Altered intracellular calcium homeostasis and endoplasmic reticulum redox state in *Saccharomyces cerevisiae* cells lacking *Grx6* glutaredoxin

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**ABSTRACT** Glutaredoxin 6 (*Grx6*) of *Saccharomyces cerevisiae* is an integral thiol oxidoreductase protein of the endoplasmic reticulum/Golgi vesicles. Its absence alters the redox equilibrium of the reticulum lumen toward a more oxidized state, thus compensating the defects in protein folding/secretion and cell growth caused by low levels of the oxidase Ero1. In addition, null mutants in *GRX6* display a more intense unfolded protein response than wild-type cells upon treatment with inducers of this pathway. These observations support a role of *Grx6* in regulating the glutathionylation of thiols of endoplasmic reticulum/Golgi target proteins and consequently the equilibrium between reduced and oxidized glutathione in the lumen of these compartments. A specific function influenced by *Grx6* activity is the homeostasis of intracellular calcium. *Grx6*-deficient mutants have reduced levels of calcium in the ER lumen, whereas accumulation occurs at the cytosol from extracellular sources. This results in permanent activation of the calcineurin-dependent pathway in these cells. Some but not all the phenotypes of the mutant are coincident with those of mutants deficient in intracellular calcium transporters, such as the Golgi Pmr1 protein. The results presented in this study provide evidence for redox regulation of calcium homeostasis in yeast cells.

**INTRODUCTION** Ion homeostasis is essential for the physiology of the cell. Cations such as K\(^{+}\), Na\(^{+}\), or Ca\(^{2+}\) are required for a large diversity of cellular processes, but at the same time they must be kept at appropriate intracellular concentrations to avoid toxicity. The yeast *Saccharomyces cerevisiae* has been used as a model to study the plasma membrane and intracellular transport mechanisms contributing to maintain cation homeostasis, as well as the responses to restore such homeostasis when this is disturbed (Ariño et al., 2010; Cunningham, 2011; Cypert and Phipcott, 2013).

Cytosolic Ca\(^{2+}\) concentration is important in signaling and regulation of many essential responses in practically all cell types (Clapham, 2007). To make this signaling possible, cells maintain cytosolic calcium at low levels and have different transporters that regulate the concentration of this cation in the intracellular compartments, causing the oscillations required for cell signaling. In yeast cells, the vacuole is the major Ca\(^{2+}\) store. Entry or exit of the cation from this organelle is mediated by different transporters (reviewed by Cunningham, 2011). Pmc1 is a vacuolar Ca\(^{2+}\) pump related to the PMCA family of plasma membrane Ca\(^{2+}\) ATPases from mammals and plants and is the main contributor to vacuolar Ca\(^{2+}\) levels. These also become determined by the activities of the Ca\(^{2+}/H^+\) exchanger Vcx1 and the Ca\(^{2+}\) channel Yvc1, which respectively import or release Ca\(^{2+}\) into or from the vacuole. As in mammalian cells, in yeast cells, Ca\(^{2+}\) is required at the endoplasmic reticulum (ER) for the correct function of the protein folding and secretory machinery (Bonilla et al., 2002). Adequate Ca\(^{2+}\) levels at the ER/early Golgi organelles mainly result from the activity of the Pmr1, a P-type Ca\(^{2+}/Mn^{2+}\) pump of the SPCA family that imports those cations into the organelle lumen (Antebi and Fink, 1992; Dürr et al., 1998). Spf1/Cod1 is another P-type ATPase that acts synergistically with Pmr1 in transporting Ca\(^{2+}\) into the ER lumen (Cronin et al., 2002) and also influences entry of Mn\(^{2+}\) inside ER vesicles (Cohen et al., 2013). Recently, Gdt1/Grc1 was characterized as a Golgi-localized transmembrane member of the SPCA family that imports Mn\(^{2+}\) into the ER lumen.
the cation/Ca\(^{2+}\) exchanger superfamily that would contribute together with Pmr1 for Ca\(^{2+}\) supply to the Golgi apparatus in yeast (Demaedt et al., 2013). On the other hand, it has been proposed that Csg2 acts as a channel that releases Ca\(^{2+}\) from ER to cytosol to keep with luminal ER homeostasis (Beeler et al., 1994; Tanida et al., 1996). At the S. cerevisiae plasma membrane, two mechanisms operate for Ca\(^{2+}\) influx—the low-affinity system (which acts only in Ca\(^{2+}\)-rich conditions) and the high-affinity system (HACS; acting in both Ca\(^{2+}\)-rich and -poor conditions). HACS is composed of three interacting proteins (Cch1, Mid1, and Ecm7) with homology to the voltage-gated Ca\(^{2+}\) channels in animals (Cunningham, 2011).

The mentioned Ca\(^{2+}\) transport systems are interrelated. Thus the absence of Pmr1, which lowers Ca\(^{2+}\) levels at the lumen of ER/Golgi compartments, activates HACS; consequently, cytosolic calcium levels increase, and this leads the cell to induce the calcineurin-dependent pathway (Locke et al., 2000; Bonilla and Cunningham, 2003). In yeast, this pathway responds to alterations in intracellular Ca\(^{2+}\) and also to high environmental concentrations of cations such as Ca\(^{2+}\) or Na\(^{+}\) and to sexual pheromones (Cyert, 2003; Thewes, 2014). Central in this pathway is the calmodulin/Ca\(^{2+}\) complex, which activates the Ser/Thr phosphatase calcineurin, which in turn dephosphorylates the Crz1 transcription factor, causing its import to the nucleus. Crz1 targets promoter elements with the CDRE motif. In this way, calcium and sodium stresses share common Crz1-dependent gene targets (Yoshimoto et al., 2002). PMC1 is one of the genes up-regulated upon alteration of Ca\(^{2+}\) homeostasis (Yoshimoto et al., 2002). Thus a Δpmr1 mutant accumulates a large amount of Ca\(^{2+}\) at the vacuole (Halachmi and Eilam, 1996) as a compensatory mechanism, and a Δpmr1Δpmc1 mutant is nonviable (Cunningham and Fink, 1996). One of the two genes responsible for the high-affinity phosphate transport system at the plasma membrane (PHO89) is also up-regulated by high-calcium stress in a Crz1-dependent manner (Yoshimoto et al., 2002). This may reflect a relationship between Ca\(^{2+}\) and phosphate influx, consistent with the fact that most of vacuolar Ca\(^{2+}\) complexes with inorganic polyphosphate (Dunn et al., 1994).

The unfolded protein response (UPR) is induced in situations causing ER stress that lead to incorrect protein folding or to protein traffic overloading at the lumen of this compartment (Walter and Ron, 2011). The response involves the induction of ER/Golgi-associated chaperones and the ERAD machinery for degradation of misfolded proteins. The UPR mediators are evolutionarily conserved, and in yeast cells, they include the signaling protein kinase Ire1 and the effector transcription factor Hac1. Among the genes induced by the UPR in S. cerevisiae (Travers et al., 2000) are PDI1 (for protein disulfide isomerase) and ERO1 for ER oxidase. These two proteins are necessary for oxidative protein folding in the ER. Partial loss-of-function ero1 mutants are hypersensitive to the reducing agent di-thiothreitol (DTT), whereas the thiol oxidant diamide rescues partially the mutated phenotype (Frands and Kaiser, 1998; Pollard et al., 1998). Ca\(^{2+}\) deprivation at the ER also activates the UPR in S. cerevisiae (Bonilla et al., 2002), supporting the important role of Ca\(^{2+}\) for the correct function of the protein secretory machinery. On the other hand, tunicamycin (an inhibitor of protein N-glycosylation that interferes with protein folding and consequently induces the UPR) provokes Ca\(^{2+}\) influx through a mechanism that is Ire1 and Hac1 independent, a phenomenon extensible to other conditions causing ER stress (Bonilla et al., 2002). This, together with the fact that the calcineurin-dependent pathway is required for long-term survival to tunicamycin treatment, indicates that the calcineurin pathway and the UPR operate in parallel for cell protection against ER stress.

Glutaredoxins (GRXs) are glutathione-dependent thiol oxidoreductases that regulate cellular redox processes involving protein sulfhydryl groups (Lillig et al., 2008; Herrero et al., 2010). S. cerevisiae contains two GRXs—Grx6 and Grx7, which are integral components of ER/Golgi membranes (Izquierdo et al., 2008; Mesecke et al., 2008b). They share extensive sequence homology between them and with the dithiol GRXs. However, Grx6 and Grx7 contain a single Cys residue at the active site. Considering the 1-Cys mechanism of action of GRXs (Lillig et al., 2008), this supports the idea that Grx6/Grx7 have a de glutathionylation function on uncharacterized targets at ER/Golgi organelles. In spite of their sequence similarities and the fact that both of them become up-regulated upon ER stress by tunicamycin (Izquierdo et al., 2008), Grx6 and Grx7 have certain specificities. Thus Grx6 is enriched at the ER, whereas Grx7 localizes preferentially at Golgi (Izquierdo et al., 2008). In addition, expression of the GRX6 gene is induced by high-calcium and sodium stresses and by oxidative stress in a Crz1-dependent manner, in contrast to GRX7 expression (Izquierdo et al., 2008). Finally, Grx6, but not Grx7, contains an iron–sulfur cluster ligand (Mesecke et al., 2008a). These differences point to specialized roles of both GRXs at the membranes of the secretory pathway compartments. In the present work, we show that the absence of Grx6 affects both redox homeostasis at the ER luminal space and Ca\(^{2+}\) homeostasis, this last alteration probably being a consequence of a deficient function of Ca\(^{2+}\) transporters present in ER membranes. Overall our study reveals the importance of GRXs to carry out redox regulatory functions at the lumen of early secretory membrane vesicles in fungal cells.

RESULTS

The calcineurin pathway is constitutively induced in Δgrx6 cells

To advance in the functional characterization of Grx6/Grx7, we analyzed the transcriptome of a double Δgrx6Δgrx7 mutant. In our study, 26 genes were constitutively induced at least twofold in the mutant compared with wild-type cells, whereas 11 were repressed (Supplemental Table S1). Among the up-regulated genes, those involved in phosphate metabolism (PHO89, PHO1, PHO12, PHO11, PHO3, PHO12) or in protein secretion/compartmentalization (ATG15, ARE1, CPR4, PER1) are predominant. We confirmed the transcriptomic results by Northern analysis of some selected genes, using in addition the single Δgrx6 and Δgrx7 mutants (Figure 1).

The results indicate that up-regulation of two genes involved in phosphate homeostasis (PHO89 and PHM6) is due to the Δgrx6 mutation, whereas up-regulation of the other genes tested depends on the Δgrx7 mutation. PHO89 and PHO84 code for the two high-affinity phosphate transporters in yeast (Persson et al., 2003; Mouillon and Persson, 2006). Therefore we also tested whether PHO84 expression was affected in the absence of Grx6 and/or Grx7. However this was not the case (Figure 1).

Expression of PHO89, but not that of PHO84, is induced by calcium stress in a Crz1-dependent manner (Yoshimoto et al. 2002; Ruiz et al., 2008). Two other genes induced in the Δgrx6Δgrx7 mutant (THR4 and CPR4) contain CDRE consensus motifs in their promoters. These observations point to alterations in the homeostasis of some ions in the absence of Grx6 and/or Grx7. In consequence, we determined whether expression from a Crz1-dependent promoter was constitutively up-regulated in the absence of the two GRXs by using the pAM5366 (wild-type CDRE promoter) and pAM5364 (mutated CDRE promoter) lacZ reporter plasmids. In the absence of Grx6, albeit not of Grx7, the CDRE-containing promoter was up-regulated, and this was dependent on the ability to bind the Crz1 factor (Figure 2A). High Ca\(^{2+}\) stress was able to induce expression from the intact CDRE promoter in wild-type and Δgrx7 mutant cells but did not cause significant additional induction over the high

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basal constitutive levels in the Δgrx6 mutant (Figure 2A). By Northern analysis, we confirmed that CMK2 and GYP7, two genes that are upregulated upon Ca²⁺ stress in a Crz1-dependent manner (Yoshimoto et al., 2002), are also constitutively induced in Δgrx6 cells and that this induction is abrogated by the Δcrz1 mutation (Figure 2B).

To confirm that the constitutive induction of the calcineurin pathway in Δgrx6 cells is biologically significant, we determined the sensitivity of wild-type and mutant cells to the calcineurin inhibitor FK506. The Δgrx6 and Δgrx6Δgrx7 mutants displayed defective growth in cultures treated with FK506 compared with wild-type or Δgrx7 cells (Figure 2C). Although the differences were moderate, they were statistically significant. To demonstrate that the growth defects were due to the absence of the enzymatic activity of Grx6 and not indirectly caused by the absence of the protein in the null mutant, we measured growth in FK506-treated cells that expressed a Grx6 active-site mutant protein with a Cys136-to-Ser substitution instead of the wild-type protein. The Δgrx6Δgrx7 cells expressing the Grx6 mutant had the same growth defects as the wild-type cells in conditions in which the calcineurin pathway is inhibited. In accordance with these results, cells expressing the enzymatically inactive Grx6 form displayed up-regulation of the CMK2 gene similarly to Δgrx6 cells (Supplemental Figure S1).

Yeast cells lacking Grx6 have altered levels of Ca²⁺ at the cytosol and ER

Taken together, the foregoing observations suggested that the absence of Grx6 causes constitutive calcium stress in the cell and subsequent induction of the calcineurin pathway, possibly by intracellular accumulation of Ca²⁺. Therefore we measured cytosolic Ca²⁺ concentration using indo-1 as a fluorescent reporter. Ca²⁺ levels were about double in the Δgrx6 mutant compared with wild-type cells, whereas the Δgrx7 mutant did not show abnormal accumulation (Figure 3A). The double Δgrx6Δgrx7 mutant behaves like the Δgrx6 mutant, confirming that the absence of Grx7 does not influence intracellular calcium homeostasis. Accumulation of Ca²⁺ in Grx6-minus cells was comparable to the accumulation in Δpmr1 cells lacking the ER/Golgi SPCA-type pump, and the absence of both Pmr1 and Grx6 has an additive effect in alteration of cytosolic Ca²⁺ (Figure 3A). As reported in earlier studies, in the absence of the P-type pump Spf1 alone, no alteration of Ca²⁺ levels occurs (Cronin et al., 2002), and elimination of Spf1 in a Δgrx6 mutant did not cause additive effects. Similarly, elimination of the Ca²⁺ exchanger Gdt1 in Δgrx6 cells had no additional effect on Ca²⁺ accumulation (Figure 3A). In summary, the absence of Grx6 causes accumulation of Ca²⁺ at the cytosol similar to (but independently of) the absence of the Pmr1 pump. To confirm that the activity of Grx6 is responsible for the foregoing phenotype, we determined Ca²⁺ levels in cells that expressed the Δgrx6 C136S active-site mutant instead of the wild-type protein. This mutant accumulated Ca²⁺ at similar levels as the Δgrx6-null mutant (Figure 3B), confirming that full activity of Grx6 is required for the homeostasis of this ion.

To discriminate between the cytosolic Ca²⁺ pool and that localized in other organelles, we also made determinations after disrupting membranes with DEAE-dextran, which therefore would correspond to total intracellular Ca²⁺ (see Materials and Methods). The DEAE-dextran treatment increased significantly the differences in Ca²⁺ levels...
Calcineurin pathway–dependent gene expression in Grx6- and Grx7-deficient strains. (A) Wild-type (W303-1A), Δgrx6 (MML842), Δgrx7 (MML887), and Δgrx6Δcrz1 (MML1723) cells were transformed with the lac2 reporter plasmid pAMS366, which contains functional (+) CDRE motifs, or with plasmid pAMS364, which contains nonfunctional (−) CDRE motifs. Cultures of transformants were exponentially grown in SC medium without or with added (0.2 M, 60 min) CaCl₂. β-Galactosidase activity was determined in three independent experiments. Bars indicate the mean ± SD, made relative to the unit value corresponding to pAMS366 transformed with type cells from SC medium without added CaCl₂. These conditions were used as reference for statistical analyses. (B) Quantification of the expression of the indicated genes from Northern blot analyses from exponential cultures in YPD medium of the same nontransformed strains indicated in A. Expression of each gene was normalized by the loading control (SNR19) and then compared with the respective expression in wild-type cells, which was given the unit value. Bars correspond to the mean of three independent experiments ± SD. (C) Effect of treatment with FK506 (2.5 μg/ml) of the following strains in YPD medium (20 h): wild type (W303-1A), Δgrx6 (MML890), Δgrx7 (MML887), and Δgrx6Δgrx7 (MML892). Bars correspond to the mean of at least five independent experiments ± SD, representing the growth yield ratio between treated and untreated cultures for each strain and then made relative to this ratio in wild-type cells. (D) As in C, with the wild-type strain or the Δgrx6 mutant nontransformed or transformed with the integrative plasmid Yiplac128 (vector), pMM1073 (GRX6), or pMM1071 (GRX6Δ, coding for the C1365 mutant form).

The absence of Grx6 causes altered sensitivity to depletion or excess of Ca²⁺

Other S. cerevisiae mutants defective in regulation of intracellular calcium homeostasis display alterations in the sensitivity to Ca²⁺ chelators (reviewed in Cunningham, 2011). Consequently, we tested the sensitivity of Δgrx6 cells to ethylene glycol tetracetic acid (EGTA), which chelates extracellular Ca²⁺ ions. In solid medium, the mutant was moderately less sensitive to EGTA than were wild-type cells (Figure 4A). We also quantified the effect of the chelator in liquid medium (Figure 4B), confirming that the Δgrx6 and the Δgrx6Δgrx7 mutants are less sensitive than wild-type cells in EGTA-treated cultures. Such growth differences were statistically significant and not observed in Δgrx7 cells. The lower sensitivity of the Δgrx6 mutant is abrogated by introduction of a Δch1 mutation. These results suggest that previous
FIGURE 3: Ca²⁺ levels are altered in Grx6-deficient strains. Analyses were done in exponential cultures in YPD medium.

(A) Relative cytosolic Ca²⁺ levels, determined by the indo-1 method, in wild type (W303-1A), Δgrx6 (MML890), Δgrx7 (MML887), Δgrx6Δgrx7 (MML892), Δpmr1 (MML1530), Δgrx6Δpmr1 (MML1535), Δspf1 (MML1716), Δgrx6Δspf1 (MML1703), Δgdt1 (MML1884), and Δgrx6Δgdt1 (MML1912). The 410/480-nm emission value for each strain (mean of three independent experiments ± SD) was made relative to the wild-type mean value. (B) As in A, with the wild-type strain or the Δgrx6 mutant nontransformed or transformed with the integrative plasmid YIpLac128 (vector), pMM1073 (GRX6), or pMM1071 (GRX6*). (C) Relative Ca²⁺ levels in cytosolic (black bars) or total intracellular (gray bars) fractions. Normalization as in A. For statistical analyses, values were compared with those of the respective compartment in wild-type cells. (D) Northern blot analysis of PMC1 mRNA in the indicated strains with SNR19 mRNA as loading control. Quantification of PMC1 mRNA levels in samples from three independent experiments (mean ± SD), once normalized by SNR19 mRNA levels and compared with expression in wild-type cells (unit value). (E) As in A, with the following strains: wild type, Δgrx6Δcch1 (MML1527), Δgrx6Δcch1 (MML1548), Δycf1 (MML1526), Δgrx6Δycf1 (MML1531), Δcsg2 (MML1529), and Δgrx6Δcsg2 (MML1538). (F) Relative ER Ca²⁺ levels, represented as luminescence units/s, before and after 1 mM Ca²⁺ reflux (time 0) in cells of the following strains carrying the STT3::3xHA::AEQ1::LEU2 cassette: wild type (MML1975), Δgrx6 (MML1977), Δpmr1 (MML1979), and Δgrx6Δpmr1 (MML1981). Represented values are the mean of at least three independent experiments, with three parallel kinetics per experiment. RLU/smax values for each strain (obtained upon final detergent permeabilization and 10 mM CaCl₂ treatment; see Materials and Methods) were the following: 12,320 (wild type), 11,970 (Δgrx6), 11,210 (Δpmr1), and 10,160 (Δgrx6Δpmr1).
overaccumulation of intracellular calcium protects the mutant cells against the calcium starvation effects caused by EGTA. The Grx6 C136S mutant has the same sensitivity phenotype to EGTA as the null Δgrx6 mutant (Figure 4C), again confirming that these effects are caused by the lack of thiol oxidoreductase activity in the mutated Grx6 molecule.

Whereas EGTA chelates extracellular calcium, BAPTA-AM is membrane permeable and is therefore able to chelate intracellular Ca\(^{2+}\) in yeast cells (Li et al., 2011). In contrast to EGTA, the Δgrx6 mutant was moderately (although significantly) more sensitive to BAPTA-AM than were wild-type cells (Figure 4D). The ER membrane Ca\(^{2+}\) transporter mutants Δpmr1 and Δspf1 also displayed increased sensitivity to BAPTA-AM, and this was especially manifested in the double Δpmr1Δspf1 mutant, consistent with the overlapping role of both transporters in regulating Ca\(^{2+}\) content in the ER lumen (Cronin et al., 2002).

Other yeast mutants in intracellular Ca\(^{2+}\) compartmentalization, in addition to being hypersensitive to chelators of the cation, also display high sensitivity to calcium excess. In accordance with the observed alterations in Ca\(^{2+}\) accumulation, the Δgrx6 mutation provoked moderate hypersensitivity to extracellular high Ca\(^{2+}\) concentration, in contrast to the Δgrx7 mutation (Figure 4E).

**The Δgrx6 mutant also overaccumulates intracellular phosphate**

Given the relationship between calcium and phosphate homeostasis in yeast cells, we measured intracellular orthophosphate levels in the Δgrx6 and Δgrx7 mutants. The Δgrx6 cells accumulate twice as much intracellular orthophosphate as wild-type and Δgrx6 mutant cells (Figure 5A). The abnormal accumulation of orthophosphate in Δgrx6 cells is totally rescued by deletion of the PHO89 gene, whereas introduction of the Δgrx6 mutation in cells lacking the other high-affinity phosphate transporter, Pho84, still causes higher orthophosphate levels compared with those in the single Δpho84 mutant (Figure 5A), pointing to Pho89 as the main agent responsible for the accumulation of orthophosphate in the absence of Grx6. We next determined where this excess of phosphate became accumulated in the Δgrx6 cells. Vacuoles were isolated from cytosolic and other membranous compartments by centrifugation in a Ficoll gradient, and a vacuolar fraction was isolated that was not detectably contaminated by cytosolic or mitochondrial proteins (Figure 5B). A fraction enriched in cytosolic proteins but contaminated by vacuolar proteins was considered as cytosolic fraction. Orthophosphate and polyphosphate levels were measured separately in both fractions, and the results showed that both forms of phosphate become accumulated in the cytosolic fraction of the Δgrx6 cells compared with the wild type, whereas the vacuolar fraction of the mutant cells exhibits reduced levels of both

**FIGURE 4**: Ca\(^{2+}\) chelating agents and Ca\(^{2+}\) excess affect growth of Grx6-deficient strains.

(A) Sensitivity of wild-type (W303-1A) and Δgrx6 (MML890) cells to EGTA. Serial dilutions (1:5) of the respective exponential cultures were spotted on YPD plates with the indicated concentrations of the agent. Growth was recorded after 2 d at 30°C. (B) Effect of treatment with EGTA (20 h) of the following strains in YPD liquid medium: wild type (W303-1A), Δgrx6 (MML890), Δgrx7 (MML887), Δgrx6Δgrx7 (MML892), and Δgrx6Δcch1 (MML1548). Bars correspond to the mean of at least five independent experiments ± SD, representing the growth yield ratio between treated and untreated cultures for each strain and then made relative to this ratio in wild-type cells. (C) As in B, with the wild-type strain or the Δgrx6 mutant nontransformed or transformed with the integrative plasmid Ylp1c128 (vector), pMM1073 (GRX6), or pMM1071 (GRX6*). (D) Effect of treatment with BAPTA-AM (20 h) of the following strains in YPD liquid medium: wild type (W303-1A), Δgrx6 (MML890), Δpmr1 (MML1530), Δspf1 (MML1716), and Δpmr1Δspf1 (MML1710). Bars correspond to the mean of at least five independent experiments ± SD, normalized as in B. (E) Sensitivity of wild-type, Δgrx6, Δgrx7, and Δgrx6Δgrx7 cells to the indicated concentrations of CaCl\(_2\). Growth on modified SD solid medium was recorded after 4 d at 30°C.
orthophosphate and polyphosphate (Figure 5C). Therefore compartmentalization of phosphate is altered in the absence of Grx6, resulting in a significant accumulation at the cytosol.

The absence of Grx6 counteracts defects in the ER protein folding machinery

The UPR is not constitutively induced in the Δgrx6 mutant (Izquierdo et al., 2008). However, we explored whether the lack of Grx6 could exacerbate the UPR in the presence of agents that by themselves interfere with the protein-folding machinery. To analyze it, we used ERO1 and PDI1 expression as reporter of the activation of the UPR (Yoshimoto et al., 2002). As expected, the protein-reductant DTT activated the UPR in wild-type cells in a transient way, but this activation was more intense in Δgrx6 cells (Figure 6A). This is in contrast with Δgrx7 cells, which displayed even a milder response than wild-type cells. The increased induction of the UPR upon interference with the protein-folding machinery in the absence of Grx6 could point to a relationship of the latter with this machinery.

The UPR target Ero1 is an essential ER oxidase required for appropriate protein folding (Frand and Kaiser, 1998; Pollard et al., 1998). To determine the possible genetic interactions between ERO1 and GRX6, we constructed a mutant in which the endogenous ERO1 promoter was substituted by the doxycycline-regulatable tetO2 promoter (Belli et al., 1998a), and we introduced the Δgrx6 mutation in that strain. As expected, the wild-type strain for Δgrx6 that expressed ERO1 under the tetO2 promoter did not grow after switching off this promoter. As occurs with the conditional temperature-sensitive ero1-1 mutation (Frand and Kaiser, 1998), the thiol oxidant diamide abolished the growth defects caused by down-regulation of ERO1 expression in the conditional tetO2–ER01 strain at 36°C (Figure 6B), although not at 30°C (unpublished data). The diamide ability to compensate for the absence of Ero1 activity seems therefore to be temperature dependent. Introduction of the Δgrx6 mutation rescued partially, although significantly, the lethality due to the absence of ERO1 expression (Figure 6B). These results therefore support a role for Grx6 in the redox conditions of the ER, which would become more oxidized in its absence. This in turn would compensate for the absence of the Ero1 oxidase.

We showed (Izquierdo et al., 2008) that lack of Grx6 and/or Grx7 in an otherwise wild-type genetic background does not affect the pool of mature carboxypeptidase Y (CPY), a vacuolar protein that is processed during secretion along ER/Golgi compartments and is used as a reporter for the functionality of the secretory machinery. In accordance with those results, in pulse and chase experiments, conversion of the precursor to the mature form of CPY followed the same kinetics in Δgrx6 and Δgrx7 cells as in wild-type cells (Figure 7A). Given the aforementioned interaction of Grx6 with Ero1 and the UPR, we analyzed CPY maturation kinetics in the Δgrx6 mutant under doxycycline-regulated conditional expression of ERO1. As expected from other work (Frand and Kaiser, 1998; Pollard et al., 1998), a significant delay in CPY maturation was observed when Ero1 function was compromised compared with the control conditions without doxycycline (Figure 7B). This was especially evident just after the pulse period (0 min), in which almost all newly synthesized CPY remained in the precursor form when Ero1 levels were depleted. However, introduction of the Δgrx6 mutation rescued the defects in CPY processing in these same Ero1-deficient conditions, as happens after diamide addition (Figure 7B). These results paralleled those on cell growth (Figure 6B) and supported the notion that the oxidant conditions created in the absence of Grx6 partially restore the previously compromised functionality of the protein folding/secretion machinery due to low Ero1 levels. To confirm this, we made real-time measurements of the redox state of the ER lumen in the foregoing strains and conditions, using a redox-sensitive eGFP form oriented to the ER lumen (Merksamer et al., 2008). Constitutive oxidizing conditions at the ER lumen and the subsequent reductive response upon DTT application were similar in GRX6 and Δgrx6 cells expressing Ero1, with slow recovery to basal conditions after application of the reductive stress (Figure 7C). In contrast, Ero1-deficient conditions constitutively created a moderate overoxidized state at the ER that was much exacerbated in cells lacking Grx6. In addition, the latter cells rapidly returned to the overoxidized conditions upon DTT treatment (Figure 7C). We can conclude that the absence of Grx6 causes over-oxidation of the pool of cysteine sulfhydryls at the ER lumen, as becomes clearly manifested in Ero1-deficient cells.

**Figure 5:** Grx6-deficient cells accumulate phosphate intracellularly. (A) Orthophosphate levels in cells (growing exponentially in YPD medium) of the following strains: wild type (W303-1A), Δgrx6 (MML890), Δgrx7 (MML887), Δgrx6Δgrx7 (MML892), Δpho84 (MML1304), Δgrx6Δpho84 (MML1337), Δpho89 (MML1306), and Δgrx6Δpho84 (MML1313). Values (mean of three independent experiments ± SD) are made relative to wild-type cells. (B) Purity of vacuolar and cytosolic cell fractions from wild-type cells growing exponentially in YPD medium, tested by Western blot with antibodies against CPY (vacuolar marker), Hxk1 (cytosolic marker), and porin (mitochondrial marker). (C) Orthophosphate and polyphosphate content in purified vacuolar and cytosolic fractions from wild-type and Δgrx6 cells growing exponentially in YPD medium. For statistical analyses, values in the mutant were compared with those of the respective compartment in wild-type cells.
Phenotypic alterations in Ca\textsuperscript{2+} homeostasis and protein secretion in \textit{Grx6}-minus cells can be rescued independently

The Ca\textsuperscript{2+} homeostasis defects in \textit{Δgrx6} cells are observed in conditions in which alterations in the protein secretion machinery are not apparent, at least for CPY (Figure 7A). To support this observation, we measured intracellular Ca\textsuperscript{2+} levels in Ero1-deficient cells with diamide, which display wild-type–like CPY processing. In these conditions, the \textit{Δgrx6} mutation still provoked increased accumulation of intracellular Ca\textsuperscript{2+} (Figure 8A), confirming that this accumulation occurs independently of the functionality of the ER protein-folding machinery. We also studied whether reducing cytosolic Ca\textsuperscript{2+} levels (by introducing a \textit{Δcch1} mutation) in \textit{Grx6}-minus Ero1-deficient cells interfered with the ability to process CPY. In fact, the \textit{tetO\textsubscript{2}-ER01Δgrx6Δcch1} mutant accumulated calcium levels similar to control \textit{Grx6} cells (Figure 8A), but CPY processing still occurred efficiently in doxycycline-treated cells in spite of the low Ero1 levels (Figure 8B). In summary, both phenotypes, respectively related to CPY processing and calcium accumulation in \textit{Δgrx6} cells, can be rescued independently of each other, indicating that one is not a direct consequence of the other. We confirmed that alterations in Ca\textsuperscript{2+} homeostasis are downstream of the general redox function of Grx6 at ER/Golgi by demonstrating that a \textit{Δpmr1} mutation does not rescue the growth defects of Ero1-deficient cells, in contrast to the \textit{Δgrx6} mutation (Supplemental Figure S2).

The previous results supported the participation of the redox activity of Grx6 in regulation of protein thiolsthe at the ER/Golgi lumen. In this context, accumulation of cytosolic Ca\textsuperscript{2+} could result from disrupting such a regulatory role of Grx6. If this were the case, reductive stress could compensate for the absence of Grx6 and return cytosolic Ca\textsuperscript{2+} to wild-type levels. Our results with DTT-treated cells (Figure 8C) confirm this hypothesis, allowing us to propose a thiol-regulatory role in Ca\textsuperscript{2+} homeostasis for Grx6.

**DISCUSSION**

Yeast Grx6 and Grx7 are the first non-PDI glutathione-dependent thiol oxidoreductases described as associated to the protein secretory machinery. On the basis of our transcriptome results with the respective mutants, we focused this study on the functional characterization of Grx6. One-cysteine GRXs like Grx6 or Grx7 lack the capacity to reduce protein disulfides. Their enzyme activity consists of deglutathionylating mixed disulfides between glutathione and protein thiols, releasing reduced glutathione (GSH; Lillig et al., 2008; Deponte, 2013). Protein deglutathionylation is a reversible mechanism for protecting protein thiols against irreversible oxidative modifications, in addition to having signaling functions (Gallogly and Mieyal, 2007). Such a protective role may be of great importance in the oxidant environment of the early secretory vesicles. In addition, the equilibrium between glutathionylated and deglutathionylated protein forms at the ER may be a mechanism to regulate the redox ratio between free GSH and oxidized glutathione. The latter may exist as dimers (GSSG) or form mixed disulfides with proteins. Because GSH is important for the activity of the Ero1/PDI machinery (Cuozzo and Kaiser, 1999; Chakravarthi et al., 2006; Sevier and Kaiser, 2008), ER/Golgi GRXs could act as general regulators of protein folding through their oxidoreductase activity on glutathione–protein mixed disulfides. In fact, the role of GRXs as facilitators of protein folding was proposed earlier (Berndt et al., 2008).

Using the ero-GFP reporter, we observed that a \textit{Δgrx6} mutant does not display altered redox homeostasis at the ER in the presence of Ero1 function. However, ERO1 expression down-regulation causes a moderate redox shift toward a higher oxidation state that is much more intense in the absence of Grx6. That is, the lack of this GRX results in a significant oxidation of the ER lumen over basal conditions, provided that Ero1 levels are under normality. In wild-type conditions for ERO1, the redox equilibrium due to balanced...
Ero1/Pdi1 activities (Kim et al., 2012) could compensate for the absence of Gn6 with no significant effect on the GSH/oxidized glutathione ratio. This would not occur in Ero1-depletion conditions. In this situation, the de-glutathionylating activity of Gn6 could restore, at least partially, the redox equilibrium by releasing GSH from the mixed disulfides. The overoxidation state created by the absence of Gn6 in Ero1-depletion conditions would be comparable to that created by the oxidant diamide in a thermosensitive ero1-1 mutant (Frand and Kaiser, 1998), with similar implications concerning rescue of Ero1 functional defects in relation to CPY secretion/maturation and general cell growth. These results support a role for the disulfide reductant activity of Gn6 in modulating the glutathionylation state of target proteins at the ER/Golgi lumen and consequent regulation of the glutathione redox balance. GRXs act as thiol reductants or oxidants, depending on the redox state of the enzyme environment (Lillig et al., 2008). The observations on Gn6-minus cells provide evidence that this GRX has a reductant activity in spite of the highly oxidant environment of the ER lumen.

Under normal Ero1 levels, secretion of the CPY reporter is not altered in a Δgrx6 mutant. However, the fact that some effects on the protein-folding machinery still occur in these conditions is supported by the more intense up-regulation of the UPR in the mutant upon treatment with protein-unfolding agents. Perhaps in Δgrx6 cells, partial depletion of GSH at the ER lumen due to the absence of the de-glutathionylating activity of Gn6 would result in intrinsic defects in the folding machinery, given the requirement of GSH for Ero1 activity (Cuozzo and Kaiser, 1999). These defects would become phenotypically manifested only upon treatment with external agents further compromising protein folding. Remarkably, such UPR up-regulation is not similarly observed in a Δgrx7 mutant, maybe due to a more secondary role of Gn7 in redox regulation of the vesicles lumen or each of the two strains, the values at each chase time in treated cultures (doxycycline and/or diamide) were compared with the respective values at the same chase time in untreated cultures. (C) Cultures of the indicated strains were grown in SC medium at 36°C with doxycycline (9 h, 2 μg/ml) or without the antibiotic. At time 0, DTT (2 mM) was added. The ratio of emission at 488 nm vs. 405 nm (made relative to the value in the samples of tetO2-ERO1 cells without doxycycline at time 0) is represented.
alternatively to the fact that Grx7 seems to have an exclusive location at Golgi, whereas UPR signaling occurs at the ER.

Although not contradicting a possible general regulatory role of ER/Golgi GRXs on the GSH/oxidized glutathione ratio at the vesicles lumen, Grx6 may have specific protein targets, as its absence results in specific phenotypes not shared with the \(\Delta grx7\) mutant. Thus we demonstrated that the \(\Delta grx6\) mutant (but not \(\Delta grx7\) cells) has higher than normal cytosolic \(Ca^{2+}\) levels, and that the mutant cells incorporate the cation from external sources. In contrast, the mutant is deficient in \(Ca^{2+}\) accumulation at the ER lumen. It is noteworthy that this depletion of \(Ca^{2+}\) at the ER lumen does not cause constitutive activation of the UPR, similar to what occurs in \(\Delta pmr1\) cells (Durr et al., 1998). The alteration in \(Ca^{2+}\) distribution occurs in \(\Delta grx6\) cells in conditions in which CPY maturation is apparently not affected, that is, in cells with normal Ero1/PDI functions, as well as in diamide-treated Ero1-depleted cells otherwise lacking Grx6 function. These results point to a specific redox function of Grx6 on some regulator of \(Ca^{2+}\) homeostasis inside the cell. We propose that this regulator would be a \(Ca^{2+}\) transporter whose activity would be modulated through the deglutathionylation activity of Grx6. In support of this hypothesis, treatment of \(\Delta grx6\) cells with the thiol reductant DTT reduces \(Ca^{2+}\) levels in the mutant to wild-type ones. The accompanying overaccumulation of phosphate inside \(\Delta grx6\) cells would be consequent of the activation of the calcineurin pathway in this mutant, one of its targets being PHS89, which encodes for one of the two high-affinity phosphate transporters at the yeast plasma membrane. In fact, our results indicate that PHS89 is the main agent responsible for the observed phosphate accumulation. Phosphate acts as a buffer in yeast cells for regulating the excess of free calcium and other cations (Dunn et al., 1994; Eide et al., 2005).

In mammalian cells, functional redox regulation has been demonstrated for several ER \(Ca^{2+}\) transporters, among them the SERCA pumps and the cardiac \(Ca^{2+}\) release channels (also known as ryanodine receptors; Zima and Blatter, 2006; Raturi et al., 2014). SERCA 2b activity is regulated by ERp57, a multifunctional ER thiol oxidoreductase member of the PDI family (Li and Camacho, 2004; Turano et al., 2011). About the redox-regulated target of Grx6, given its ER/Golgi location, attention should be initially focused on the \(Ca^{2+}\) transporters in these organelles: the Pmr1 and Spf1 pumps, the Gdt1 exchanger, and the Csg2 channel. Pmr1 is the main controller of intra-ER/Golgi \(Ca^{2+}\) necessary among other functions for mannosylation of secretory proteins (Cunningham, 2011). In case Grx6-mediated deglutathionylation of Pmr1 were required for activation of the pump, this would result in lack of Pmr1 function in \(\Delta grx6\) cells. However, this is not compatible with some of the observed phenotypes in the respective single and double mutants. Thus 1) \(\Delta pmr1\) cells accumulate \(Ca^{2+}\) preferentially at the vacuole (through activation of the calcineurin-dependent pathway and consequent up-regulation of PMC1), whereas in \(\Delta grx6\) cells, \(Ca^{2+}\) accumulation occurs at the cytosol; 2) \(\Delta pmr1\) cells display retarded CPY maturation/secretion (Vashist et al., 2002), in contrast to the \(\Delta grx6\) mutant; and 3) cytosolic accumulation of \(Ca^{2+}\) and depletion at the ER are additive between the \(\Delta pmr1\) and \(\Delta grx6\) mutations, definitively pointing to independent roles for both proteins. A scenario in which Pmr1 deglutathionylation by Grx6 would result in its inhibition would also be incompatible with the foregoing phenotypes.

The fact that the \(\Delta grx6\Delta spf1\) mutant accumulates intracellular \(Ca^{2+}\) to the same levels as the single \(\Delta grx6\) mutant and in contrast to the single \(\Delta spf1\) mutant (Cronin et al., 2002; this study) argues against Grx6 as a direct regulator of Spf1, either as activator (in which case, both single mutants would have similar phenotypes) or deactivator (which would result in similar phenotypes for \(\Delta spf1\) and \(\Delta grx6\Delta spf1\) cells). Similar arguments apply for Grx6 as potential regulator of the \(Ca^{2+}\) exchanger, Gdt1. In summary, not Pmr1, Spf1, or Gdt1 appears to be a direct redox target of Grx6, at least when separately considered. Another possible interpretation would result in...
from considering compensatory effects of the absence of some of them on the activity of the remaining transporters in order to maintain robust control of Ca\(^{2+}\) homeostasis or alternatively by considering the existence of an intermediate regulation level between Grx6 and Ca\(^{2+}\) entry at the ER. The primary role of Csg2 as a channel exporting Ca\(^{2+}\) from the ER to the cytosol is more controversial, as it has also been characterized as a Ca\(^{2+}\)-binding protein involved in the synthesis of sphingolipids (Zhao et al., 1994). The absence of Csg2 does not provoke intracellular Ca\(^{2+}\) accumulation (Tanida et al., 1996; this study) and does not suppress the cytosolic accumulation of the cation in Δgrx6 cells, eliminating a direct regulatory function of Grx6 on Csg2 as the explanation for the observed phenotype of the mutants. The possibility is open that other, still-unknown proteins involved in Ca\(^{2+}\) movement to or from the cytosol exist that could be the direct targets of Grx6 redox activity. We cannot discard Mid1 (one of the three components of the plasma membrane HACS complex) as a possible Grx6 target. In fact, Mid1 has been reported to localize also at the ER (Yoshimura et al., 2004), and it contains a cysteine-rich domain (similar to the α28 subunits of mammalian voltage-gated Ca\(^{2+}\) channels; Martin et al., 2011), which could be implicated in regulation of its activity. This hypothesis would be compatible with reversion of the Δgrx6 calcium-related phenotypes by abolishing HACS activity in a Δcc1 mutant.

GRXs of the Grx6/Grx7 subfamily are present only in fungi. In higher eukaryotes, their function might be carried by PDI family members, as experimentally demonstrated for the SERCA 2b pump. In fact, this family displays larger diversification of functionally specialized members in mammals than in yeast and other fungi (Oka and Bulleid, 2013). In any case, modulation of intracellular Ca\(^{2+}\) homeostasis through thiol redox regulation of transporters may be evolutionarily conserved in eukaryotes.

**MATERIALS AND METHODS**

**Strains and plasmids**

Strains used in this study derive from *S. cerevisiae* wild-type W303 and are listed in Supplemental Table S2.

Plasmids pAMS366 and pAMS364 drive expression of the *lacZ* reporter gene respectively from four wild-type copies in tandem of *CDRE* or from mutated *CDRE* forms unable to bind Crz1 (Stathopoulos and Cyert, 1997). Plasmid pJS79 carries a *STT3:3×HA::AEG1::LEU2* cassette that can be mobilized into the chromosomal *STT3* locus as a *Spel–Spel* fragment (Strayle et al., 1999). It allows expression of a 3HA-aequorin protein with the Ca\(^{2+}\)-sensitive site targeted to the ER lumen. Plasmid pMM1071 derives from pMM822 (Izquierdo et al., 2008) by mutagenizing the active-site Cys-136 codon of the GRX6 open reading frame (*ORF*) to a Ser codon using the ExSite method (Weiner and Costa, 1995), followed by subcloning of a BamHI–PstI fragment containing the mutated *GRX6* ORF plus promoter and downstream regions into the polylinker of the LEU2 vector Yplac128 (Gietz and Sugino, 1988). Mutation was confirmed by DNA sequencing. In parallel, pMM1073 was obtained by direct subcloning of the BamHI–PstI fragment of pMM822 containing wild-type *GRX6* plus adjacent sequences into Yplac128. Plasmids were integrated at the LEU2 locus of the transformed strain after linearization by EcoRV digestion.

**Growth media and culture conditions**

YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic SC medium (Sherman, 2002) was usually used for *S. cerevisiae* cell growth at a temperature of 30°C unless otherwise indicated. Media were solidified with 2% agar. Plate assays of growth sensitivity were done by spotting serial 1:10 dilutions of exponential cultures onto medium plates containing the corresponding agent and recording growth after 2 or 3 d of incubation at 30°C. For sensitivity to CaCl\(_2\) stress, a modified synthetic SD medium was used (Demaegd et al., 2013).

Growth in liquid medium of several strains under parallel separate treatments (in 0.5-ml cultures initially inoculated with 2 × 10\(^5\) cells) was automatically recorded (OD\(_{600\text{nm}}\)) at 1-h intervals, using shaken microtiter plates sealed with oxygen-permeable plastic sheets, in a PowerWave XS (BioTek, Winooski, VT) apparatus at controlled temperature. Treatments were applied to cells that had been growing previously during at least 10 generations in exponential conditions. For the tested strains, the ratio of the automatically recorded optical density values (growth yield) between treated and untreated cultures after different time periods was calculated and then made relative to the same ratio in the wild-type strain (unit value).

**Genetic methods**

Standard protocols were used for DNA manipulations and transformation of yeast cells. Single-null mutants were generated using the short-flanking homology approach after PCR amplification of the natMX4 or kanMX4 cassettes and selection for resistance to nourseothricin (Goldstein and McCusker, 1999) or Geneticin (Wach et al., 1994), respectively. Disruptions were confirmed by PCR analysis. The endogenous *ERO1* promoter was substituted by the tetO\(_2\) promoter using the kanX4-based cassette from plasmid pCM224 as described by Bellì et al. (1998a). Multiple mutants were obtained by crossing the parental mutant strains, followed by diploid sporulation, tetrad analysis, and selection of the mutant combinations.

**Microarray analyses**

RNA purification, microarray characteristics, and other experimental steps, as well as data analyses, have been described previously (Ferrezuelo et al., 2009).

**Isolation of vacuolar fraction**

Isolation of vacuoles was based on the method described by Li et al. (2001), with modifications. About 10\(^10\) cells from an exponentially growing culture were centrifuged, resuspended in 30 ml of 0.1 M Tris-H\(_2\)SO\(_4\) buffer, pH 9.3, with 10 mM DTT, and incubated at 30°C for 30 min. After two washings with 20 mM potassium phosphate buffer, pH 7.4, with 1.2 M sorbitol, cells were resuspended in 4 ml of this buffer containing Zymolyase 20T (Seikagaku Corp., Tokyo, Japan; at 3 mg/g of cells). Spheroplast formation was followed microscopically, and, once formed, spheroplasts were recovered by low-speed centrifugation (1800 × g, 5 min) and resuspended in 3.5 ml of a solution of 15% Ficoll (Sigma, St. Louis, MO) in 10 mM 1,4-piperazinediethanesulfonic acid (PIPES)–KOH, pH 6.8, plus 0.2 M sorbitol. After addition of DEAE-dextran (50 μg/ml, final concentration), the spheroplast lysate was kept for 2 min at 0°C and then for 5 min at 30°C with mild shaking. Magnesium chloride was added at a final concentration of 1.5 mM; the lysate was transferred to a centrifuge tube and then overlaid with 3 ml of the PIPES/sorbitol buffer with 8% Ficoll, 4 ml of buffer with 4% Ficoll, and 1 ml of buffer alone. Samples were centrifuged at 110,000 × g for 90 min with a Beckman SW55Ti rotor. Fractions of 0.4 ml were taken, an aliquot of each was separated for acetone precipitation of proteins, followed by Western analysis of compartment reporters (CPY for vacuole, porin for mitochondria, and hexokinase 1 for cytosol), and the remainder of each fraction was kept for phosphate determination. Vacular fraction was collected at the 0/4% Ficoll interphase.
**Determination of intracellular ion levels**

Cytosolic Ca\(^{2+}\) concentration was determined using the pentapotassium salt of indo-1 as described in Halachmi and Elam (1989), after determining the fluorescence ratio at 410/480 nm according to Grynkiewicz et al. (1985). In this method, indo-1 (Sigma) is loaded in the cytosolic compartment by incubating the yeast cells at an acidic pH. To determine intracellular total Ca\(^{2+}\) levels (including membra nous vesicles stores), cellular membranes were permeabilized with DEAE-dextran (2 mg/ml) as described in Halachmi and Elam (1989).

Free Ca\(^{2+}\) levels at the ER lumen were determined using a spe cifically ER targeted version of aequorin, as described by Strayle et al. (1999). The protocol reported in the original work was essen tially used, except that light emission was measured in a liquid sus pension of cells and colenterazine n (Anaspec, Fremont, CA) was used as prosthetic group. Light recording was done before and after refilling with 1 mM CaCl\(_2\). Subsequent cell lysies with 0.1% digiton in plus addition of 10 mM CaCl\(_2\) allowed confirmation that previous measurements had been done in nonlimiting conditions for aequorin (Strayle et al., 1999).

Orthophosphate was measured by molybdate reactivity (Reddi et al., 2009). For determination of total intracellular phosphate, sam ples were treated with 1 N sulfuric acid for 10 min at 100°C before analysis. Polyphosphate levels were calculated as the difference be tween total P\(_i\) and orthophosphate levels.

**Northern blot analyses**

RNA isolation and electrophoresis, probe labeling with digoxigenin, hybridization, and signal detection were done as described previ ously (Belli et al., 1998a). Gene probes were generated by PCR from genomic DNA, using oligonucleotides designed to amplify internal open reading frame sequences. SNRT19 mRNA was used as loading control. Signals were quantified using the ChemiDoc Imaging System (Bio-Rad, Hercules, CA) software. Background values were determined for a region lacking visible signal of the same size as the measured band and adjacent to it, and such background was subtracted for the respective band signal value.

**Pulse-chase labeling and immunoprecipitation of CPY**

Samples (~6 × 10\(^8\) cells) from cultures in SC medium were resus pended in 10 ml of the same medium lacking methionine and cysteine and incubated for 30 min at the appropriate temperature before adding 500 μCi of a [\(^{35}\)S]methionine/[\(^{35}\)S]cysteine cocktail (Perkin Elmer, Waltham, MA). After a 5- or 6-min pulse, cold methi onine and cysteine were added at 2 mM final concentration each. At successive 7-min intervals, samples of 1.5 ml were taken, and 20 mM (final concentration) sodium azide was added. Cells were pelleted, resuspended in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS, 6 M urea, and 1× protease inhibitor complete [Roche, Basel, Switzerland]) and lysed with glass beads by shaking and treatment at 100°C. The supernatant was carefully taken, and IP buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5%Tween-20, and 1× protease inhibitor complete) was added to a 1-ml final volume. The suspension was mixed with 50 μl of sheep anti-mouse immunoglobin Dynabeads (Invitrogen, Carlsbad, CA) coupled to 3 μg of mouse monoclonal anti-CPY anti bodies (Molecular Probes, Carlsbad, CA). After mixing for 2 h at 4°C, the supernatant was eliminated and the beads were washed three times with 350 μl of IP buffer and then eluted with 20 μl of 1× Laemmli buffer, boiled, and separated by SDS–PAGE. Gels were au toradiographed, and x-ray film signals were quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

**Measurement of ER redox state**

Real-time ER redox measurements were done as described in Merksamer et al. (2008) in cultures of transformants with plasmid pPM28, which expresses a redox-sensitive erO-GFP form.

**Western blot analyses**

Western blot analyses were done as described in Belli et al. (1998b). The following primary antibodies were used: mouse monoclonal anti-CPY (1:500), rabbit polyclonal anti-Hxk1 (1:5000; USBiological, Salem, MA), or mouse monoclonal anti-porin (1:1000; Molecular Probes).

**Determination of β-galactosidase activity**

Enzyme activity (Miller units) was determined in permeabilized cells as described in Ausubel et al. (1987).

**Statistical analyses**

The Mann–Whitney U test was used, using the software JMP 10. Unless otherwise indicated, values in the mutant strains were com pared with those of wild-type cells. **p < 0.05, 0.01, 0.001,** respectively.

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