cells. Gates for discriminating the different cell populations were positioned on the BL-1-BL-3 plot (log scale) according to manufacturer's recommendations. The data were then processed using the Attune<sup>®</sup> cytometer software. The percentage of cells in each physiological state was calculated from cell counts within the gates (*red* and *blue*, respectively), relative to the total number of cells determined from the forward scatter-side scatter plot. Cytometry-based viability assays presented in this work were repeated at least three times, from independent cultures. The data were then exported to Excel for graphics preparation and to XL-Stat or Statgraphic software for statistical analysis. Viability was also determined by measuring cfu after 2 days on YP Gal plates at 30 °C to assess the correlation between the cytometry-based viability assay and cfu results.

**Assay of Trehalose-6P Synthase**—Crude extracts were prepared according to Ref. 29. Practically, 10 optical density units were collected in a centrifuge tube and washed once with sterile water, and cell pellets were frozen at -20 °C. For protein extraction, 200  $\mu$ l of extraction buffer (20 mM Hepes, pH 7.1, 1 mM EDTA, 100 mM KCl, 1 mM DTT, and 1 mM PMSF) and 0.3-g glass beads (0.5-mm diameter; BioSpec 11079105) were added to the cell pellets. Crude extracts were kept on ice before use, and total proteins were quantified using the Bradford assay (Bio-Rad protein assay dye reagent concentrate, 500-0006). The activity of trehalose-6P synthase was carried out according to Ref. 29.

**Western Blot Analysis of *Tps1***—Crude extract preparations were carried out as for trehalose-6P synthase assay. For protein electrophoresis, 300 ng of proteins were loaded on Stain-Free precast SDS-PAGE gels 10% (Bio-Rad; 456-8033), and the migration was done at 180 V for 25 min. Transfer to nylon membrane (Bio-Rad; 170-4158) was done using the Trans-Blot<sup>®</sup> Turbo<sup>™</sup> blotting system. Membrane saturation was done in 50 ml of 10× TBS and 0.1% Tween 20, plus 2.5 ml of

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blocking reagent for TBS (Thermo; 37535), for 1 h with gentle agitation and at room temperature. Primary antibodies were raised against the 56-kDa subunit of *Tps1* (KH-1142, rabbit serum, kind gift from J. Londesborough, Technical Research Center of Finland, Espoo, Finland (6)) and diluted 1/10,000 times in TBS Tween solution. Hybridization with secondary antibodies was performed with anti-rabbit alkaline phosphatase-conjugated antibodies (diluted 1/2,000 in 1× TBS) (Sigma; A3687). Revelation was monitored by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma; B3679).

**Western Blot Analysis of *Hsf1***—For crude extract preparation, 50 optical density units were collected from cells grown to exponential phase, before (T0) and during heat shock to 40 °C at the indicated times. Cells were washed once with cold water and frozen in liquid nitrogen. The extraction was carried out immediately after at 4 °C in a buffer containing 50 mM Tris-HCl, pH 6, 0.5 M NaCl, 1 mM EDTA, pH 7, 0.1% SDS, 1% Triton, 1% mixture protease inhibitor, 20 mM NaF, 1 mM Na<sub>2</sub>VO<sub>3</sub>, 50 mM β-glycerophosphate, and 1 mM PMSF. For the control lane with phosphatase, the buffer contained 50 mM Tris-HCl, pH 6, 0.5 M NaCl, 1 mM EDTA, pH 7, 0.1% SDS, 1% Triton, and 10 units of alkaline phosphatase. The cells were broken in the presence of 0.5-g glass beads for 30 runs of 30 s at 4 °C. The samples were centrifuged for 5 min at 500 × *g*, and the supernatant was transferred to 1.5-ml microcentrifuge tubes for an additional centrifugation (1 min, 13,000 × *g*). Crude extracts were kept on ice before their use, and total proteins were quantified using the Bradford assay (Bio-Rad protein assay dye reagent concentrate; 500-0006).

For Western blot analysis of *Hsf1*, 15 μg of crude extract proteins were loaded on a 4–15% precast polyacrylamide gel; Bio-Rad; 456-8083), and the migration was carried out at 4 °C at 100 V. Procedure was done according to *Tps1* Western blotting. Revelation for *Hsf1* was monitored by chemiluminescence (Pierce; 34075), using the ChemiDoc™ XRS imaging system from Bio-Rad. Primary antibodies against the *Hsf1* (kind gift from Dr. H. Sakurai, Graduate School of Medical Science, Kanazawa, Japan) were diluted 1/1,000-fold. Secondary antibodies used were anti-mouse peroxidase-conjugated antibodies (Thermo; 32430) and diluted 1/2,000-fold.

**RNA Sampling and cDNA Synthesis for Quantitative PCR Assays**—The cells were grown in YN Gal medium to A<sub>600</sub> ~1.0 and submitted to heat shock as described above. Samples (~10<sup>8</sup> cells) were collected (2,200 × *g*, 4 °C, 3 min) before (T0 control, calibrator sample) and 15 and 30 min after the shift to 40 °C. The pellets were immediately transferred in 2-ml microcentrifuge tubes, washed with 1 ml of cold water, frozen in liquid nitrogen, and stored at –80 °C until RNA extraction. Frozen cells were mechanically disrupted for 3 min using the TissueLyserII apparatus from Qiagen, with one stainless steel bead per tube. Total RNA was extracted using the SV Total RNA purification kit from Promega. The quantification of the RNA samples was assessed by using the ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies), whereas the Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent) was used to certify RNA integrity. Only RNA samples with a 260/280-nm wavelength ratio of ~2 and a 260/230-nm wave-

length ratio >2 were retained for analysis. One microgram of total RNA was reverse-transcribed into cDNA in a 20-μl reaction mixture, using the iScript cDNA synthesis kit (Bio-Rad).

**Quantitative PCR Assay on the Biomark**—High throughput quantitative PCR was performed using the Fluidigm® Biomark microfluidic system. Every sample-gene combination is quantified using 96.96 Dynamic Array™ integrated fluidic circuits (IFC), which ensure performing up to 9,216 parallel qPCRs<sup>3</sup> in nanoliter scale volumes. Pre-amplification of cDNA samples, chip loading, and quantitative PCR were performed according to the manufacturer's protocols. cDNA sample preparation was as follows: 5 ng of each cDNA were initially pre-amplified (activation at 95 °C for 10 min and 14 PCR cycles (95 °C for 15 s and 60 °C for 4 min)) with PreAmp Master Mix (Fluidigm) and a primer mix at 200 nM containing all the primers targeting all the genes analyzed on the array. Pre-amplified samples were then diluted after an exonuclease treatment (NEB M02935). To finalize samples preparation for IFC loading, a reagent mix consisting of 440 μl of 2× TaqMan Master Mix (Applied 430976), 44 μl of 20× DNA binding dye sample loading reagent (Fluidigm; 100-3738), 44 μl of 20× Evagreen (Biotium; 31000) plus 132 μl of Tris-EDTA was prepared, and 6-μl aliquots were dispensed into the 96 wells of the Sample plate. Two microliters of pre-amplified cDNA samples were finally added to this reagent mix, and the sample plate was briefly vortexed and centrifuged. Relative to primer sets preparation, each pair of primers was prepared as a 5 μM solution (each primer) in the assay loading reagent (Fluidigm) and dispensed into the 96 wells of the assay plate. Following priming of the Dynamic Array™ IFC in the IFC Controller HX, 5 μl of cDNA samples and primer sets were pipetted from the sample and assay plates, respectively, and dispensed into the inlets of the IFC. After loading the cDNA samples and primer sets into the IFC on the IFC Controller HX, the IFC was placed into the BioMark for thermal cycling with the following protocol: thermal mix steps at 50 °C for 2 min, 70 °C for 30 min, and 25 °C for 10 min and hot start at 50 °C for 2 min and 95 °C for 10 min, followed by 35 PCR cycles (95 °C for 15 s and 60 °C for 60 s). After completion of these cycles, melting analysis was performed to verify PCR specificity, contamination, and the absence of primer dimers.

**qPCR Assays Quality Control and Data Analysis**—Most of the primer sets for qPCR, especially the reference genes, were previously described and validated (30). New pairs of primers were designed using Vector NTI advance v11 (Life Technologies, Inc.). A BLAST analysis against the *S. cerevisiae* genome sequence was included for specificity confidence. Reaction efficiency for each primer pair was evaluated by the dilution series method using a mix of cDNA samples as the template. The absence of genomic DNA in RNA samples was checked by qPCR before cDNA synthesis. All these quality control assays were performed on a classical qPCR machine (MyIQ real time PCR system from Bio-Rad) as previously described (30). The qPCR assays on the Biomark were performed in technical duplicates. A negative control (No Template Con-

<sup>3</sup> The abbreviations used are: qPCR, quantitative PCR; IFC, integrated fluidic circuit.

trol) was incorporated in each assay. Assay efficiencies of all primer sets analyzed on the IFC were re-evaluated by the dilution series method and applied to final calculations of transcript levels.

For normalization purposes, the transcript levels of six putative reference genes were evaluated in each sample (data not shown). For data analysis, individual  $C_q$  values were exported from the Fluidigm real time PCR analysis software version 4.1.2. They were used to assess normalized fold change values, using the wild type (*TPS1*) strain before heat shock as the calibrator sample, and robust normalization factors calculated from the geometric averaging of the four most stable reference genes in our experimental setup (*i.e.* *TAF10*, *ALG9*, *IPP1*, and *UBC6*). Final fold-change values are given as means  $\pm$  S.D. from the two biological replicates (independent cultures) that were carried out for transcript level analysis in the CEN.PK strain background.

**Other Analytical Procedures**—Cell sampling and metabolites extraction were carried out as previously described (31), and ATP measurement was realized as described (32). The enzymatic measurement of ATP was carried out at 30 °C in Tris-HCl buffer with addition of 0.4 mM  $\text{NAD}^+$ , 0.1 mM glucose, 4.5 units/ml glucose-6-phosphate dehydrogenase, and 1.5 units/ml hexokinase. NADH production was then assessed at 340 nm using a fluorescence spectrophotometer (Agilent G1103A). Trehalose measurement was done according to Ref. 33.

**Statistical Analysis of the Data**—Statistical analyses of “drop of viability” data were conducted by using the STATGRAPHICS Centurion 16 software. This included the analysis of variance, the Tukey’s test on the means, the nonparametric Kruskal-Wallis procedure on medians, and drawing of the box and whiskers plots to visualize these data sets.

## Results

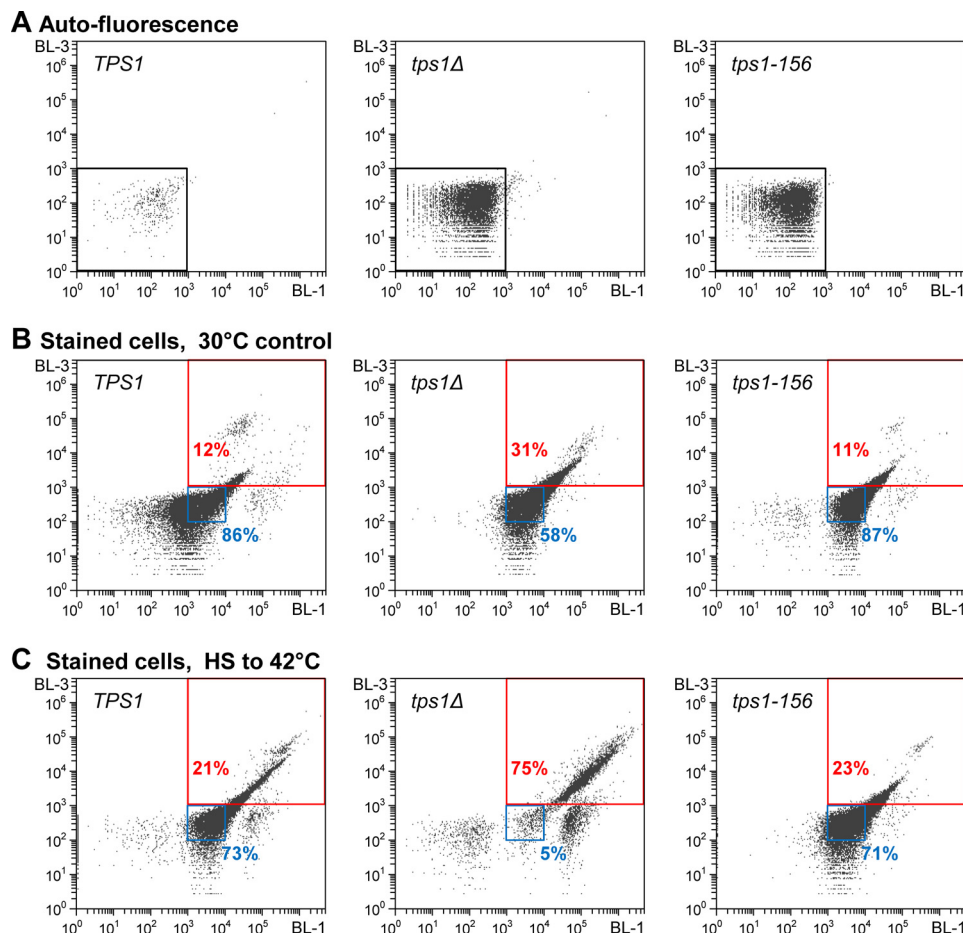
**Construction and Validation of Inactive Catalytic Variants of the *Tps1* Protein**—To construct catalytically inactive variants of the *S. cerevisiae* *Tps1* protein, we followed the strategy employed earlier by Wilson *et al.* (23) for *Magnaporthe grisea* *Tps1*. We mutated three key residues required for the interaction with glucose-6P substrate in the catalytic site of the protein, leading to *Tps1*<sup>Y102V</sup>, *Tps1*<sup>W111S</sup>, and *Tps1*<sup>D156G</sup> variants, respectively. After transformation of a *tps1Δ* mutant with CEN plasmids carrying the corresponding *tps1* alleles, the loss of function of these strains relative to trehalose metabolism was validated in four ways. First, Western blotting against *Tps1* validated that expression and stability of *Tps1* variants were identical to wild type (not shown). Second, none of these strains exhibited measurable trehalose-6P synthase activity, whereas wild type (*TPS1*) cells led to a specific activity of 6.5 nmol/min/mg protein. Third, none of these strains could lead to trehalose-6P accumulation upon glucose addition to trehalose-grown cells according to experimental conditions described in (31). Contrary to the control strain that showed a burst of trehalose-6P 5 min after the glucose pulse (8  $\mu\text{mol/g}$  DW), trehalose-6P was below the detection level in cells expressing the different *tps1* alleles. As a final proof for trehalose-6P synthase deficiency *in vivo*, we assessed the inability to produce trehalose

under conditions known to readily trigger the accumulation of this sugar, such as heat shock (6, 34, 35). When the cells were incubated for 2 h at 42 °C, which is the optimum temperature for *Tps1* catalytic activity and trehalose accumulation, wild type cells accumulated a huge amount of trehalose ( $22 \pm 5 \mu\text{g}$  eq-glucose/ $10^7$  cells), whereas no trace of this disaccharide could be detected in cells expressing the catalytic variants. Because the three variants gave identical results, including for stress experiments carried out below (data not shown), we arbitrarily used and thoroughly present results obtained with the *Tps1*<sup>D156G</sup> variant.

To directly assess the role of trehalose in response to various stresses (see below), we used the *MAL*<sup>+</sup> CEN.PK strain, able to import trehalose from the medium by the constitutively expressed *Agt1* transporter (26, 36). The function of the catalytically inactive variants of *Tps1* in yeast survival to stress was therefore examined in this CEN.PK strain background, as well as in the *mal*<sup>-</sup> BY4741 strain unable to import trehalose. The impact of different stresses was assayed on exponentially growing cells in a galactose synthetic medium, permissive for growth of the *tps1* null mutant, supplemented or not with 1% exogenous trehalose. The viability of these strains was precisely measured by the use of a flow cytometric method that allowed counting up to 100,000 cells/sample (Fig. 1). For each strain, a correlation with cfu counting was first realized and confirmed the robustness of this quantitative flow cytometric method (not shown). The data presented in Table 4 indicated a 30–40% decrease in cell viability of exponentially growing cells deleted for *TPS1*, independent of the carbon sources used as the growth substrate. This drop of viability was not prevented by preloading *tps1Δ* cells with trehalose. More remarkably, it was not observed in *tps1-156* cells expressing the catalytically inactive *Tps1*<sup>D156G</sup> variant.

***Tps1*, Not Trehalose, Is Important for Yeast Viability in Response to Various Kinds of Stresses**—In response to temperature upshift to 42 °C, the viability of the *tps1Δ* mutant sharply dropped by several decades, whereas the viability of the wild type *TPS1* strain remained almost unchanged as could be seen from this logarithmic graph (Fig. 2A). Interestingly, the viability pattern of the *tps1-156* strain was exactly the same as wild type. In none of these strains, preload of trehalose (see Table 5 for intracellular trehalose content) had a protective effect and changed strain sensitivity to heat. We then investigated the impact of the catalytically inactive *Tps1*<sup>D156G</sup> variant on heat resistance of a *tps2Δ* mutant (Fig. 2A), defective in trehalose-6P phosphatase and also known to exhibit temperature-sensitive growth (37, 38). When exposed to 42 °C, we found that this mutant was much less sensitive than the *tps1Δ* mutant, whereas heat sensitivity of the *tps1Δ tps2Δ* double mutant was almost the same as *tps1Δ*. In contrast, upon expression of the *tps1-156* allele, the *tps2Δ* mutant recovered the survival rate of wild type *TPS1* cells. These data indicated that the heat sensitivity of a *tps2Δ* mutant strain was likely due to the overaccumulation of trehalose-6P during heat shock and not to the absence of the *Tps2* protein nor to the lack of trehalose.

We then analyzed cell viability in response to desiccation, another relevant adverse condition for yeast. As is shown in Fig. 2B, the loss of *TPS1* gene markedly increased the sensitivity of



**FIGURE 1. Monitoring cell viability by flow cytometry.** Representative BL-1–BL-3 scatter plots (log scale, BL-1 (525-nm filter), BL-3 (670-nm filter)), showing gates positioning, after voltage calibration of the Attune<sup>®</sup> acoustic focusing cytometer (Life Technologies, Inc.). Plots obtained with exponentially growing cells of the three seminal strains of this work, i.e. *TPS1* (left column), *tps1Δ* (middle column), and *tps1-156* (right column). Autofluorescence plots (A), or stained cells fluorescence plots for viability determination, before (B) or after exposure of the cells to 42 °C for 2 h (C). A, cell autofluorescence plots. Voltage calibration was set for optimal positioning of the whole cell population below 10<sup>3</sup> thresholds (black gate), for both the x axis (BL-1, 525-nm filter) and the y axis (BL-3, 670-nm filter). B, viability determination plots of exponentially growing cells, after staining with the Guava<sup>®</sup> ViaCount<sup>®</sup> reagent. x axis, fluorescence intensity of the nuclear dye, which stains only nucleated cells; y axis, fluorescence intensity of the viability dye, which brightly stains dying cells. Positioning of gates for “living” cells (blue gate) and “nonviable” cells (red gate), according to manufacturer’s recommendations. Numbers (in %) indicate the fraction of the population in the gate. C, viability determination plots of cells exposed for 2 h to 42 °C. Legend is as in B.

**TABLE 4**

**Influence of *TPS1* alleles on the viability of exponentially growing cells**

Shown are results from flow cytometric analysis of viability of exponentially growing cells ( $A_{600} \sim 1.0$  unit) cultivated in YN synthetic medium supplemented with different carbon sources at 30 °C. The values are the means  $\pm$  S.D. of five independent experiments. Gal Tre, 2% galactose + 1% trehalose; Gly Lac Eth, 3% glycerol + 2% lactate + 2% ethanol; ND, not determined; NG, no growth.

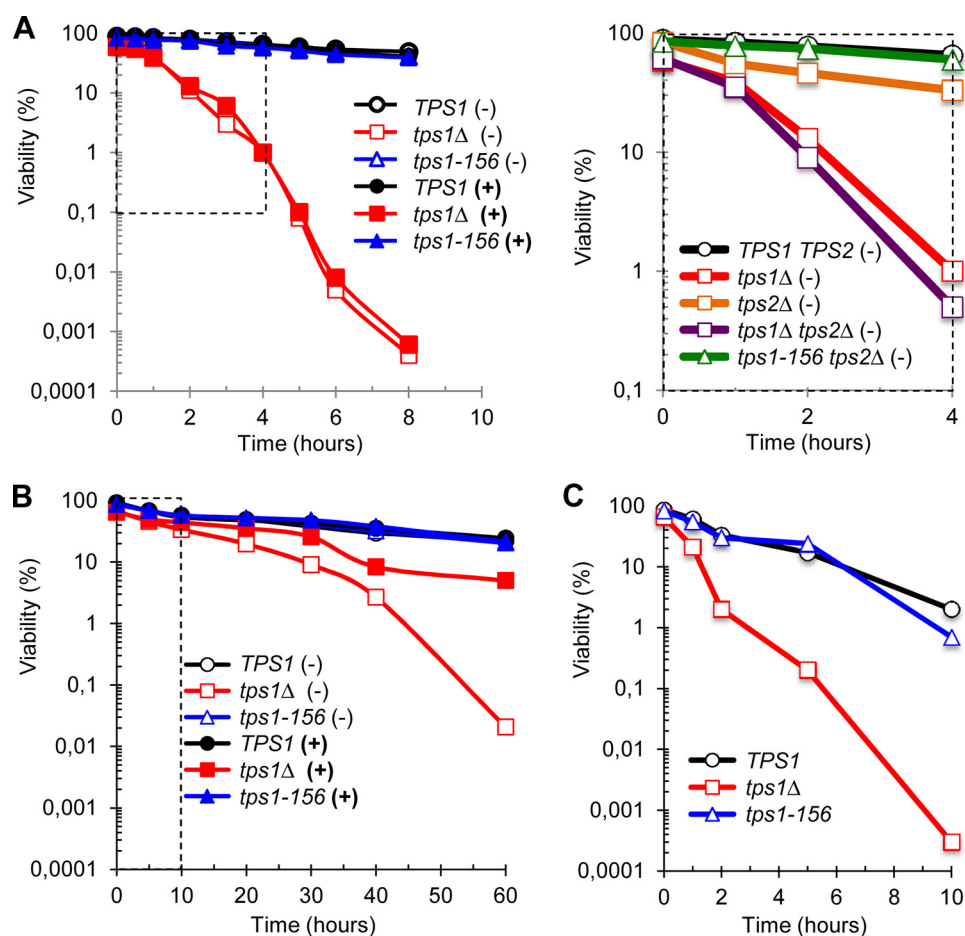
| Carbon source | CEN.PK113–7D background |              |                 | BY4741 background |              |                 |
|---------------|-------------------------|--------------|-----------------|-------------------|--------------|-----------------|
|               | <i>TPS1</i>             | <i>tps1Δ</i> | <i>tps1-156</i> | <i>TPS1</i>       | <i>tps1Δ</i> | <i>tps1-156</i> |
| Galactose 2%  | 91 $\pm$ 4              | 63 $\pm$ 10  | 89 $\pm$ 4      | 86 $\pm$ 5        | 61 $\pm$ 12  | 83 $\pm$ 6      |
| Trehalose 2%  | 84 $\pm$ 5              | 61 $\pm$ 8   | 86 $\pm$ 5      | NG                | NG           | NG              |
| Gal Tre       | 88 $\pm$ 6              | 66 $\pm$ 7   | 86 $\pm$ 4      | 81 $\pm$ 6        | 55 $\pm$ 8   | 84 $\pm$ 3      |
| Gly Lac Eth   | 84 $\pm$ 5              | 59 $\pm$ 11  | 85 $\pm$ 7      | ND                | ND           | ND              |

yeast cells to dehydration, with less than 2% residual viability after 40 h. As for heat shock to 42 °C, this marked sensitivity was not observed in *tps1-156* cells. However, when the strains were loaded with trehalose prior to desiccation, a protective effect of the disaccharide was observed only in the *tps1Δ* mutant ( $p$  values  $< 10^{-5}$ ).

These unpredicted results led us to extend investigation into the impact of the *Tps1*<sup>D156G</sup> variant and/or of trehalose on the drop of viability in response to various stress conditions. As is

shown in Fig. 3, no statistically significant difference could be noticed between *TPS1* (wild type) and *tps1-156* cells. In none of these stresses, preload of trehalose had a protective effect, contrary to what was observed during desiccation for *tps1Δ* fragile cells. However, different patterns could be observed according to the stress applied. As previously seen in Fig. 2A, the drop of viability of a *tps1Δ* mutant was 5 times higher than that of wild type *TPS1* and *tps1-156* in response to temperature upshift to 42 °C (50% versus 10% drop of viability after 2 h). Likewise, the *tps1Δ* mutant was two times more sensitive than wild type *TPS1* and *tps1-156* cells, following a 5 mM H<sub>2</sub>O<sub>2</sub> stress. Unexpectedly, we found that exposure to a mild temperature shock at 37 °C for 2 h was more detrimental than at 42 °C, even for the wild type *TPS1* strain. Also, this drop of viability at 37 °C was comparable between *TPS1*, *tps1-156* and *tps1Δ* cells. Similar results were obtained upon treatment of these three strains with 5% ethanol for 6 h.

Very similar profiles were obtained with strains from the BY4741 background (data not shown). A notable exception was the extreme sensitivity to desiccation stress of the BY derived



**FIGURE 2. Time-dependent evolution of viability during exposure to heat shock or desiccation.** The figure shows the viability of CEN.PK background strains (A and B) in the absence (YN Gal medium, (-)) or in the presence of trehalose (YN GalTre medium, (+)). BY background strains (C) were only grown on YN Gal medium because their *mal<sup>-</sup>* genotype precluded the import of trehalose from the medium. The data are represented as means  $\pm$  S.D. of at least three independent biological replicates. S.D. not plotted on the log scale graphics. A, viability (log scale) as a function of time, in response to heat shock to 42 °C. The dotted line-delimited area in the left panel highlights the scaling for data presented in the right panel. B, viability (log scale) as a function of desiccation time. After desiccation at 30 °C for the indicated time, cell viability was measured after 1 h of rehydration in PBS solution (see Ref. 18 for further details). Control at time 0 corresponds to exponentially growing cells at 30 °C. The dotted line-delimited area highlights the scaling for data presented in C. C, same as in B with BY background strains.

**TABLE 5**

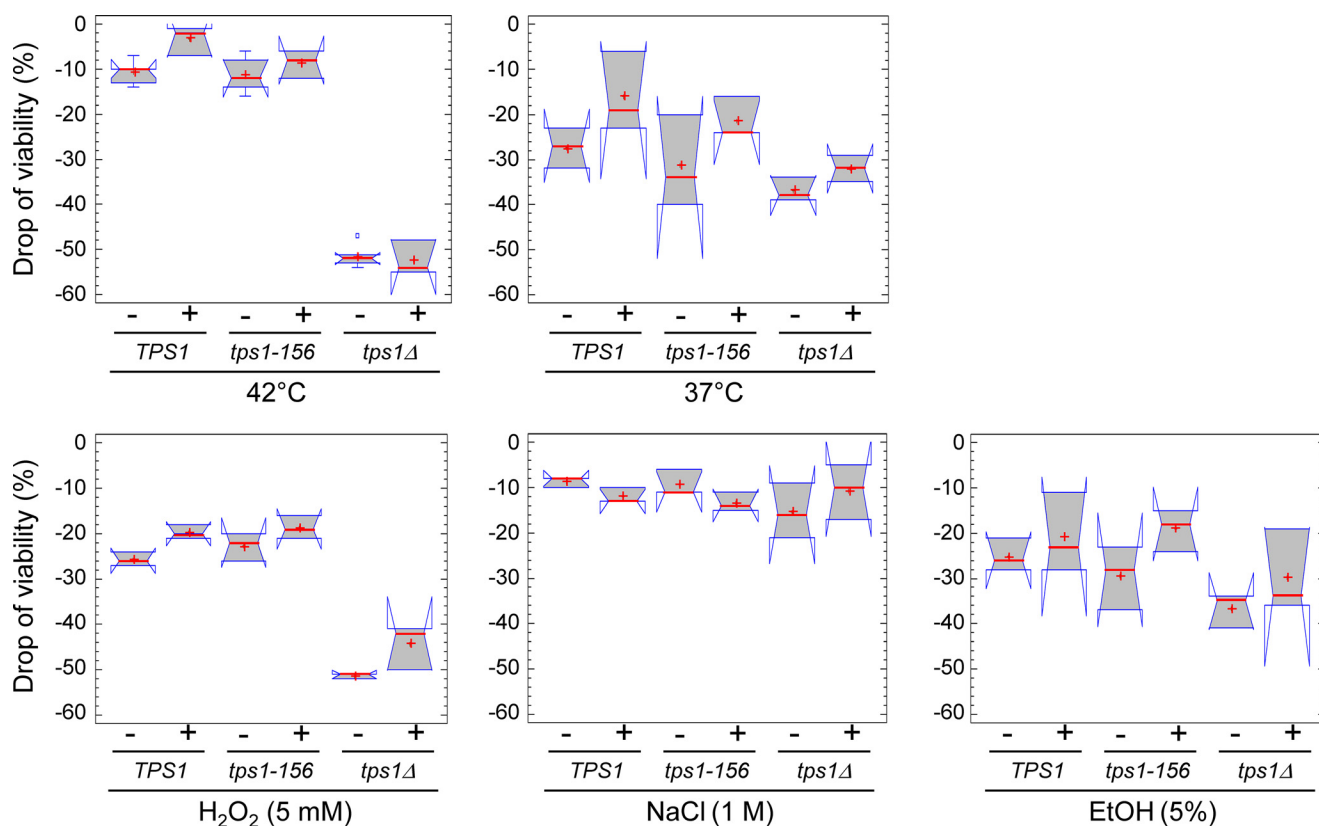
**Intracellular trehalose quantification in the CEN.PK113-7D background strains**

-Tre and +Tre indicate whether growth was performed in YN Gal or in YN GalTre medium, respectively. The values are the means  $\pm$  S.D. of three biological replicates and are expressed in  $\mu\text{g eq}\cdot\text{glucose}/10^7$  cells.

| Condition   | <i>TPS1</i> |            | <i>tps1Δ</i> |             | <i>tps1-156</i> |             |
|---|-------------|------------|--------------|-------------|-----------------|-------------|
|   | -Tre        | +Tre       | -Tre         | +Tre        | -Tre            | +Tre        |
| <b>2 h after upshift to the indicated temperature (unless otherwise stated in parentheses)</b>      |             |            |              |             |                 |             |
| Control (30 °C; $T_0$ )   | 0           | 28 $\pm$ 8 | 0            | 20 $\pm$ 7  | 0               | 19 $\pm$ 6  |
| 37 °C (1 h)   | 0           | 46 $\pm$ 6 | 0            | 50 $\pm$ 7  | 0               | 49 $\pm$ 7  |
| 37 °C   | 0           | 40 $\pm$ 7 | 0            | 45 $\pm$ 5  | 0               | 42 $\pm$ 8  |
| 42 °C (1 h)   | 35 $\pm$ 6  | 45 $\pm$ 6 | 0            | 47 $\pm$ 8  | 0               | 41 $\pm$ 9  |
| 42 °C   | 22 $\pm$ 5  | 33 $\pm$ 7 | 0            | 51 $\pm$ 10 | 0               | 46 $\pm$ 9  |
| 45 °C   | 13 $\pm$ 4  | 36 $\pm$ 7 | 0            | 25 $\pm$ 9  | 0               | 32 $\pm$ 7  |
| 48 °C   | 3 $\pm$ 2   | 39 $\pm$ 9 | 0            | 33 $\pm$ 7  | 0               | 32 $\pm$ 6  |
| 50 °C   | 0           | 22 $\pm$ 8 | 0            | 8 $\pm$ 7   | 0               | 15 $\pm$ 6  |
| 52 °C   | 0           | 25 $\pm$ 6 | 0            | 18 $\pm$ 6  | 0               | 17 $\pm$ 3  |
| <b>2 h after temperature upshift to 50 °C, following a preheat for 1 h at the given temperature</b> |             |            |              |             |                 |             |
| Preheat at 37 °C  | 0           | 35 $\pm$ 8 | 0            | 41 $\pm$ 12 | 0               | 35 $\pm$ 11 |
| Preheat at 42 °C  | 3 $\pm$ 2   | 21 $\pm$ 8 | 0            | 26 $\pm$ 9  | 0               | 31 $\pm$ 5  |
| <b>Under peroxide, salt or ethanol exposure for the indicated time</b>                              |             |            |              |             |                 |             |
| Control (30 °C; $T_0$ )   | 0           | 25 $\pm$ 7 | 0            | 23 $\pm$ 11 | 0               | 27 $\pm$ 8  |
| 50 mM $\text{H}_2\text{O}_2$ for 2 h  | 0           | 23 $\pm$ 6 | 0            | 9 $\pm$ 6   | 0               | 26 $\pm$ 9  |
| 1 M NaCl for 2 h  | 0           | 26 $\pm$ 5 | 0            | 19 $\pm$ 4  | 0               | 23 $\pm$ 5  |
| 5% EtOH for 6 h   | 8 $\pm$ 3   | 9 $\pm$ 6  | 0            | 4 $\pm$ 3   | 0               | 7 $\pm$ 5   |



## Role of *Tps1* in Yeast Stress Tolerance



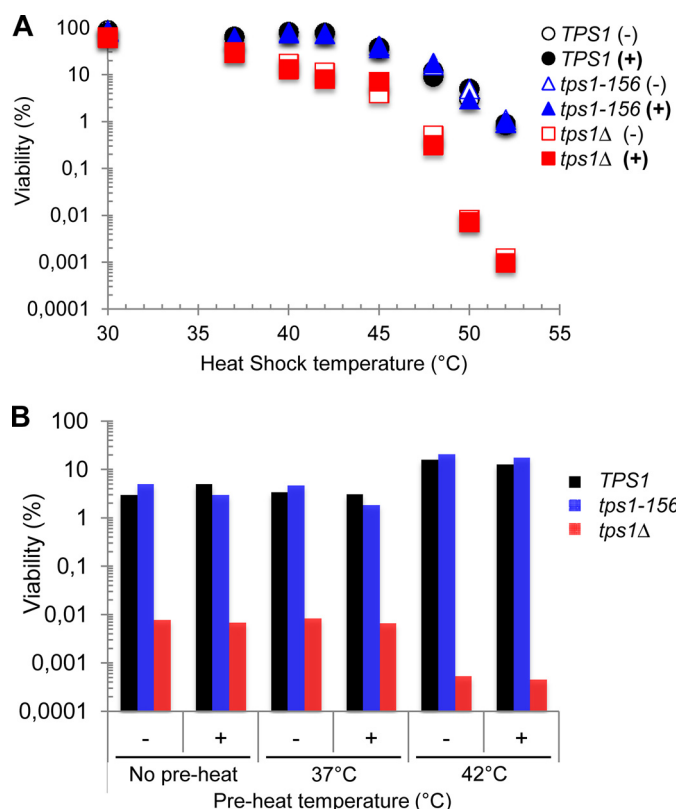
**FIGURE 3. Overview of the impact of the different stresses on the drop of viability.** The “drop of viability” under stress exposure was calculated as the difference between the initial viability (exponentially growing cells in the YN Gal or YN GalTre medium) and the viability after exposure to a stress of interest for the desired time. To better determine the impact of the stress in this box plot representation, we plotted the negative of “drop of viability values. —, median with median notches; +, mean. The range covered by each notch on the median shows the uncertainty associated with the estimate of the median of that group. The notches are scaled in such a way that any two samples whose notches do not overlap can be declared to have significantly different medians at the default system significance level (5%). Except for the ethanol treatment that was performed for 6 h, residual viability of the cells was assessed after 2 h of stress exposure, in the presence (+) or in the absence (–) of trehalose. Control was viability of exponentially growing cells before stress exposure.

strains (Fig. 2, compare *C* and *B*). Altogether, these results strengthened the contribution of the *Tps1* protein, but not of trehalose, in the tolerance of yeast to oxidative stress and high temperature.

*Tps1* Is Required for Thermotolerance and Acquired Thermotolerance—Literature data are relatively inconsistent with respect to the conditions carried out to investigate physiological responses to heat shock, because temperature upshifts ranging from moderate (*i.e.* 37 °C) to extreme temperatures (*i.e.* up to 52 °C) can be found. Thus, based on the above results, we sought to reassess the potential role of either trehalose or *Tps1* protein in yeast subjected to different temperature upshifts. Results presented in Fig. 4A showed that the deletion of *TPS1* rendered the cells extremely sensitive to temperature stress above 40 °C. They also established the importance of the *Tps1* protein in protecting the cells, because the sole presence of the *Tps1*<sup>D156G</sup> variant allowed yeast cells to resist like wild type to these heat shocks. These results finally confirmed the ineffectiveness of trehalose to protect cells against high temperature stress. Preloading *TPS1*, *tps1Δ*, and *tps1-156* cells with the disaccharide did not provide any positive impact on the heat resistance of these cells (Fig. 4A), despite similar accumulation of trehalose in the different strains for all tested temperatures (Table 5). Comparable behavior was obtained with strains from the BY4741 back-

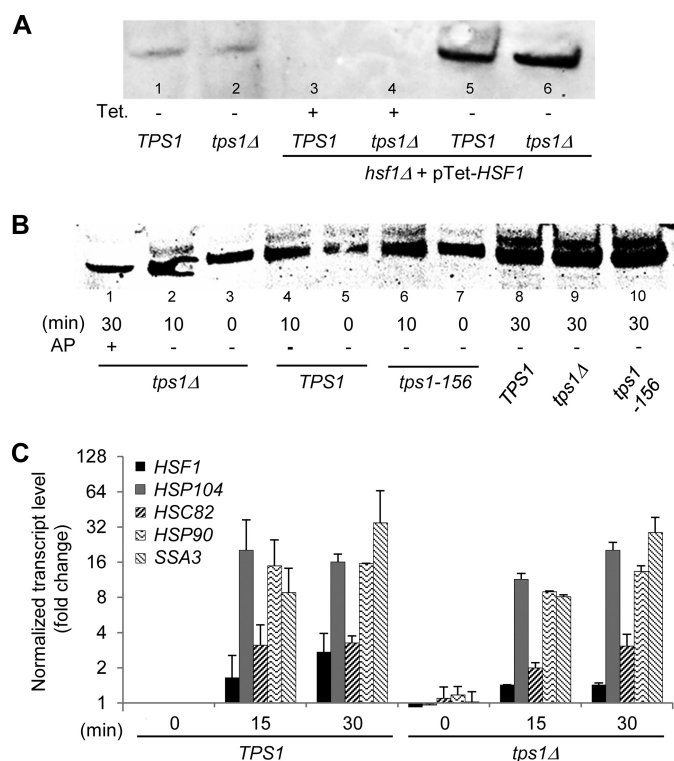
ground (data not shown), but, as reported above with other stresses, these BY strains showed a slightly higher thermo-sensitivity than the CEN.PK strains.

We then investigated whether the adaptive or acquired thermotolerance required *Tps1* rather than trehalose. Acquired thermotolerance is defined as the ability of proliferating yeast cells to withstand a potentially lethal heat shock (*e.g.* 50 °C), provided they are previously submitted to gentle stress such as a moderate temperature rise (39). As compared with a direct shift of yeast cells from 30 °C to 50 °C, a preheat at 37 °C for 1 h did not help the cells to better survive at this high temperature (Fig. 4B). These data reinforced the observation made above that the *TPS* system hardly contributes to cell resistance in response to a mild temperature shift to 37 °C. In contrast, a preincubation of yeast cells at 42 °C for 1 h clearly increased the potency of both wild type *TPS1* and *tps1-156* cells to endure exposure to 50 °C, whereas the viability of *tps1Δ* cells remained extremely low under this condition (Fig. 4B). Again, preloading the cells with trehalose did not bring any positive effect on the adaptive thermotolerance of these cells. Similar results were obtained using the BY4741 strain background (data not shown). We could therefore conclude that the *Tps1* protein itself, and not trehalose, is part of the molecular machinery necessary to survive to high temperature stresses.



**FIGURE 4. Contribution of the *Tps1* protein to thermotolerance and acquired thermotolerance.** Culture conditions were as in Fig. 2, with CEN.PK cells growing exponentially in YN-Gal in the absence (–) or in the presence (+) of trehalose. The data represented are the mean of three independent biological replicates. S.D. values are not plotted on these log scale graphics. *A*, viability (log scale) in response to different heat shock temperatures. The viability was determined at 30 °C (control) and 2 h after the shift to 37, 40, 42, 45, 48, 50, or 52 °C. *B*, viability (log scale) of yeast cells exposed to 50 °C for 2 h, after preheat for 1 h at 37 or 42 °C. “No preheat” condition was control with direct transfer to 50 °C.

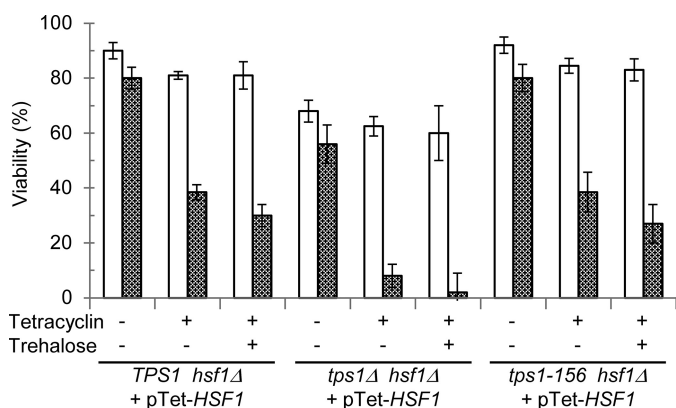
*The Contribution of Tps1 to Thermotolerance Is Independent of Hsf1-dependent Transcription of HSPs*—Our finding that the *Tps1* protein played a major role in resistance to high temperature upshifts raised the question of whether this role could be mediated through the heat shock transcriptional factor encoded by *HSF1*. *Hsf1* is an essential protein in yeast, ascribed to be the primary regulator for heat-induced transcription of heat shock proteins (HSP). It is also involved in diverse cellular processes such as protein degradation, detoxification, energy generation, carbohydrate metabolism, and maintenance of cell wall integrity (40–42). To investigate the interaction between *Hsf1* and *Tps1*, we used strains deleted for the essential *HSF1* gene and rescued by expression of this gene under the tetracyclin-Tet- $O_7$ -repressible promoter. Western blot analysis confirmed that *Hsf1* protein was no longer detected in cells treated with tetracyclin, whereas it was highly abundant in untreated cells because of the strength of Tet- $O_7$  promoter (27) (Fig. 5*A*). Then we revisited a previous report, suggesting that trehalose is a positive regulator of the heat-induced phosphorylation and activity of *Hsf1* (43, 44). Using yeast cells transformed with *pTet-HSF1*, we showed that a temperature upshift to 40 °C for 10 and 30 min led to the appearance of two bands on the SDS-PAGE detected by the anti-*Hsf1* antibodies (Fig. 5*B*). As already shown, the upper band corresponded to the phosphorylated



**FIGURE 5. Contribution of *Tps1* to thermotolerance is not mediated by *Hsf1* transcription factor.** *A*, Western blot analysis of *Hsf1* in yeast crude extracts of CEN.PK background strains. In lanes 1 and 2 are shown the native level of *Hsf1* in *TPS1* and *tps1Δ* strains. In lanes 3–6 are shown *Hsf1* levels expressed from *pTet-HSF1* in *TPS1 hsf1Δ* and *tps1Δ hsf1Δ* strains. Cells were cultivated in the presence of 0.5 μg/liter tetracyclin (+, expression of *HSF1* is down) or in the absence of the antibiotic (–, expression of *HSF1* is up). *B*, heat-induced mobility shift of *Hsf1* upon exposure of yeast cells to 40 °C for 10–30 min. Strains were transformed with *pTet-HSF1*, leading to high expression of the protein. The 30-min *tps1Δ* sample (lane 1) was also treated by 1 unit of alkaline phosphatase (AP). *C*, RT-qPCR analysis of *HSF1* and *HSP* transcript levels after exposure of wild type *TPS1* and *tps1Δ* cells to 40 °C for 15–30 min. The values give normalized fold changes (log<sub>2</sub> scale) relative to the *TPS1* strain before heat shock (time 0), used as the calibrator sample. Normalization was carried out using multiple, validated reference genes (*TAF10*, *ALG9*, *IPP1*, and *UBC6*). The data are represented as means ± S.D. of two biological replicates.

form of this protein, because it disappeared after incubation of the cell extract with alkaline phosphatase prior to gel electrophoresis. The intensity of this upper, phosphorylated band of *Hsf1*, hardly visible in the control condition before heat shock, significantly raised with the time spent at 40 °C, to be clearly and equally visible in all the strains, including the *tps1Δ* mutant. Therefore, our results indicated that, in contrast to a previous report (43), the phosphorylation state of *Hsf1* does not depend on trehalose. In agreement with this result and as shown in Fig. 5*C*, we found that the transcriptional induction of several *HSP* genes upon exposure of cells to 40 °C for 15 and 30 min, a condition known to be *Hsf1*-dependent (45, 46), was not affected by the deletion of *TPS1*. Identical results were obtained with a *tps1Δ* mutant made in the BY4741 background (data not shown). Our results are therefore at variance to previous reports showing down-regulation of *HSP* genes in *tps1Δ* mutants (43, 47). The reason for this discrepancy could be explained by differences in methodology to quantify mRNA levels, which in our case employs a robust RT-qPCR protocol

## Role of *Tps1* in Yeast Stress Tolerance

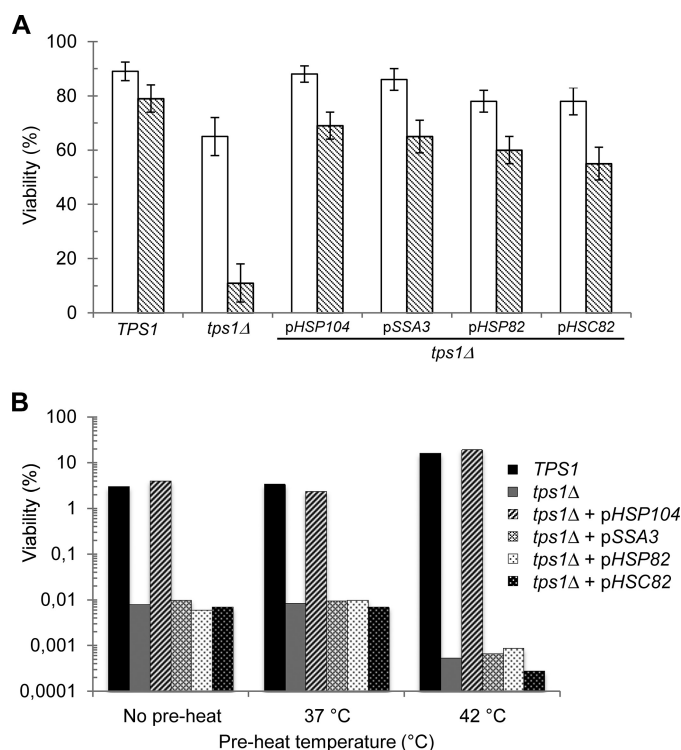


**FIGURE 6. Genetic interaction between *TPS1* and *HSF1*.** Viability of the *TPS1 hsf1Δ*, *tps1Δ hsf1Δ*, and *tps1-156 hsf1Δ* strains rescued by pTet-*HSF1* and exposed to 40 °C for 2 h. Viability measured before heat shock (30 °C, open bars) or after 2 h at 42 °C (gray bars). Tetracyclin, stress exposure in the absence (–) or presence (+) of tetracyclin. Trehalose, growth in YN Gal without (–) or with 1% trehalose (+). The data are represented as means ± S.D. of three independent biological replicates.

(30), whereas previous works were done by Northern blot analysis, a much less accurate and sensitive method.

Finally, we explored a genetic interaction between *TPS1* and *HSF1* on the cell viability in response to 40 °C heat shock (Fig. 6). The strong expression of Hsf1 (*TPS1 hsf1Δ* pTet-*HSF1* strain) led to similar results as for the *TPS1* strain (compare data in Figs. 3 and 6). Lowering the expression of *HSF1* by extinction of Tet- $O_7$  promoter with the antibiotic barely affected the viability of these cells at 30 °C but considerably sensitized them to heat shock as illustrated by a 40% drop of viability after 2 h at 40 °C. Interestingly, viability profiles of the *tps1-156 hsf1Δ* pTet-*HSF1* strain were strictly identical to the control strain (*TPS1 hsf1Δ* pTet-*HSF1*). Remarkably, the *tps1Δ* strain sensitivity to heat shock was fully suppressed by high expression of Hsf1, as illustrated by a moderate 10% drop of viability in the *tps1Δ hsf1Δ* strain carrying pTet-*HSF1* cultivated in the absence of tetracyclin. Conversely, down-expression of *HSF1* in the *tps1Δ hsf1Δ* pTet-*HSF1* strain led to similar thermosensitivity than the single *tps1Δ* mutant. Finally, loading yeast cells with trehalose prior to heat shock had statistically no effect on their reduced viability upon *HSF1* extinction. Taken together, these results further indicated that trehalose had no role in thermotolerance and adaptive thermotolerance, which actually implicated *Tps1*, independently of the Hsf1 transcriptional activity.

***Tps1* Protects Cells from ATP Depletion upon Exposure to Heat Shock**—Based on the above results, we investigated whether overexpression of different heat shock proteins can suppress the *tps1Δ* sensitivity to heat shock. Accordingly, cell survival of this mutant after exposure to 42 °C for 2 h increased from 10 to ~70% upon overexpression of *HSP104*, *SSA3*, *HSP82*, and *HSC82* (Fig. 7A). Among the different HSPs found to suppress this *tps1Δ* phenotype, *HSP104* turned out to be the most efficient suppressor able to restore thermotolerance and adaptive thermotolerance similar to wild type cells (Fig. 7, A and B). However, upon longer exposure, the cell viability of the *tps1Δ* transformed with a high copy vector containing *HSP104* dropped significantly to reach less than 10% viable cells after 8 h (Fig. 8A). Because of the nature of the Hsp104 protein that

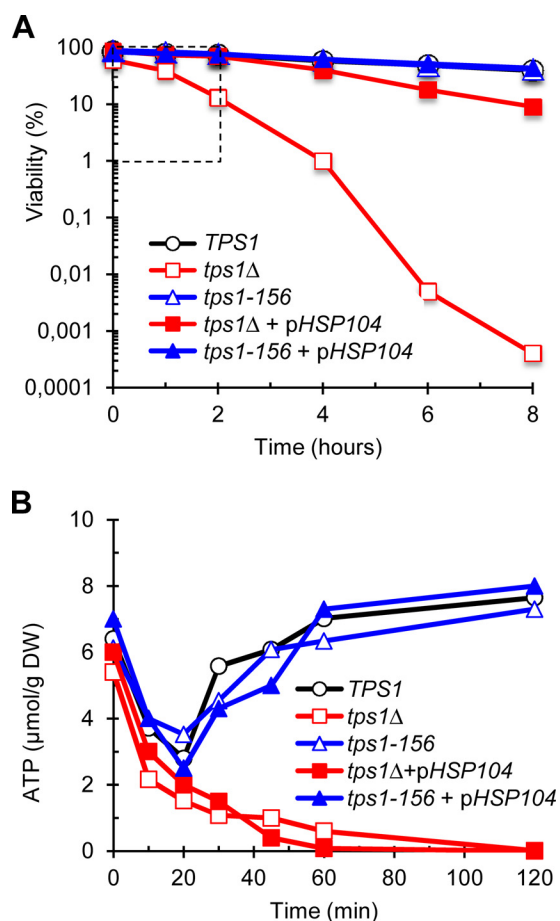


**FIGURE 7. The thermotolerance of *tps1Δ* cells can be suppressed by over-expression of HSP genes.** A, viability of the CEN.PK *tps1Δ* strain transformed with a 2 $\mu$  plasmid bearing *HSP104*, *SSA3*, *HSP82*, or *HSC82*. Control strains are the wild type *TPS1* and *tps1Δ* cells from the CEN.PK background. Exponentially growing cells on YN Gal medium at 30 °C (open bars) and cells exposed to 42 °C for 2 h (gray bars) are shown. B, viability of the same strains preheated at 37 or 42 °C prior to their transfer to 50 °C for 2 h. “No preheat” condition was control with direct transfer to 50 °C. The data are represented as means ± S.D. of three independent biological replicates.

belongs to the Clp/Hsp100 family of AAA+ proteins (ATPases associated with various cellular activities) (48) and because energy deficiencies in the *tps1Δ* mutant have been reported (49), we determined the ATP content in yeast cells during the heat shock response. Remarkably, we found that the *tps1Δ* mutant overexpressing or not *HSP104* became irreversibly depleted of its ATP content in less than 1 h at 42 °C (Fig. 8B). In contrast, in both wild type and *tps1-156* cells, the ATP transiently dropped by ~50% during the first 20 min to regain initial levels after 40–60 min. The complete loss of ATP coincided with the time when the viability of the *tps1Δ* overexpressing *HSP104* started to decline. However, this drop of viability was very slow as compared with that of *tps1Δ* alone, suggesting a protective effect of *HSP104* overexpression, independent of ATP levels.

## Discussion

Trehalose has long been proposed to play an important role during stress response, mainly as a chemical chaperone (2, 50–52). However, doubts about this stress-protecting function have been recently raised from contradictory results, notably in thermotolerance and desiccation (51–53). A main reason for this ambiguity was the inability to directly assess the physiological function of trehalose in yeast cells without interfering with its metabolism, as well as with stress applied. In this report, we achieved this goal by exploiting our previous finding that yeast



**FIGURE 8. The *tps1Δ* mutant rapidly loses its ATP content in response to heat shock.** *A*, loss of viability (log scale) as a function of time, in response to heat shock to 42 °C. The dotted line-delimited area highlights the time scaling for ATP levels data presented in *B*. *B*, levels of ATP during the first 2 h of exposure to 42 °C. The strains *tps1Δ* and *tps1-156* were transformed with a 2- $\mu$  empty plasmid or with *pHSP104*. The data are represented as means  $\pm$  S.D. of three independent biological replicates.

cells can accumulate exogenous trehalose from the culture medium by the Agt1  $\alpha$ -glucoside transporter (26, 36). According to this strategy, we clearly showed that trehalose had no role in protecting cells against heat shock and oxidative stress. With respect to desiccation, we found that trehalose has no protective effect when wild type cells are subjected to this stress. This is in line with results from Calahan *et al.* (53) showing that mutants defective in trehalose biosynthesis retained wild type levels of desiccation tolerance, whereas a more recent report from the same group (54) proposed that trehalose is only needed for survival during long term (>2 days) desiccation. Our results also agreed with a previous work of Ratnakumar and Tunnacliffe (52) that pointed out a lack of correlation between trehalose levels and survival of the yeast *S. cerevisiae* during short-term desiccation. Collectively, these results led to the conclusion that the accumulation of trehalose in yeast cells exposed to various stresses is purely coincidental and is the result of the genetic and metabolic regulation of the trehalose pathway (20). Instead, they strengthen the view that the principal function of trehalose is to provide an enduring source of energy that helps yeast cells to survive very long period of resting state, as well as to drive cell cycle progression upon return to growth conditions (10, 11, 20).

The most remarkable finding in this report was to show that the Tps1 protein is indispensable in yeast to withstand high temperature and oxidative stresses. Indeed, contrary to a *tps1Δ* mutant that is stress hypersensitive, a mutant strain expressing a catalytically inactive Tps1 variant exhibited the same sensitivity to stresses as wild type cells. We nevertheless made the observation that Tps1 had no role in protecting cells against a mild heat stress carried out at 37 °C or a severe stress to 50 °C after a preconditioning at 37 °C, whereas this protein became determinant in yeast survival to temperature upshift to 40 °C or higher. This result indicated that the function of Tps1 to protect cells against severe heat stress relies on low amounts of the protein present in exponentially growing cells before stress exposure (8) and does not require its transcriptional activation, which only takes place at temperature below 40 °C (6). As a conclusion, our finding strongly supports the notion that the yeast *S. cerevisiae* Tps1 protein, similar to what was found for the *M. grisea* protein (23), bears regulatory functions, independent from its enzymatic role in the trehalose biosynthetic pathway.

A system for wild type cells to sustain heat shock could be an interplay between *TPS1* and key genes in the heat shock machinery, such as *HSF1* encoding the heat shock transcription factor. Conlin and Nelson (43) suggested that trehalose could play this role by directly regulating the heat-induced activity of Hsf1. However, our results refute this hypothesis. We showed that the heat shock-induced transcription of *HSP* genes is similar between wild type and a *tps1Δ* mutant, which does not accumulate trehalose, in full agreement with the total independence of heat shock-induced Hsf1 phosphorylation upon Tps1. Also, the loss of *TPS1* rendered cells extremely more sensitive to heat stress than the sole down-expression of *HSF1*. Conversely, the hypersensitivity to heat of a mutant lacking Tps1 was suppressed by overexpression of *HSP* genes, *HSP104* being the most effective. These results also agreed with findings that thermotolerance trait was impaired far more in a double *tps1Δ hsp104Δ* mutant than in either single mutant (38, 55). Taken together, these results favored a model in which Tps1 and Hsf1 contribute together, but in an independent manner, to the thermotolerance.

The question was therefore to know how Tps1 contributes to thermotolerance of the cells in interaction with Hsf1-dependent heat shock proteins. Part of the explanation came from the finding that the ATP content in a mutant lacking Tps1 protein was completely depleted within 1 h after heat shock to 42 °C, whereas this loss of ATP was fully prevented in cells expressing the catalytically inactive Tps1 variant. The Tps1 dependence of ATP maintenance could therefore explain that the suppression of the heat sensitivity of *tps1Δ* by overexpression of *HSP104* did not persist upon long term exposure to 42 °C, because ATP is indispensable for the disaggregation and refolding activity of Hsp104 protein (56–58). In addition, this function of Tps1 in the maintenance of energy may reconcile previous reports showing that the conformational repair of a glycoprotein in ER lumen or the solubilization of a mutant huntingtin protein in heat shocked cells is compromised in a *tps1Δ* mutant (59, 60).

Finally, we found that the absence of the Tps1 protein resulted in a dramatic 30–40% drop of viability in exponentially

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growing cells without exposure to any harmful conditions. Because these *tps1Δ* cells exhibited an ATP content similar to wild type cells in all permissive growth conditions for *tps1Δ* mutant (Ref. 49 and unpublished data), it is likely that Tps1 must have additional regulatory function(s) indispensable for cell viability in dividing cells. Moreover, Tps1 is a relatively low abundance protein in exponentially growing yeast cells on glucose (6, 8, 61), with similar levels to Pfk26, which synthesizes the glycolytic effector Fru-2,6-P<sub>2</sub> (62). We then favor the idea that Tps1, in addition to its catalytic function in trehalose synthesis, is a sensing/signaling intermediate with regulatory function(s), at least in energy homeostasis. Such function in preventing energy depletion should be essential to withstand adverse conditions.

To conclude, beyond the simple metabolic function of Tps1 via trehalose-6P and trehalose synthesis, our results may allow considering Tps1 as a new, attractive example of “moonlighting” proteins, which are characterized by their ability to perform completely unrelated tasks utilizing regions outside the active site for other functions, mostly regulatory and structural (63). Also, in light of this crucial role of Tps1 in stress resistance, probably through mechanisms that maintain energy homeostasis, our view of survival mechanisms to stress has to be revised and will likely be extendable to other organisms that express Tps1 homologs. These findings finally appear strategic for two relevant yet divergent perspectives. The first one fits with the current interest of many biotechnologically oriented efforts aiming at optimizing the microbial cell factories, particularly yeasts. In addition to their use in traditional fermentation processes, yeasts face growing interest in the exploitation of new C-sources, not only for bioethanol production but also for the white (synthetic) biotechnologies (64–66). All these processes are sources of many adverse situations for these microbial cell factories. Identifying the keys for maintaining or boosting stress resistance is a prerequisite for the robustness of industrial strains, the interplay between Tps1 and energy homeostasis being an appealing target for strain selection and/or engineering. The second perspective relies on the urgent need for new antifungal therapies. The trehalose synthase pathway is widespread in the fungal kingdom where it can be part of the virulence system (23, 67–69), thus making Tps1 a potential antimicrobial target for weakening mammalian and plant pathogenic microorganisms.

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*Note Added in Proof*—The autofluorescence scatter plots shown in Fig. 1A were not correct in the version of this article that was published on May 1, 2015 as a Paper in Press. Specifically, data from the *TPS1* strain was mistakenly duplicated and used to represent results

from the *tps1-156* strain. Furthermore, the scatter plots shown in Fig. 1 (B and C) represented results of independent experiments with possible experimental variation. The corrected version of Fig. 1 presents data obtained for *TPS1*, *tps1Δ*, and *tps1-156* strains from experiments performed on the same day to avoid variations caused by the environment (medium and culture conditions). This correction does not affect the interpretation of the results or the conclusions.

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**Metabolism:**

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