The monothiolic glutaredoxin Grx5 of *S. cerevisiae* interacts with Isa 1 and its cellular localisation is different to Grx3 and Grx4

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Abstract

Grx5 is the most important monothiolic glutaredoxin that protects against oxidative stress in *Saccharomyces cerevisiae*. It is located in the mitochondrial matrix, being involved in the biosynthesis of Fe/S clusters that takes place in mitochondria. Thus, the *grx5* mutant shows phenotypes similar to other mutants in components of the biosynthetic machinery of Fe/S clusters. Grx3 and Grx4 also are monothiolic glutaredoxins, although they seem to develop different functions in the cell relative to Grx5. Here we demonstrate (using protein-GFP fusions) that both Grx3 and Grx4 are located at the nucleus. We have also used the two-hybrid approach to show that Grx5 physically interacts with Isa1, a component of the Fe/S cluster biosynthetic machinery. Using the same approach, we observed that Grx4 displays interactions with a number of proteins, whose significance remains unknown.

Introduction

Glutaredoxins are oxidoreductases that protect cells against oxidative stress (Grant, 2001) and are required for the activity of specific enzimes (Holmgren, 1990). They contain active cysteine residues in highly conserved regions. Glutaredoxins have been found in bacteria, yeasts and higher eukaryotes (Holmgren, 1990).

Two families of glutaredoxins has been identified in *Saccharomyces cerevisiae*. Dithiolic glutaredoxins, Grx1 and Grx2, have two cysteine residues at their active sites (Cys-Pro-Tyr-Cys) and play different roles in protecting cells against oxidants such as hydrogen peroxide and menadione (Luikenhuis *et al*., 1998). Monothiolic glutaredoxins, Grx3, Grx4 and Grx5, contain only one cysteine at the putative active site (Pro-Lys-Cys-Gly) (Rodríguez-Manzaneque *et al*., 1999). While the absence of Grx3 or Grx4 does not cause dramatic effect on sensitivity to oxidants, the lack of Grx5 results in high sensitivity to hydrogen peroxide and menadione, increased protein damage, growth defects in minimal medium and inability for respiratory growth (Rodríguez-Manzaneque *et al*., 1999). Previously, it has been demonstrated that Grx5 has a mitochondrial target at its N-terminus that localises the protein in the mitochondrial matrix (Rodríguez-Manzaneque *et al*., 2002). Moreover, the biological function of Grx5 depends on this localisation.

In yeast cells, the biosynthesis of Fe/S clusters takes place in the mitochondria (Lill and Kispall, 2000). Fe/S-containing proteins may be located in mitochondria (aconitase and subunits of respiratory complexes I, II and III), in cytosol (glutamate sintase and isopropil malate sintase) and in nucleus (endonuclease Ntg2 in *S. cerevisiae*) (Lill *et al*., 1999). Fe/S clusters are specially sensitive to oxidants and liberate free iron that enhances the production of reactive oxygen species (ROS) via the Fenton reaction (Imlay *et al*., 1988). ROS damage cellular components such as DNA, lipids and proteins (Wolf *et al*., 1986), and cells have developed different systems to eliminate ROS, such as glutaredoxins (Grant, 2001).

The biosynthesis of Fe/S clusters in *S. cerevisiae* involves different proteins called iron-sulfer cluster assembly complex (ISC) (Fig. 1, see Mühlenhoff and Lill, 2000, for a review). The components of the machinery show a significant homology with bacteria and are highly conserved in eucariota (Mühlenhoff and Lill, 2000). Mutants in some components of the ISC machinery accumulate iron in mitochondria

and have an enzimatic deficience in Fe/S proteins. The absence of Grx5 causes similar effects in the cell, showing that Grx5 could play a role in the biosynthesis/assembly of Fe/S clusters (Rodríguez-Manzaneque *et al*., 2002). Moreover, the overexpression of *SSQ1* or *ISA2* suppresses some of the *grx5* phenotypes. The products of these genes are components of the ISC machinery. Ssq1 is a Hsp70-type chaperone that could protect proteins from oxidation (Mühlenhoff and Lill, 2000). Isa2 could bind iron and transfer it to the Fe/S cluster (Lill and Kispal, 2000).

PSORT analysis predicts no mitochondrial localation for Grx3 or Grx4 (Rodríguez-Manzaneque et al., 2002) and the respective mutants in *GRX3* or *GRX4* do not show defective phenotypes such as the *grx5* mutant. Therefore, the Grx3 and Grx4 monothiolic glutaredoxins could develope different functions in the cell relative to Grx5.

Here we demonstrate that Grx5 is directly involved in protein-protein interactions with other components of the machinery for the biosynthesis of Fe/S clusters. Moreover, we demonstrate interactions between Grx4 and some proteins that could help us to understand the function of this glutaredoxin. Finally, we localise monothiolic glutaredoxins by GFP targeting.

Materials and Methods

Two-hybrid assays

GRX5 and the control bait genes were cloned into a vector carrying the Gal4 DNA-binding domain (pGBT9, with *TRP1* as a gene marker), by gap-repair. The prey genes (proteins involved in the biosynthesis of Fe/S clusters) were cloned into a vector containing the Gal4 activation domain (pACT2, with *LEU2* as a gen marker), by usual restriction procedures.

pGBT9 constructions were used to transform into the *MAT* α type *S. cerevisiae* strain, PJ69-4α, and pACT2 constructions into the *MAT* **a** type strain, PJ69-4a. Both strains are identical but with opposite mating types. The genotype is: *MAT* **a** or α *trp1-109 leu2-3, 112 ura3-52 his3-200 gal4*Δ*gal80*Δ *GAL2:ADE2 LYS2::GAL1:HIS3 met2::GAL7:lacZ*. They contain three reporter genes, *ADE2*, *HIS3* and *lacZ*, that are expressed by an active Gal4 protein. We selected for activation of *ADE2* and *HIS3* by growth of white colonies on SD plates supplemented with uracil (40mg/l), adenine (2mg/l) and 3-aminotriazole (3mM).

To reveal putative interactions between Grx4 and other proteins, *GRX4* was cloned into pGBT9 as it is explained for Grx5, and the construction was also transformed into PJ69-4α. To find preys for Grx4, we used a library containing genomic DNA from the *S. cerevisiae* strain UM41705, which lacks the *GAL4* gene. The DNA had been fractionated through sonication to a size range between 0.3-3Kb. The fragments were cloned into the pACT2 vector via *XhoI* restriction site. The library DNA was transformed into PJ69-4a. Identification of positive interactions was made by activation of *ADE2* and *HIS3* as before. To identify the proteins interacting with Grx4, PCR analysis was performed directly on the growing colonies to amplify the DNA in the library plasmid. Through sequence analysis of the PCR products and search of yeast genome sequence databases, we determined whose proteins were encoded by the respective plasmid inserts.

β-galactosidase assay in liquid cultures

We quantified the two hybrid interactions of two-hybrid positive clones by the activation of the *lacZ* gene, using the β-galactosidase assay protocol described in Ausubel *et al*. (1989).

GFP fusions

We amplified *GRX3*, *GRX4* and *GRX5* by a PCR reaction, and the PCR products were cloned into the pRS426 vector by gap-repair. This vector codes for the Green Fluorescent Protein (GFP) and constructions were made to obtain frame fusions (at the N-terminus) with the desired proteins. Recombinant plasmids were transformed into PJ69-4a cells. Transformants were grown in SD medium (with adequate supplements) at 30ºC, and cells were visualised in the fluorescence microscope Nikon Eclipse E600 with the 60x1.4 objective. Images were taken with a LSR AstroCam System and analized and integrated with the Pixel Software.

Results and discussion

Grx5 physically interacts with Isa1

We used Grx5 as a bait in a two hybrid assay. The proteins used as a prey are indicated in red in Figure 1. As a positive control, we used the interaction between Snf1 and Snf4, that are proteins involved in glucose metabolism. Empty vectors were used as negative controls. Among all the mitochondrial proteins involved in the synthesis of Fe/S clusters in yeast, only Isa1 showed interaction with Grx5 using the genetic assay (Figure 2). Interestingly, such interaction was not observed with Isa2. To quantify interactions, β-galactosidase assays were performed (Figure 3). We observed that β-galactosidase levels obtained through the Isa1/Grx5 interactions are as high as through the well-documented interaction between Snf1 and Snf4. Enzyme assays confirmed the absence of interaction between Isa2 and Grx5.

Isa1 and its homologous Isa2 protein display sequence similarity to bacterial IscA and contain three cysteine residues that are conserved in IscA-type proteins in bacteria, yeast, plants and mammals (Jensen and Culotta, 2000; Kaut *et al*., 2000). The three cysteines are essential for the activity of Isa1/2 and could be an iron binding motiv. Simple mutants of *ISA* genes are viable but accumulate iron in mitochondria (Jensen and Culotta, 2000) and are necessary for normal activity of mitochondrial and cytosolic Fe/S proteins (Jensen and Culotta, 2000; Kaut *et al*., 2000; Pelzer *et al*., 2000). It has been proposed that Isa1 and Isa2 could form heterodimers and develop similar function in the cell because the single mutants show similar phenotypes (Mühlenhoff and Lill, 2000). However, there are evidences that they do not exactly play the same roles in the biosynthesis of Fe/S clusters. Thus, overexpression of *ISA2*, but not *ISA1*, partially supresses some phenotypic defects in a *grx5* mutant (Rodríguez-Manzaneque *et al*., 2002). Here, we have shown that Isa1, but not Isa2, directly interacts with Grx5.

Two-hybrid assay with Grx4

Grx4 was used as a bait to characterise interacting proteins from a library containing genomic DNA. The proteins that were obtained in the screening are shown in Table 1.

NTH1 encodes for the neutral trehalase that catalyses the hydrolysis of trehalose (Van Dijck *et al*., 1995). In yeast, trehalose is accumulated under adverse conditions as a compatible solute that protects proteins and membranes against damage during a

variety of stress conditions (Nwaka and Holzer, 1998). The expression of *NTH1* is induced by different stress conditions, such as heat shock, oxidative stress and toxic chemicals, and also under starvation conditions (Zähringer *et al*., 1998). The function of Nth1 during stress recovery is not well understood. Under the stress conditions, for instance heat stress, trehalose may function as a chemical chaperone, binding to proteins to prevent their denaturation. However, during recovery from heat stress, trehalose apparently must be hydrolysed rapidly by the trehalase, liberating the cellular structures from bound trehalose (Singer and Lindquist, 1998). This would make them again accessible to chaperone proteins, enabling renaturation. A second function of trehalose could be to provide energy for the renaturation of cellular structures (Nwaka and Holzer, 1998). This indicates that both synthesis and hydrolysis of trehalose have to be well regulated in response to a stress conditions. The promoter of *NTH1* contains three STREs (stress responsive elements) and the stress induced expression of *NTH1* proceeds via the Msn2/Msn4/STRE pathway (Zähringer *et al*., 2000).

Six genes encoding Msh1-6 have been identified in *S. cerevisiae* that are involved in mismatch repair (Reenan and Kolodner, 1992; New *et al*., 1993; Marsischky *et al*., 1996). *MSH1* is thought to function in the supression of mutations in mitochondrial DNA (Reenan and Kolodner, 1992). *MSH2/3/6* are thought to function in mismatch repair in the nucleus (Reenan and Kolodner, 1992; Marsischky *et al*., 1996). *MSH4/5* do not appear to be required for mismatch repair but rather are required for efficient crossing over during meiotic recombination (Winand *et al*., 1998). *MSH2* was demonstrated to interact with both *MSH3* and *MSH6* (Marsischky *et al*., 1996) and mismatch repair was proposed to involve two different heterodimeric complexes, *MSH2-MSH3* and *MSH2-MSH6*. Both complexes has been demonstrated to bind to misrepaired bases in DNA (Macpherson *et al*., 1998).

Prp42 is a protein that shares 50% sequence similarity with Prp39 U1 snRNP protein (McLean and Rymond, 1998). Both proteins are not functionally redundant, because each is needed for cellular pre-mRNA splicing. Prp39 and Prp42 are required to assemble a stable U1 snRNP capable of productive interaction with cellular pre-mRNA. *PRP42* is an essential gene.

Cells can modulate the expression of nuclear genes in response to alterations in mitochondrial function, a response termed retrograde regulation (Liao and Butow, 1993). In yeast, one member of this response is *CIT2*, which encodes a peroxisomal isoform of citrate synthase that catalyzes the first step in the glyoxylate cycle and

generates intermediates that can enter the mitochondrial tricarboxylic acid (TCA) cycle. Thus, activation of the *CIT2* retrograde response allows for a more efficient use of carbon for biosynthetic processes. Levels of *CIT2* expression are dependent on three genes, *RTG1*, *RTG2* and *RTG3*. *RTG1* and *RTG3* encode basic helix-loop-leucine zipper (bHLH-Zip) transcription factors (Liao and Butow, 1993; Jia *et al*., 1997). Rtg1 and Rtg3 activate transcription by binding as a heterodimer to a site called an R box (GTCAC), two of which are located in the *CIT2* promoter (Jia *et al*., 1997). Only Rtg3 has been found to contain transcriptional activation domains (Rothermel *et al*., 1997). The *RTG* genes are also involved in the retrograde control of expression of the TCA cycle genes *CIT1*, *ACO1*, *IDH1* and *IDH2* (Liu and Butow, 1999). In normal mitochondrial function (low expression of *CIT2*), Rtg1 and Rtg3 exist as a complex in the cytoplasm. In cells with disfunctional mitochondria (*CIT2* expression is elevated) these transcription factors accumulate in the nucleus (Sekito *et al*., 2000).

S. cerevisiae has two tropomyosin isoforms, *TPM1* and *TPM2* (Drees *et al*., 1995). Tropomyosin stabilizes actin filaments against fragmentation. *TPM1* is the predominant isoform. Both Tpm1 and Tpm2 modulate myosin binding to actin in the presence and absence of ATP and each isoform has distinct effects (Strand *et al*., 2001).

Our observations suggest that Grx4 could modulate the function of some proteins through direct interaction with them and regulation of the redox state of some key sulphydryl groups. Further work, using additional approaches, is needed to confirm the observed interactions.

GFP targeting of monothiolic glutaredoxins

To confirm the diferential location of the three monothiol glutaredoxins in yeast, *GRX3*, *GRX4* and *GRX5* were cloned separately into a vector that contains the GFP gene. Pictures of the respective GFP-Grx tranformants were taken with a fluorescence microscope (Figure 4). Grx4 is largely located in the nucleus, while Grx5 is exclussively located in mitochondria. Grx3 showed the same fluorescence pattern as Grx4 (not shown), indicating that it is also nuclear. The presence of glutaredoxins in the nucleus the is not rare. Human glutaredoxin Grx2 has been identified predominantly in the nucleus but also in mitochondria (Lundberg et al., 2001). The nuclear location of Grx3 and Grx4 adds further significance to the physical interactions between Grx4 and nuclear proteins (Table 1).

Acknowledgments

We thank collegues in Lundberg Laboratory for their technical assistance and collegues in Departament de Ciències Mèdiques Bàsiques for their comments on the manuscript.

This work was made in the Department of Cell and Mollecualr Biology (Lundberg Laboratory) in Göteborg (Sweden) and was supported by a Marie Curie fellow-ship to M.T.R-M.

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Legends to the figures

Fig1. Hypothetical model for Fe/S cluster assembly in mitochondria. In red are shown proteins used as a preys in the two-hybrid assay.

Fig2. Isa1 interacts with Grx5 in a two-hybrid assay. Left panel corresponds to non-selective medium, right panel to selective medium. AD vector is pACT2; BD vector is pGBT9.

Fig3. β-galactosidase assays show an interaction between Isa1 and Grx5 similar to the positive control Snf1-Snf4. Numbers above bars indicate β-galactosidase (Miller) units.

Fig4. Cellular location of Grx5 and Grx4, using GFP fusions.

Figure 1

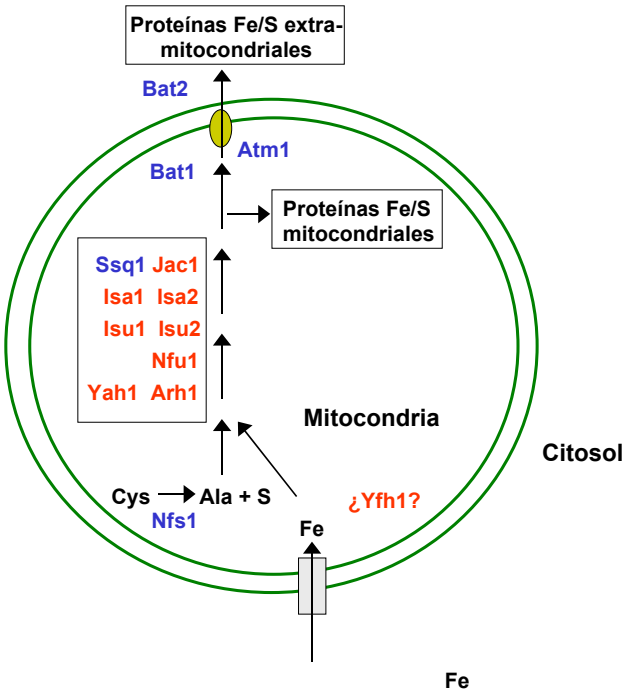


Figure 2

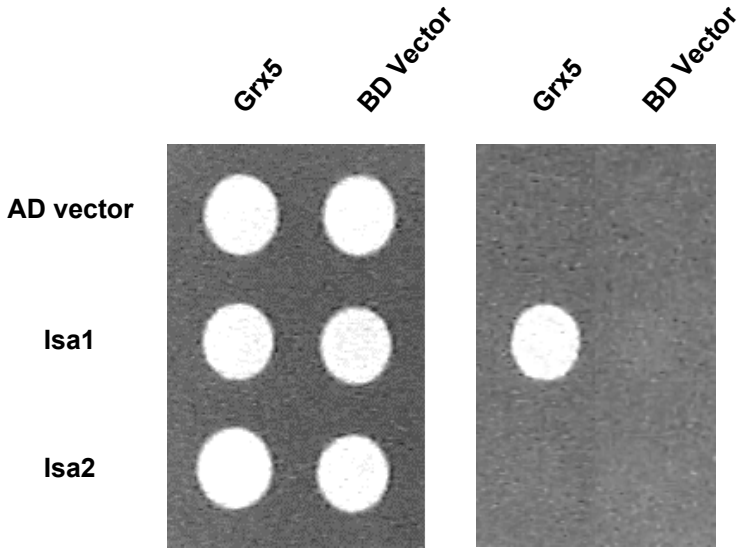


Figure 3

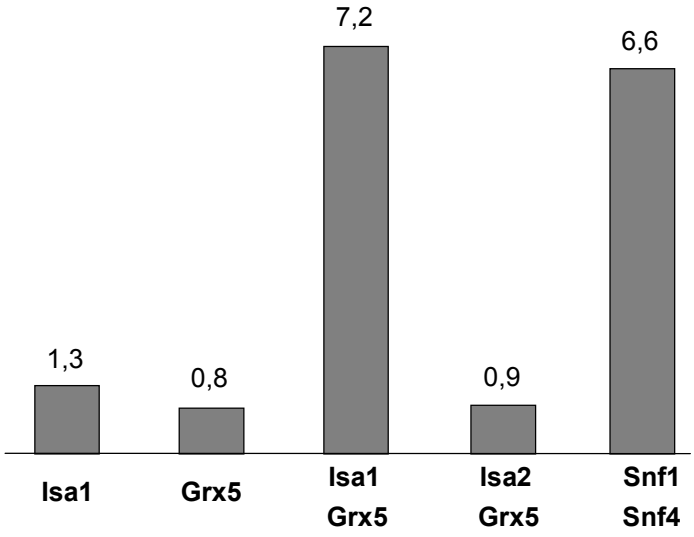
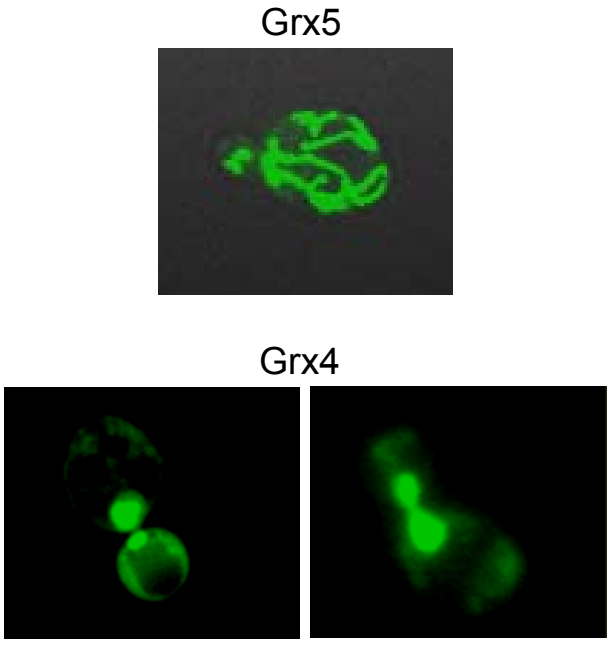


Figure 4



Gene	Chromosome	Gene product (location)	Function
NTH1	IV	Neutral trehalase (cytosol)	Trehalose hydrolisis
MSH6	IV	Human GTBP homolog (nucleus)	Mismatch repair in mitosis and meiosis
PRP42	IV	U1 sn RNP protein (nucleus)	Pre-mRNA splicing
RTG3	II	BHLH/Zip transcription factor (nucleus)	*CIT2* expression regulation
TPM1	XIV	Tropomyosin I (actin cytoskeleton)	Actin filament organization

Table 1. Gene products that interact with Grx4 in a two-hybrid screening.

Yeast
Yeast 2002; **19**: 1261–1276.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/yea.916

Yeast Functional Analysis Report

Functional analysis of yeast gene families involved in metabolism of vitamins B_1 and B_6

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Received: 7 May 2002
Accepted: 30 July 2002

Abstract

In order to clarify their physiological functions, we have undertaken a characterization of the three-membered gene families *SNZ1–3* and *SNO1–3*. In media lacking vitamin B_6, *SNZ1* and *SNO1* were both required for growth in certain conditions, but neither *SNZ2, SNZ3, SNO2* nor *SNO3* were required. Copies 2 and 3 of the gene products have, in spite of their extremely close sequence similarity, slightly different functions in the cell. We have also found that copies 2 and 3 are activated by the lack of thiamine and that the Snz proteins physically interact with the thiamine biosynthesis Thi5 protein family. Whereas copy 1 is required for conditions in which B_6 is essential for growth, copies 2 and 3 seem more related with B_1 biosynthesis during the exponential phase. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: ***Saccharomyces cerevisiae*; thiamine; pyridoxal; functional analysis; *SNZ*; *SNO***

Introduction

Paralogous gene families, which cover the three domains of life (Galperin, 2001; Goffeau *et al*., 1996; Henikoff *et al*., 1997; Rubin *et al*., 2000) represent an important part of all genomes sequenced so far. They comprise ca. 40% of the yeast genome (Blandin *et al*., 2000). This gene redundancy can be associated with an exact functional redundancy (Brookfield, 1997). Examples are the rDNA genes, the histone genes in species having a rapid early embryonic development, such as the sea urchins (Tartof, 1975) and the *CUP1* gene from *Saccharomyces cerevisiae*. However, in many cases, paralogous genes have undergone functional specializations and are only partially redundant or even functionally non-redundant. The *HSP70* paralogous gene family of *S. cerevisiae* is an interesting example (Boorstein *et al*., 1994). On the one hand, some of its members have overlapping functions, such as *SSA1, SSA2* and *SSA3*. None of them is essential, but the triple deletion mutant exhibits a synthetic phenotype and is not viable. On the other hand, other members have acquired essential functions, such as *KAR2* and *SSC1*, although they encode proteins very similar to the Ssa proteins. To obtain an exhaustive overview of these relationships in *S. cerevisiae*, we and others have undertaken a systematic functional characterization of 35 gene families containing two to four members with uncharacterized or poorly characterized functions (Dujon *et al*., in preparation).

Here we report a study of the *SNZ* and *SNO* gene families of *S. cerevisiae*, each consisting of three

members (called 1, 2 and 3), located adjacently in chromosomes XIII, XIV and VI, respectively (Figure 1). *SNO2* and *SNO3* nucleotide sequences are almost identical (99%), as well as *SNZ2* and *SNZ3* sequences. *SNO1* and *SNZ1* sequences are more divergent from their respective counterparts (around 81% identical). Copies 2 and 3 are located within large subtelomeric duplicated regions that encompass other genes, including two members of a family of thiamine (vitamin B_1) putative biosynthetic enzymes: *THI5* and *THI12*. Homologues of *SNZ* and *SNO* genes have been found in a wide range of microorganisms and plants (Galperin, 2001; Mittenhuber, 2001), thereby making their functional analysis of general interest. The *SNZ* genes in yeast were originally discovered as expressed in stationary phase (Braun *et al*., 1996), and the *SNO* genes were found as proximal and coordinately regulated with the *SNZ* genes (Padilla *et al*., 1998). Three different studies have revealed that homologues of the *SNZ* and *SNO* genes from *Aspergillus nidulans* (Osmani *et al*., 1999), *Neurospora crassa* (Bean *et al*., 2001) and *Cercospora nicotiniae* (Ehrenshaft *et al*., 1999; Ehrenshaft and Daub, 2001) were related to the biosynthesis of pyridoxal (vitamin B_6).

In this study we demonstrate that *SNO1* and *SNZ1* are required for growth of yeast in the presence of low level of intracellular vitamin B_6. We also show that transcripts of *SNO2, SNO3, SNZ2* and *SNZ3* are accumulated in the absence of external thiamine, as well as *THI5* and *THI11* transcripts, and that Snz proteins can interact with Thi5 and Thi12 proteins.

Materials and Methods

Yeast strains

The yeast strains used or constructed in this work are listed in Table 1 BY4741, BY4742, CML235 and CML236 are the wild-type strains from which the *snz* and *sno* mutants were obtained. All strains, except for W303-1A, are from the S288c genetic background.

Generation of multiple mutants

Single null mutations in the genes were generated by disruption with the cassettes described by Wach *et al*. (1994) or Brachman *et al*. (1998). Multiple mutants were generated by successive

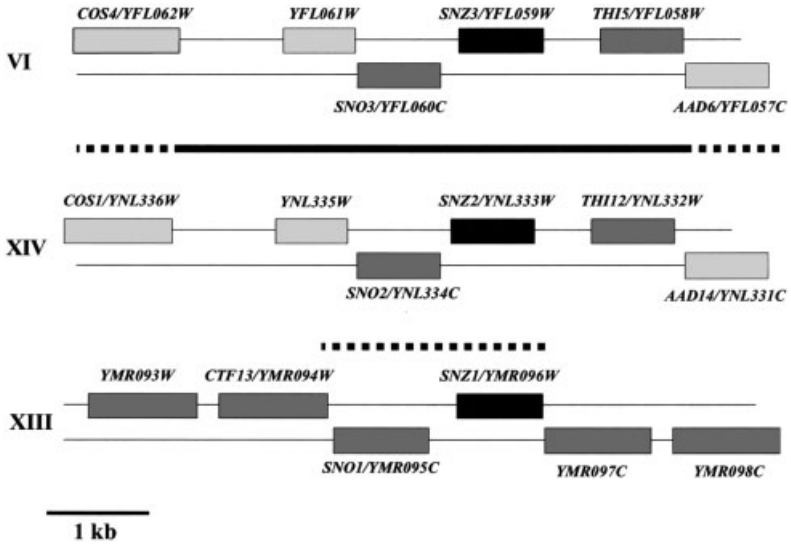

Figure 1. Chromosomal organization of *SNZ, SNO* and *THI5/12* families. Chromosomal regions from chromosomes VI, XIV (both subtelomeric) and XIII, including the gene families, are shown. Genes transcribed in 'Watson' orientation are shown in the upper line and those transcribed from the 'Crick' strand in the lower one. Relevant genes are named. The black bar between chromosomes VI and XIV marks a region of 6350 bp that has only 45 nucleotide changes, mostly single nucleotide transitions. The dotted bars between chromosomes mark regions with partial similarity (50–90%). Genes *THI11* and *THI13* (members of the *THI5* family) are both subtelomeric on chromosomes X and IV, respectively, but do not have neighbouring *SNZ/SNO* genes

transformations with different markers or by conventional crosses. Disruptions were tested by analytical PCR using the adequate oligonucleotide sets (Rodríguez-Navarro *et al*., 1999; Wach *et al*., 1994).

In the cases of *SNZ2* (YNL333W) and *SNZ3* (YFL059W), their almost identical sequences precluded targeted disruption of each copy. *SNZ2* and *SNZ3* are located in different chromosomes (see Figure 1). We designed a 'blind' disruption and crossing strategy using two different markers, *URA3* and *LEU2*, in both *MAT***a** and *MAT*α. Crosses between randomly selected **a** and α clones with different marker were made and diploids were sporulated to obtain, in some cases, Leu^+Ura^+ spores that, therefore, corresponded to a *snz2 snz3* double mutant. Thus, the parental strains that had been used for that particular cross should correspond to single *snz2* and *snz3* mutants. The identity of the single and double mutants was corroborated by Southern blot after pulsed-field electrophoresis (not shown). Double and triple mutants, including Δ*snz1* deletion, were made from the single or the *snz2 snz3* double mutants by disrupting *SNZ1* with the *KanMX4* cassette (Wach *et al*., 1994).

Table 1. Yeast strains used and constructed

Strain	Genotype	Source or reference
FY1679	*MAT***a**/α, *ura3-52/ura3-52, leu2-*Δ*1/LEU2, trp1-63/TRP1, his3-*Δ*200/HIS3*	Thierry and Dujon, 1992
BY4741	*MAT***a**, *leu2-*Δ*0, his3-*Δ*1, met15-*Δ*0, ura3-*Δ*0*	Brachmann *et al*., 1998
BY4742	*MAT*α, *leu2-*Δ*0, his3-*Δ*1, lys2-*Δ*0, ura3-*Δ*0*	Brachmann *et al*., 1998
CML235	*MAT***a**, *ura3-52, leu2-*Δ*1, his3-*Δ*200*	Spore from FY1679
CML236	*MAT*α, *ura3-52, leu2-*Δ*1, his3-*Δ*200*	Spore from FY1679
W303-1A	*MAT***a**, *ade2-1, leu2-2, 122, ura3-1, his3-11, trp1-1a can100*	H. Ronne
PJ69-4a	*MAT***a**, *ade2, trp1-109, leu2-3, 112, ura3-52, his3-200 gal4* Δ, *gal80*Δ, *GAL2:ADE2, LYS2::GAL1:HIS3 met2::GAL7:lacZ*	James *et al*., 1996
PJ69-4α	*MAT*α, *ade2, trp1-109, leu2-3, 112, ura3-52, his3-200 gal4*Δ, *gal80*Δ, *GAL2::ADE2, LYS2::GAL1:HIS3, met2::GAL7:lacZ*	James *et al*., 1996
BQS1029	(BY4742) *snz1-*Δ*0::LEU2*	This work
BQS1037	(BY4741) *snz1-*Δ*0::MET15*	This work
BQS1067	(BY4741) *snz3-*Δ*0::LEU2*	This work
BQS1068	(BY4742) *snz2-*Δ*0::URA3*	This work
BQS1148	(BY4742) *snz1-*Δ*0::KanMX4, snz2-*Δ*0::URA3*	This work
BQS1149	(BY4741) *snz1-*Δ*0::KanMX4, snz3-*Δ*0::LEU2*	This work
BQS1060	(BY4742) *snz3-*Δ*0::LEU2, snz2-*Δ*0::URA3*	This work
BQS1073	(BY4742) *snz3-*Δ*0::LEU2, snz2-*Δ*0::URA3, snz1-*Δ*0::KanMX4*	This work
FYBL1-8B	*MAT***a**, *ura3-*Δ*851, leu2-*Δ*1, his3-*Δ*200, lys2-*Δ*202*	Fairhead *et al*., 1996
FYBL119-5B	*MAT*α, *ura3-*Δ*851, leu2-*Δ*1, his3-*Δ*200, yol055c-*Δ*::KanMX2, ypl258c-*Δ*::KanMX2, ypr121w-*Δ*::KanMX2*	Llorente *et al*., 1999
FYBL1-8B/BL138	*MAT***a**, *ura3-*Δ*851, leu2-*Δ*1, his3-*Δ*200, lys2-*Δ*202, thi2-*Δ*::HIS3*	Llorente *et al*., 1999
FYBL1-8B/BL142	*MAT***a**, *ura3-*Δ*851, leu2-*Δ*1, his3-*Δ*200, lys2-*Δ*202, thi3-*Δ*::HIS3*	Llorente *et al*., 1999
MML21	(CML235) *sno1-*Δ*0::KanMX4*	This work
MML23	(CML235) *sno2-*Δ*0::KanMX4*	This work
MML25	(CML235) *sno3-*Δ*0::KanMX4*	This work
MML27	(CML235) *sno1-*Δ*0::KanMX4 sno2-*Δ*0::KanMX4 sno3-*Δ*0::KanMX4*	This work
MML49	(CML236) *sno1-*Δ*0::KanMX4 sno2-*Δ*0::KanMX4*	This work
MML50	(CML236) *sno2-*Δ*0::KanMX4 sno3-*Δ*0::KanMX4*	This work
MML259	(CML235) *sno1-*Δ*0::KanMX4 sno3-*Δ*0::KanMX4*	This work

In the case of *SNO2* and *SNO3*, the *KanMX4* cassette was used to disrupt both genes. Individual mutants in each of them were distinguished by Southern analysis after *Sca*I-*Xho*I digestion of genomic DNA (a *Xho*I site is present upstream of *SNO3* that is absent in the corresponding *SNO2* region).

Growth conditions

The *S. cerevisiae* strains were routinely grown on YPD (1% yeast extract, 2% peptone and 2% glucose), minimal SD medium [0.67% yeast nitrogen base (YNB without amino acids, DIFCO), 2% glucose, supplemented with auxotrophic requirements]; or minimal SC medium [0.67% yeast nitrogen base (YNB without amino acids, DIFCO), 2% glucose, supplemented with Drop-out mix (DIFCO)]. Vitamin B_6-deficient medium (SC-B6) was prepared by substituting the pre-mixed YNB for a mixture of the same components except from vitamin B_6 [biotin, pantothenic acid, nicotinic acid, thiamine, inositol, H_3BO_3, $CuSO_4$, KI, $MnSO_4$, $NaMoO_3$, $ZnSO_4$, H_2KPO_4, $(NH)_2SO_4$, $MgSO_4$, $CaCl_2$, $FeCl_3$, Na_2MoO_4] at the same concentrations as the DIFCO medium in 0.5 M, pH 6, 2-[N-morpholine]ethanesulphonic acid buffer. For control experiments this medium was supplemented with vitamin B_6 to 2 μg/ml. For complementation analysis with pCM plasmids, SC-B6 in the absence (derepressing conditions) or in the presence (repressing conditions) of doxycycline was used.

Thiamine-deficient medium was prepared as described (Llorente *et al.*, 1999). It is identical to the B_6-deficient medium but without thiamine and plus vitamin B_6 to 2 μg/ml. Geneticin-resistant strains were grown on YPD plates containing 200 mg/l geneticin (Gibco BRL). Meiosis induction was carried out by growing cells in YPD to saturation and then in YPA (1% yeast extract, 2% peptone and 2% acetate) to 2×10^7 cells/ml. After washing cells twice with water they were resuspended in sporulation medium (0.5% potassium acetate).

For phenotypic analysis, the growth of haploid mutants was checked on YP 2% glycerol and in YPD containing 1.2 M NaCl, 0.8 M KCl, or 1.8 M sorbitol. Cells were grown at 15 °C, 28 °C and 37 °C for 2–3 days or longer when necessary.

Effects of overexpression of *SNZ1* and *SNZ2/3* genes on growth curves were made after transformation of strain W303-1A to uracil prototrophy with plasmids pCM262SNZ1, pCM262SNZ2 or empty pCM262 vector. At all times before the actual experiment, expression from the $tetO_7$ promoter was turned off by the presence of 2 μg/ml doxycycline in the culture medium. To measure growth curves, transformed cells were initially cultivated in liquid SC medium lacking uracil and containing doxycycline. The culture was then split in two aliquots, one of which was rinsed free of doxycycline and then cultured without doxycycline. Growth of cells at 30 °C in 400 μl microchambers with continuous shaking was measured as turbidity in a Labsystems Bioscreen C Microbiology Workstation, using a wide-band visible light filter.

Analysis of sensitivity to menadione

Cell cultures in YPD medium at 30 °C and at the indicated growth stage (exponential or post-diauxic) were directly added with 20 or 40 mM menadione. After the indicated times, 1 : 5 serial dilutions were made and drops spotted onto YPD plates. Growth was recorded after 2 days of incubation at 30 °C.

Northern analysis

Isolation of total RNA, electrophoresis, radioactive or non-radioactive probe labelling, hybridization and signal detection were all done as previously described (Llorente *et al.*, 1999; Gallego *et al.*, 1997). Probes were generated by PCR amplification from genomic DNA, using oligonucleotides designed to amplify the entire ORF without adjacent sequences (Table 2).

Two-hybrid

A library of genomic *S. cerevisiae* DNA from the *his3 ade2 gal4* strain JB974 in the Gal4 activation domain (Gal4-AD) fusion vector pACT2 (13) was used. The construction of this library will be described elsewhere (Ramne A, Sunnerhagen P *et al.*, in preparation).

Bait clones encoding fusions of Gal4 DNA-binding domains (Gal4-DB) and proteins of interest were constructed using homologueous recombination *in vivo* (Muhlrad *et al.*, 1992). Briefly,

Table 2. Oligonucleotide primers used for PCR and cloning

Name	Sequence 5′-3′
3′pGBT9SNZ2	AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG CCC AAT TTC GGA AAG TC
5′pGBT9SNZ2	AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG ATG TCA GAA TTC AAG GTT AAA AC
3′pGBT9SNZ1	TAA GAA ATT CGC CCG GAA TTA GCT TGG CTG CCC AAT TTC GGA AAG TC
5′pGBT9SNZ1	AAC AAA GGT CAA AGA CAG TTG ACT GTA TCG ATG ACT GGA GAA GAC TTT AAG
3′pCMSNZ2	C GTA TGG GTA ACC TGG TGA TCC GTC GAC CTG CAG CCA TCC GAT TTC AGA AAG TCT TGC
5′pCMSNZ2	C CGG ATC AAT TCG GGG GAT CAG TTT AAA CGC GGC CGC ATG TCA GAA TTC AAG GTT AAA AC
3′pCMSNZ1	C GTA TGG GTA ACC TGG TGA TCC GTC GAC CTG CAG CCA CCC AAT TTC GGA AAG TC
5′pCMSNZ1	C CGG ATC AAT TCG GGG GAT CAG TTT AAA CGC GGC CGC ATG ACT GGA GAA GAC TTT AAG
SNZ1-R	TCA CCC TTG GTA CGA ATC ATA
SNZ1-D	GGT GGC GTT ATT ATG GAT GT
SNO1 lo	TTA ATT AGA AAC AAA CTG TC
SNO1 up	AAC CCA CAG TAC AAT GTC CG
SNO2 lo	AGA ACA AAT TCT CTG ATG AA
SNO2 up	ATG TCA GAA TTC AAG GTT
SNZ1 lo	TCA CCA CCC AAT TTC GG
SNZ1 up	GGA GAA GAC TTT AAG ATC A
SNZ2 lo	CTA CCA TCC GAT TTC AG
SNZ2 up	ATG TCA GAA TTC AAG GTT
SNZ2s1	ACT ATA ATA GAA AAA TAA GTA TAT CGT AAA AAA GAC AAAA
SNZ2s2	AAG GAA ACA AAT TAG CGT TGT GTG AGC ATC GCT AGT TCTA
SNZ2A1	CGA CGG TCA TTT TTG AGA
SNZ2A4	CAT AGT TCA TGA GCC GTT
SNZ1A1	TTT CAT CGA CTT TCC GGA
SNZ1A4	TGC CGT TTC AGA TCA TAA
SNZ1s1	AGC AAA TAT ACA CAG TAC TAA TAT TCA GTT AAT TAT CACG
SNZ1s2	AAA GTG TTA TGC TCA AAA TAC CTG TTC AAA GAA ATC ACTG

full-length coding sequences of genes were PCR amplified from total genomic *S. cerevisiae* FY1679 DNA, using the Roche Expand High Fidelity™ system and hybrid primers with 30 nucleotides of homology to the gene and 17–21 nucleotides of homology to sequences flanking the cloning site (Table 2) of the Gal4-DB vector pGBT9 (Bartel *et al*., 1993). PCR products were co-transformed with pGBT9 restricted with *Bam*HI and *Eco*R1 into *S. cerevisiae* PJ69-4α (James *et al*., 1996). In our hands, >90% of plasmids from tryptophan prototrophs obtained in this manner contained an insert of the correct size.

Transformants (50–100, picked at random from each transformation) were pooled and used for subsequent mass mating with PJ69-4a transformed with the *S. cerevisiae* genomic DNA Gal4-AD fusion library. Diploid cells with a functional two-hybrid interaction were selected on medium lacking tryptophan, leucine and histidine, and containing 3 mM 3-aminotriazole (3-AT) and 2 mg/l adenine. The identity and reading frame of genes in prey plasmids was verified by partial sequencing.

Plasmid construction

Recombinant clones containing *SNZ1* or *SNZ2* open reading frames in the pCM262 plasmid vector were constructed by gap repair (see Table 2) in *S. cerevisiae* similarly to the construction of two-hybrid bait clones. pCM262 is an episomal plasmid derived from pCM190 (Garí *et al*., 1997), designed to overexpress genes tagged at the C-terminus with three haemaglutinin (HA) epitopes and six histidine residues in tandem, under the control of the $tetO_7$ promoter. The synthetic 3HA-6His cassette was introduced as a *Pst*I-*Asc*I fragment in the polylinker of pCM190 (Rodríguez-Manzaneque MT, Herrero E, to be described elsewhere). After co-transformation into FY1679 of pCM262 (restricted with *Pst*I and *Not*I) and PCR products containing the respective ORFs, uracil prototrophs were picked and checked for expression of full-length protein product by Western analysis, using anti-HA antibodies. Plasmids were then recovered into *E. coli* from such yeast transformants and the correctness of their restriction patterns verified.

Macroarray analysis

We used the hybridization membranes produced by J. Hoheisel (Hauser *et al.*, 1998) and followed his recommendations for use. Briefly, single-stranded α^{33}P dCTP-radiolabelled complex cDNA samples were synthesized by reverse transcription of the same RNA extracts as for Northern blots. An equimolar mix of the 12 anchored 17-mer oligonucleotides dT_{15}(A,C,G)N was used to prime for the reverse transcription reaction. 1/20th of the sample was run on a 5% denaturing polyacrylamide gel and then exposed for 30 min onto X-ray film (Kodak) to check the efficiency of labelling and the extent of the reverse transcription reaction. Samples that gave good results displayed a smear ranging approximately from 80 to >600 nucleotides. The samples were used to hybridize the membranes in the same conditions as for Northern blots. Hybridization signals were revealed with a Phosphorimager (Molecular Dynamics 445SI) after 24 h exposure. Images were analysed using the XDotsReader program commercialized by COSE. For each hybridization, the intensities were normalized by the mean intensity of the membrane, for comparative purposes. Only genes that showed more than a three-fold increase ratio in the absence of thiamine vs. its presence, and that were differentially expressed in several experiments, were considered.

Results

Requirement for vitamin B_6 of the different mutants

The *SNZ* and *SNO* homologues *SOR1* and *PDX2*, respectively, from *C. nicotiniae* (Ehrenshaft *et al.*, 1999; Ehrenshaft and Daub 2001) and *pyroA* from *A. nidulans* (Osmani *et al.*, 1999) have been described as required for vitamin B_6 biosynthesis in those organisms, suggesting that the *SNZ* and *SNO* genes could be related to the same pathway in yeast. To check this hypothesis, we constructed all the combinations of single, double and triple deletion mutants for each family. All these mutants grew as well as the control strain on YP or complete synthetic media at either 15 °C, 28 °C or 37 °C and with glucose or glycerol as sole carbon sources (not shown). Because vitamin B_6 is a common compound of standard complete and minimum culture media for yeast, we assayed the growth in a synthetic medium without it. We did not see any major growth defect when the inoculum was precultured in YPD or SC. However, when those cells were pre-grown in SC-B6 medium, single *snz1, sno1* mutants and *snz* triple mutants showed a strong growth defect (Figure 2A, B, C). This defect was more acute in *snz1* than in the *sno1* mutant. Neither single *snz2, snz3, sno2* or *sno3* mutants (not shown) or double *snz2 snz3* mutants showed any growth defects under these conditions. Triple *sno* mutant behaved similarly to *snz* triple mutant (not shown).

Defects of the *snz1* and *sno1* mutants were further analysed with regard to the growth phase of the pre-culture. When the culture time was extended to 7 days, the *snz1* mutant was more severely defective than the *sno1* mutant and the defect worsened if the pre-culture proceeded from stationary phase (Figure 2B). Although, at first sight, the triple mutant *snz1 snz2 snz3* behaved identically to the single *snz1* mutant (not shown), very long incubation times (2 weeks, Figure 2C) revealed a stronger growth defect for the triple mutant. It is worth noting that the growth level seen in YPD control plates is similar for the wild-type and for all the mutants tested, suggesting that the viability of the cells is unaffected by the absence of those gene products during stationary phase.

The growth defect of *snz1* mutant can be complemented by the overexpression of both *SNZ1* and *SNZ2* (see Figure 2D). This result suggests that *SNZ2* and *SNZ3* code for a protein with a similar activity to Snz1p. As expected, the addition of vitamin B_6 alone also restores the growth (data not shown).

In conclusion, *SNZ1* and *SNO1* are both required for growth when cells are depleted in vitamin B_6. The residual growth in SC-B6 observed for *snz1 snz2 snz3* triple mutants pre-cultured until the exponential phase probably reflects traces of vitamin B_6 in the cells. On the other hand, *SNZ2* and *SNZ3* have no complete functional redundancy with *SNZ1*, despite similar biochemical properties of their products.

Menadione sensitivity

It has been shown previously that some of the *snz* and *sno* mutants are sensitive to methylene

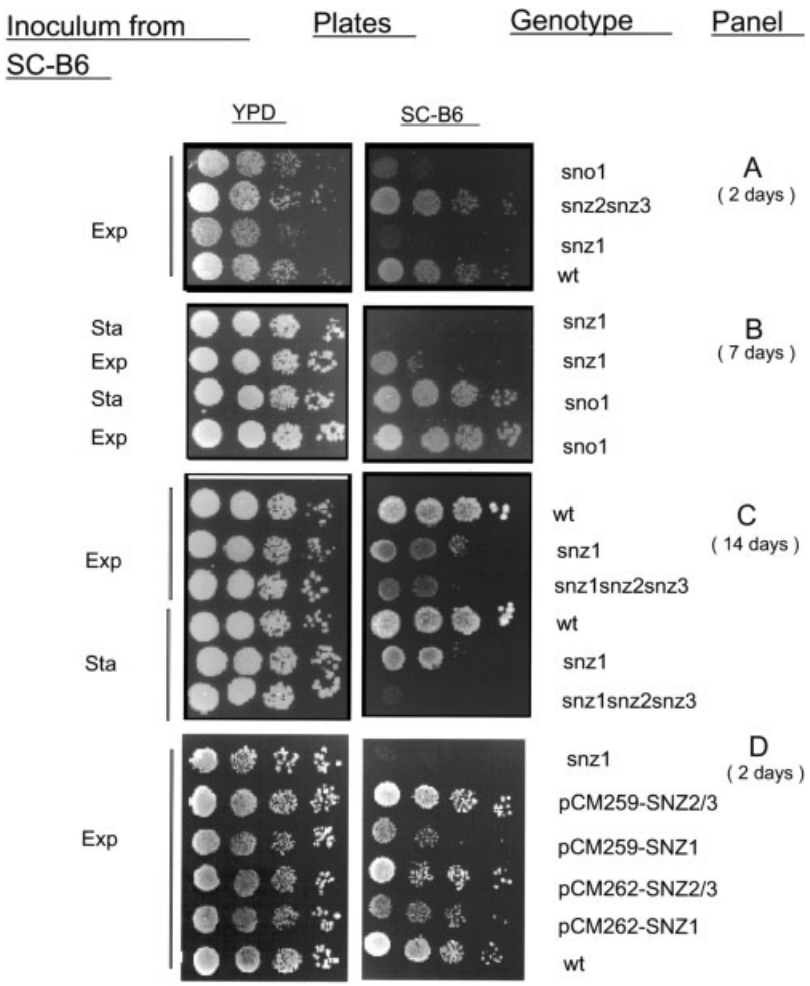

Figure 2. Growth of *snz* and *sno* mutant strains in a vitamin B_6-lacking medium. Serial four-fold dilutions of exponentially (Exp) or stationary (Sta) phase cultures (inoculum) were spotted on to YPD (control) and SC-B6 plates. Plates were incubated at 28 °C for 24–36 h (all YPD plates), 2 days [SC plates (A, D)], 7 days [SC plates (B)] or 14 days [SC plates (C)]. Complementation of the auxotrophy for vitamin B_6 in the *snz1* mutant by a pCM259/262–SNZ1 or pCM259/262–SNZ2 plasmids is shown in (D). Two independent transformants for each plasmid are shown. The relevant genetic background of the strains is indicated

blue, a generator of singlet oxygen (Padilla *et al*., 1998), one of the most active ROS (reactive oxygen species). Similarly, the *SNZ*-homologous genes *pyroA* and *SOR1* genes are known to protect *A. nidulans* and *C. nicotiniae*, respectively, against singlet oxygen (Ehrenshaft *et al*., 1999; Osmani *et al*., 1999).

We tested the sensitivity of *snz* and *sno* mutants to the superoxide generator, menadione. Menadione sensitivities of all the mutants were similar to that of the control strain when treated during the exponential phase for 30 min (Figure 3) or longer (not shown). However, when treated during the post-diauxic phase for 180 min with 20 mM menadione, the triple *sno1 sno2 sno3* and, especially, *snz1 snz2 snz3* mutants, were extremely sensitive to it. By using a higher drug concentration (40 mM) it was possible to observe that the single *snz1* or *snz3* and, not surprisingly, the double *snz1 snz3* and *snz2 snz3* mutants were more sensitive than other single or double mutants. All mutants, as well as wild-type cells, were highly resistant to both drug concentrations when treated during the stationary phase (not shown).

These results confirm that *SNO*, and especially *SNZ* genes, confer resistance to ROS to the cells.

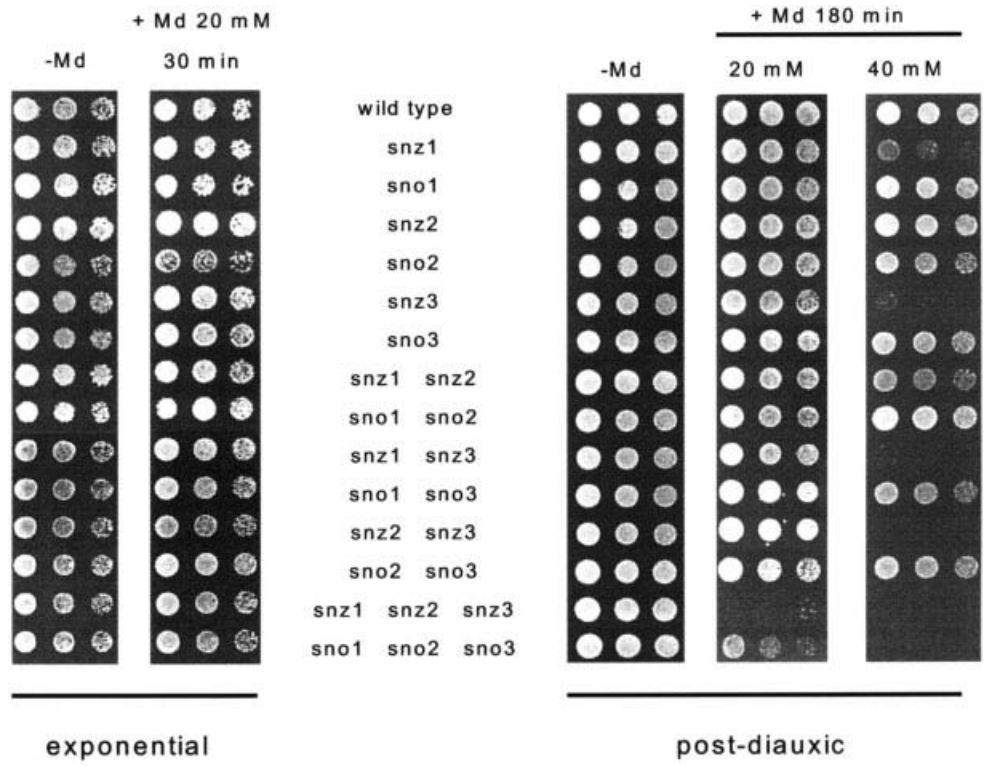

Figure 3. Menadione sensitivity of *snz* and *sno* mutant strains. Sensitivity of *snz* and *sno* mutants to menadione treatment. Cells were grown in YPD medium at 30 °C, to either exponential phase (2×10^7 cells/ml) or 20 h later (post-diauxic phase) and were treated with the indicated menadione concentrations for 30 min (exponential cells) or 180 min (post-diauxic cells). After treatments, cells were diluted in fresh YPD medium (1 : 5 serial dilutions) and 2 μl drops were spotted on YPD plates. Growth was recorded after 2 days of incubation at 30 °C. The apparent higher resistance of the *snz1 snz2* mutant compared with *snz1* single mutant is due to a higher cell number in this particular experiment. It was not observed in other experiments

These results also show that the protection effect is more dependent on *SNZ1* and, surprisingly, *SNZ3* than on *SNZ2*, and that this effect is masked by the intrinsic resistance of advanced stationary cells to environmental stresses (Werner-Washburne *et al.*, 1996).

Vitamin B_6 effects on expression of *SNZ* genes

The role of *SNZ* and *SNO* genes in vitamin B_6 biosynthesis suggested possible regulation of their expression by this vitamin. Figure 4 shows that *SNZ1* expression in exponential phase is not dependent on either the absence or the presence of a high vitamin B_6 concentration (0.2 mg/ml), although it is repressed in YPD. *SNZ2–3*, however, are expressed approximately at the same rate in all the four conditions.

Overexpression

In the course of these experiments, we noted that overexpression (from the doxycycline-regulated $tetO_7$ promoter) of Snz1–3HA–6His or Snz2–3HA–6His in *S. cerevisiae* strain W303-1A caused a marked delay in recovery from stationary phase (Figure 5). A similar response was seen in strain FY1679 (not shown). When cells overexpressing these proteins were diluted 1 : 100 from saturated overnight cultures into fresh medium, resumption of logarithmic growth occurred up to 16 h later than for cells not overexpressing either protein. This effect was clearly stronger for the Snz2 fusion protein (Figure 5C) than for Snz1p (not shown). Once logarithmic growth had resumed, only a minor effect on growth rate was seen after the longest periods in stationary phase. Density at saturation was also largely unaffected.

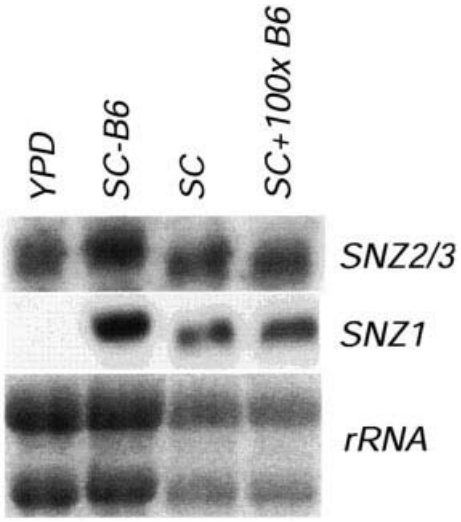

Figure 4. Influence of vitamin B_6 in *SNZ* expression. Total RNA from log phase cultures in YPD, SC (vitamin B_6 is 2 μg/ml), SC − B_6 and SC + B_6 (vitamin B_6 is 0.2 mg/ml) was analysed in a wild-type strain with a Northern experiment using *SNZ1* and *SNZ2/3* probes (made with oligonucleotides SNZ2A1 and SNZ2A4). Total rRNA is included as loading control

As can be seen (Figure 5D), this effect is clearly dependent on gene expression: at a high concentration of doxycycline, 2 μg/ml (transcription repressed), no delay was observed. At intermediate concentrations (0.05 or 0.5 μg/ml), the delay was less pronounced (not shown). The addition of vitamin B_6 at any stage of the experiment did not change the results (not shown).

Two-hybrid screens

In order to find more clues to the specific roles of *SNZ* genes, we conducted two-hybrid screens. In the first one, with the *SNZ1* bait, out of 10 positive clones obtained that encoded *bona fide* in-frame proteins, three contained sequences of the *YHR198c* ORF. These represented two independent clones with a common overlapping segment, encoding a short central portion of the Yhr198c protein (amino acids 167–180).

When *SNZ2* was used as the bait, *YJR156c* (*THI11*) was found as reactive prey in the correct reading frame. The *THI11* gene product is probably involved in the biosynthesis of the pyrimidine precursor of thiamine (Hohmann and Meacock, 1998), and it is homologous to the *Schizosaccharomyces pombe* Nmt1 protein (Van Dyck *et al*., 1995). Thi11p has three paralogues in the yeast genome, with almost identical sequences, Thi5p, Thi12p and Thi13p. In similar screens using *SNO1* or *SNO2/3* as baits, no preys were found that corresponded to proteins involved in vitamin biosynthesis (not shown).

Vitamin B_1 effects on gene expression

The close proximity on chromosomes VI and XIV of the putative thiamine biosynthetic genes *THI5* and *THI12*, respectively, to the *SNZ3/SNO3* and *SNZ2/SNO2* loci (see Figure 1) together with the results of our two-hybrid experiments (see above) may suggest a possible functional link between these two classes of genes. Therefore, we investigated the effects of vitamin B_1 depletion on the transcription of the *SNZ* and *SNO* families, and on the prototrophy of the corresponding null mutants.

It is known that the concentration of exogenous thiamine influences the transcript amounts of several genes involved in its metabolism, such as *THI4, 5, 6, 10, 11, 12, 13, 20, 21, 22* and *PHO3*. Moreover, this regulation is under the positive control of one or both of the two regulators Thi2p and Thi3p (Hohmann and Meacock, 1998). In order to have an exhaustive list of genes whose transcripts are regulated by the extracellular concentration of thiamine, we used macroarrays of genes produced by the J. Hoheisel laboratory (Hauser *et al*., 1998). Hybridizations were performed using complex cDNA samples synthesized from RNAs of the wild-type strain FYBL1-8B, grown in the presence of high concentration of extracellular thiamine (1 μM) and in the absence of extracellular thiamine. We also performed hybridizations using complex cDNA samples synthesized from RNAs of the strains FYBL138 and FYBL142 deleted for *THI2* and *THI3*, respectively, grown in the presence of low concentration of extracellular thiamine (10^{-2} μM). All the genes we found to be upregulated in the absence of exogenous thiamine are indicated in Table 3. We confirmed the already published upregulation of *THI4, 5, 6, 10, 11, 12, 13, 20, 21, 22, PET18, YLR004c* and *PHO3*. But, in addition, we found that transcripts of *SNO2/3, SNZ2/3, THI2* and *ECM15* also accumulated in the absence of exogenous thiamine. Since Thi2p and Thi3p do not regulate the transcription of *ECM15*, this gene has not been studied further. The accumulation of the

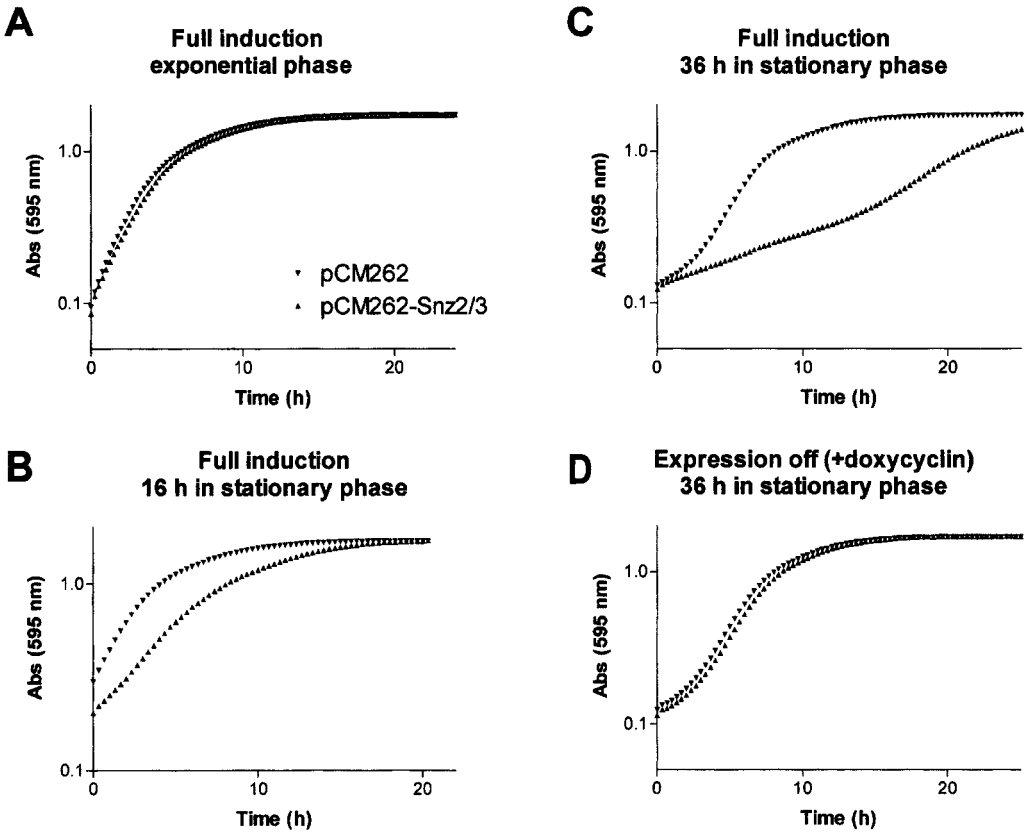

Figure 5. Growth of *SNZ2* overexpressing strains after dilution in fresh medium. W303-1A cells transformed with empty vector pCM262 (inverted triangles), pCM262-SNZ1 (not shown), or pCM262-SNZ2 (triangles) were grown in liquid SC − ura medium in the presence or absence of 2 μg/ml doxycycline, as indicated in Materials and methods. From the logarithmic pre-culture ($A_{600} = 0.5$), the cells were either transferred directly (A), or allowed to proceed to stationary phase (B, C, D). For B, C and D, cells were then kept for the indicated times at stationary phase. Next, the cell suspension was diluted to OD = 0.1 in fresh medium (with or without doxycycline, as indicated), and growth with shaking and aeration in 400 μl chambers was recorded. Values are the mean of two independent experiments; for clarity, error bars are omitted. Absorbance values (*y* axis) are shown on a logarithmic scale

other transcripts is dependent on the presence of either Thi2p or Thi3p or both. These results were confirmed by Northern blot experiments, as shown in Figure 6 for *SNO2/3* and *SNZ2/3*. Other known examples of thiamine regulation, such as *PDC5* and *PDC1* (Muller *et al.*, 1999), are not listed here because of the very stringent criteria used for significance levels (see Materials and Methods) but were detected as induced by Northern blot. It is impossible to conclude whether both the transcripts of *SNO2* and *SNO3* are regulated in the same way because their nucleotide sequences are nearly identical, which must generate cross-hybridization. This is also the case for *SNZ2* and *SNZ3*, as well as for the *PHO* gene family, of which only the transcripts of *PHO3* have been described as being regulated by extracellular thiamine concentration (Nishimura *et al.*, 1992).

The Northern blot experiments shown in Figure 6 illustrate that the *SNO1* transcripts are undetectable with this approach during exponential growth phase, and remain unaffected by the extracellular concentration of thiamine. *SNO2–3* transcripts are detectable during the exponential growth phase only in the absence of extracellular thiamine, in a *THI2–3*-dependent manner. *SNZ1* transcripts are slightly more abundant during the exponential growth phase in the presence of low concentration of extracellular thiamine with respect to high concentration, but this accumulation is not dependent on either Thi2p or Thi3p. *SNZ2–3* transcripts accumulation occurs clearly in the absence of

Table 3. Yeast transcripts regulated by extracellular thiamine

Genes		Induction	Δ*thi2*	Δ*thi3*	Northern	Conclusion
YNL332w	*THI12*	+++	–	–	NT	Confirmation
YJR156c	*THI11*	+++	+	–	NT	Confirmation
YDL244w	*THI13*	+++	+	–	NT	Confirmation
YFL058w	*THI5*	+++	+	–	NT	Confirmation
YAR071w	*PHO11*	+++	–	–	NT	Confirmation
YHR215w	*PHO12*	+++	–	–	NT	Confirmation
YBR092c	*PHO3*	+++	–	–	NT	Confirmation
YBR093c	*PHO5*	+++	–	–	NT	Confirmation
YLR237w	*THI10*	+++	+++	–	NT	Confirmation
YGR144w	*THI4*	+++	+	+	NT	Confirmation
YPL214c	*THI6*	+++	–	+	NT	Confirmation
YNL334c	*SNO2*	+++	–	–	Yes	New
YFL060c	*SNO3*	+++	–	–	Yes	New
YNL333w	*SNZ2*	+++	–	–	Yes	New
YFL059w	*SNZ3*	+++	–	–	Yes	New
YOL055c	*THI20*	+++	–	–	Yes	Confirmation
YPL258c	*THI21*	+++	–	–	Yes	Confirmation
YPR121w	*THI22*	+++	–	–	Yes	Confirmation
YCR020c	*PET18*	+++	–	–	Yes	Confirmation
YLR004c		+++	–	–	Yes	Confirmation
YBR240c	*THI2*	+++		–	Yes	New
YBL001c	*ECM15*	+++	+++	+++	NT	New

This table indicates the results from macroarray hybridization experiments. The Induction column represents the relative levels of transcripts observed for the wild-type strain FYBL1-8B grown in the absence of extracellular thiamine with respect to those found in the presence of extracellular thiamine (only ratios above or equal to a three-fold increase have been considered). Columns Δ*thi*2 and Δ*thi*3 represent the same ratios but for the strains FYBL138 and FYBL142 deleted for *THI2* and *THI3*, respectively. These strains are auxotrophic for thiamine and have thus been grown in the presence of a low extracellular thiamine concentration (10^{-8} M) instead of no thiamine. 'Yes' indicates that the regulation has been confirmed by Northern blot experiments. NT, non-tested by Northern blot experiment.

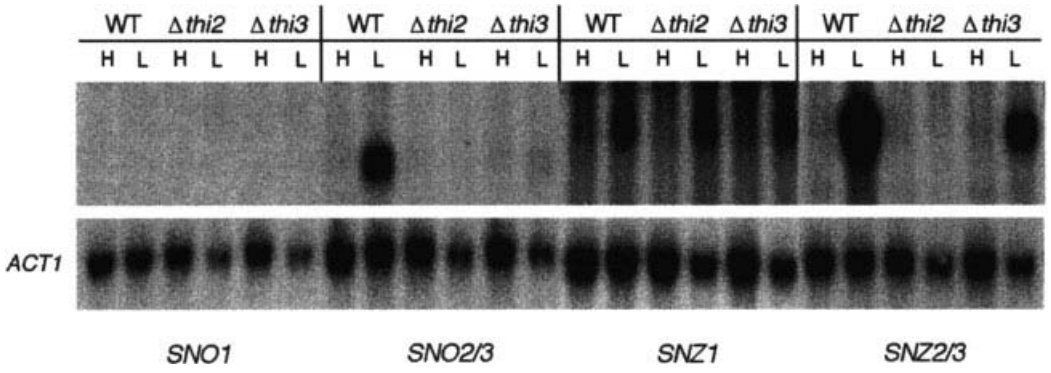

Figure 6. Gene expression analysis in thiamine-limiting conditions. 10 μg total RNA extracted from cells grown in SC to exponential phase in the presence of high (H) or low (L) extracellular thiamine concentration were loaded and run on a 1.5% agarose gel containing 0.6% formaldehyde, transferred to Hybond N^+ membranes (Amersham) and hybridized with specific probes (upper panel; made by PCR with oligonucleotide pairs called 'lo' and 'up' for each case; see Table 2) indicated at the bottom of the figure. H, 1 μM extracellular thiamine; L, no thiamine for the wild-type strain (WT) FYBL1-8B, and 10^{-2} μM for the auxotrophic strains Δ*thi*2 (FYBL1-8B/BL138) and Δ*thi*3 (FYBL1-8B/BL142). *ACT1* was used as loading control for all the samples

extracellular thiamine, and is completely dependent on Thi2p and only partially dependent on Thi3p.

Discussion

Role of *SNZ* and *SNO* genes in vitamin B_6 biosynthesis

Most microorganisms and plants possess at least one pathway leading to pyridoxine and pyridoxal 5′-phosphate synthesis. The *Escherichia coli* pathway has been extensively studied (see Drewke and Leistner, 2001) but the corresponding pathway in yeast is not well defined. Nevertheless, the fact that the L-[amide-^{15}N]glutamine label is incorporated efficiently into pyridoxine in *S. cerevisiae* but not in *E. coli* indicates that vitamin B_6 biosynthesis must be significantly different in either organism (Tazuya *et al*., 1995).

Other eukaryotic organisms, such as *A. nidulans, Mucor racemosus* and *N. crassa* and prokaryotes, such as *Staphylococcus aureus* and *Bacillus subtilis*, may have biosynthetic pathways for pyridoxine similar to that of *S. cerevisiae* (Tanaka *et al*., 2000). It has recently been shown that the *pyroA* gene from *A. nidulans* (Osmani *et al*., 1999) and the *PDX1* (*SOR1*) gene from *C. nicotiniae* (Ehrenshaft *et al*., 1999) are involved in *de novo* biosynthesis of vitamin B_6 in those fungi. It has been suggested that mutations in *pdx-1* and *pdx-2* (*SNZ* and *SNO* homologues, respectively) cause pyridoxine auxotrophy in *N. crassa* (Bean *et al*., 2001). A more recent study shows an involvement of *PDX2* of *C. nicotianae* in the pyridoxine biosynthesis pathway (Ehrenshaft and Daub, 2001). The fungal genes are 58–67% (*SNZ*) and 36–38% (*SNO*) identical to the yeast genes. The *SNZ* and *SNO* families are widely represented in eubacteria, archaea and eukaryotes (Braun *et al*., 1996; Ehrenshaft *et al*., 1999; Galperin, 2001; Mittenhuber, 2001). Our results indicate that the *SNZ* and *SNO* gene families are also involved in the vitamin B_6 biosynthesis in *S. cerevisiae*. We identified a functional specialization within these two gene families, since the absence of copy 1 leads to a more severe growth phenotype than the absence of copy 2 and 3 when cells are grown in SC-B6. Although the *SNZ2–3* genes seem to be dispensable for any condition tested, they should code for proteins with a similar activity to Snz1p because they can complement, at least as efficiently as *SNZ1* itself (Figure 2D), the *snz1* null phenotype when overexpressed. This suggests that all the three *SNZ* genes code for enzymes involved in vitamin B_6 biosynthesis, probably at the last step, the ring closure, as suggested by Ehrenshaft *et al*. (1999). Vitamin B_6, however, seems not to be a regulator of *SNZ* gene transcription (Figure 4), although the high transcription levels already present in synthetic medium might mask the effect of the absence of the vitamin.

The phenotypes of *sno* mutants are less pronounced than that of the corresponding *snz* mutants. It has been suggested that *SNO* genes are involved in the first step of pyridoxal biosynthesis (Ehrenshaft and Daub, 2001; Osmani *et al*., 1999). Thus, it is conceivable that, if the *SNZ* genes code for pyridoxine biosynthetic enzymes, the substrate for Snz proteins could be produced in the absence of Sno proteins, although in very minor amounts, by alternative pathways.

SNZ and *SNO* genes have an interesting effect on sensitivity to oxidative radicals. Padilla *et al*. (1998) have shown that *sno1* or *snz1* mutations are very sensitive to the singlet oxygen generator methylene blue. Furthermore, it has been shown that B_6 vitamers are efficient quenchers of singlet oxygen *in vitro* (Bilski *et al*., 2000; Ehrenshaft and Daub, 2001) and that externally added vitamin B_6 effectively suppresses the toxicity of methylene blue in *A. nidulans* (Osmani *et al*., 1999). We have shown here that a different kind of ROS, the superoxide radical, produced by menadione, has a similar effect on *snz/sno* mutants. It has been argued that B_6 can act in active oxygen resistance and that such as a protective effect is more necessary in stationary phase, when cells are subjected to increased oxidative stress (Ehrenshaft *et al*., 1999). Vitamin B_6 may be just an antioxidant or, perhaps, its destruction by ROS causes deficiency of B_6 vitamers that are necessary for other metabolic uses (Osmani *et al*., 1999). This last hypothesis is supported by the fact that neither H_2O_2 nor menadione induce the transcription of any of these genes (Gasch *et al*., 2000). Furthermore, preliminary data from the groups of Joaquim Ros and Enrique Herrero (University of Lleida, Spain) indicate that addition of vitamin B_6 reduces the level of protein carbonylation (a parameter measuring protein oxidation) induced by addition of oxidants such as menadione or hydrogen peroxide. Finally,

the striking difference between menadione sensitivity of *snz2* and *snz3* (see Figure 3) suggests that the two genes have somewhat different roles, in spite of their high sequence similarity.

Integration of biosynthetic pathways for vitamins B_1 and B_6

It is known that in some prokaryotes (e.g. *E. coli*), some precursors and enzymes (e.g. the *pdxK* gene product) are shared by biosynthetic pathways for the vitamins B_1 and B_6 (Begley *et al*., 1999; Mittenhuber, 2001). In *S. cerevisiae* the fact that the transcription of the *SNZ2-3* and *SNO2-3* genes is induced by the absence of thiamine in a Thi2p/Thi3p-dependent manner, suggests that those genes have a function related with the biosynthesis of vitamin B_1 as well. Two additional facts support this view.

First, the finding of interactions between Thi and Snz proteins by two-hybrid analysis (see below) provides an independent suggestion for a common functional pathway for Snz and Thi proteins, i.e. a putative role for the Snz, and also Sno, proteins in thiamine biosynthesis, and corroborates the idea of Hohmann and Meacock (1998). Our results suggest, however, that Snz2-3 and Sno2-3 proteins, but not Snz1p and Sno1p, are related to vitamin B_1 biosynthesis: *SNZ1* and *SNO1* transcription is not induced by thiamine depletion. Although all the members of Snz and Sno protein families can reasonably be considered as putative enzymes acting on the same reactions, it cannot be dismissed that they may act at different times or cellular compartments, similarly to other cases of isoenzymes (Matthews *et al*., 2000). Several pathways for thiamine biosynthesis should exist because none of the single, double and triple deletion mutants of the *SNO* and the *SNZ* gene families display auxotrophy for thiamine (Llorente B and Peréz-Ortín JE, not shown). This is consistent with the fact that under anaerobic conditions the pyrimidine moiety of thiamine is not derived from pyridoxal, therefore a different pathway must act instead (Tanaka *et al*., 2000).

Second, there is close map proximity between these genes and some members of the *THI5/11/12/13* family (see Figure 1). These *THI* genes are highly similar: the proteins differ only in one amino acid out of 340. The corresponding protein is only detected in the absence of thiamine (Muller *et al*., 1999), *THI5* and *THI12* transcripts accumulate in the absence of extracellular thiamine (Meacock PA, personal communication) and they putatively code for an enzyme involved in the biosynthesis of the pyrimidine moiety of the thiamine molecule (Hohmann and Meacock, 1998). It is therefore interesting that three consecutive genes repeated twice in the genome have a common transcriptional response. The three couples *SNO/SNZ* share divergently transcribed promoters, something that supports the notion of common regulatory sequences (Padilla *et al*., 1998). This head-to-head arrangement has been also found in the yeasts *Candida albicans* and *S. kluivery* (Llorente B, unpublished), in the fungus *N. crassa* (Bean *et al*., 2001), and in the sponge *Suberites domuncula* (Seack *et al*., 2001) that suggests an ancient origin for *SNO-SNZ* co-regulation. The close association of *THI* genes with *SNZ-SNO*, however, is not so common. We have analysed the genomes of *Schizosaccharomyces pombe, Candida albicans* and the hemiascomycetes group of the Genolevures project (Souciet *et al*., 2000). Most of them possess only one orthologue of *SNZ, SNO* and *THI5*. None of the *THI5* orthologues has been found to be syntenic with the *SNO-SNZ* orthologues. In addition, copies 2 and 3 of the *SNZ-SNO* genes seem to be the product of a recent subtelomeric chromosome duplication, since they are not detected in some *S. cerevisiae* strains (Padilla *et al*., 1998). The functional specialization of copies 2 and 3 and their association with *THI5* locus may thus have developed recently.

A protein complex of Snz, Sno and Thi proteins

As discussed above, our two-hybrid results show that Snz1p interacts with Yhr198p and Snz2/3p with Thi11p. Previous two-hybrid analysis showed interactions between the Snz and Sno proteins themselves and with other proteins (DIP database, 2001; Ito *et al*., 2000; Padilla *et al*., 1998; Uetz *et al*., 2000). A scheme of the putative interactions is shown in Figure 7.

Examples of multi-enzymatic complexes comprising enzymes that catalyse successive steps in a metabolic pathway are well known (Matthews *et al*., 2000). It seems that every one of the Snz and Sno proteins can interact with itself and with the other members. Because the transcription profiles of copies 2 and 3, on the one hand, and copy

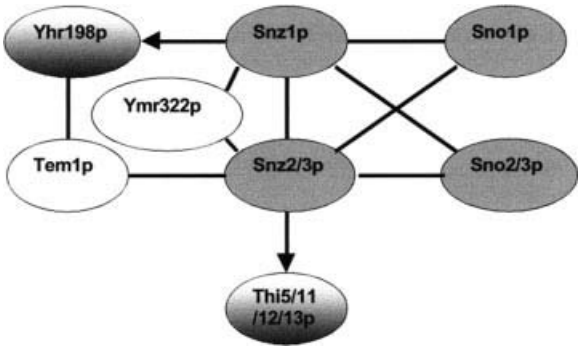

Figure 7. Summary of two-hybrid interactions of Snz, Sno and Thi families. Protein preys detected in this work are represented in degraded grey. Interacting proteins a linked by bars and arrows. Sno and Snz proteins are highlighted. See text for further discussion

1, on the other hand, are so different that it is possible that the protein complex changes depending on the physiological circumstances of the cell. In fact, Padilla *et al*. (1998) described a 230 kDa complex dependent on Snz1p and which only appears during stationary phase. Because the predicted molecular weight for these proteins is between 25 kDa (Sno) and 32 kDa (Snz), the complex found should include several copies of them and/or additional proteins. The two-hybrid experiments show candidates for those interacting proteins.

The first candidate is Yhr198p. No definitive function has yet been assigned to *YHR198c*, but it carries a purine/pyrimidine phosphoribosyl transferase signature, indicative of a role in the purine/pyrimidine salvage pathway. It has been described to interact with Temlp by two-hybrid analysis which itself, in turn, interacts with Snz2/3p (Uetz *et al*., 2000). Another protein that has been described to interact with the Sno-Snz proteins is Ymr322p (DIP database, 2001).

The existence of a multiprotein complex is also supported by our overexpression studies. We found that Snz1p and, specifically, Snz2p when overexpressed as 3HA-6His-tagged fusions in a non-regulated way, caused a delay in the recovery from the stationary phase. The most direct explanation for the delay is that some metabolite(s) that are exhausted during this phase but necessary to resume growth are, in this condition, more difficult to synthesize. However, the supplementary addition of the obvious candidate, pyridoxal, does not reduce the delay (Sunnerhagen P, not shown). Furthermore, the delay occurred after growth in standard (i.e. not vitamin-depleted medium). The defect caused by extra copies of tagged Snz proteins may be caused by an imbalance of the protein subunits of the complex due to of the withdrawal of some important subunit(s), which is caused by the excess subunits of Snz protein or by the 3HA tag. It has been argued that an imbalance between the putative subunits of the complex explains the dominant-negative effect of the *snz1-Δ2* mutation in 6-AU sensitivity (Padilla *et al*., 1998).

In summary, our results show that these two families, *SNZ* and *SNO*, contain genes that are only partially redundant in function in spite of their high sequence similarity. This seems to be another case in which gene duplicates have different roles in yeast (Blandin *et al*., 2000; Brookfield, 1997; Llorente *et al*., 1999).

Acknowledgements

We thank Dr J. Hoheisel for the gift of yeast macroarrays, Dr S. Hohmann for his critical reading of the manuscript and A. Llopis for her excellent technical work. B.D. is a member of the Institut Universitaire de France. This work was funded by the European Commission as part of the Eurofan 2 project under contract BIO4-CT97-2294 to B.D., J.E.P.-O., E.H. and P.S. Funding was also provided to P.S. by the Swedish Natural Science Research Council (2000-5471); to J.E.P-O. by the Spanish C.I.C.Y.T. (BIO98-1316-CE), and to E.H. from Generalitat de Catalunya (1999SGR 00170).

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Grx5 es una glutaredoxina monotiólica cuya ausencia provoca estrés oxidativo en la célula

Saccharomyces cerevisiae contiene una familia de glutaredoxinas ditiólicas, formada por Grx1 y Grx2, que intervienen en la protección contra estrés oxidativo (Luikenhuis *et al*., 1998). Dicha protección implica la reducción de los grupos tiol de las proteínas oxidadas debido al estrés, mediante un mecanismo en el que intervienen las dos cisteínas del centro activo de las glutaredoxinas (Bushweller *et al*., 1992). Las tioredoxinas Trx1 y Trx2 de levadura son también oxidoreductasas implicadas en el equilibrio redox celular. Aparentemente se podría pensar en una redundancia funcional de ambos sistemas tioredoxina y glutaredoxina. Sin embargo, al menos una tioredoxina o una glutaredoxina ditiólica es necesaria y suficiente para el crecimiento, ya que el cuádruple mutante es inviable (Draculic *et al*., 2000). Recientemente se ha demostrado que las tioredoxinas son más importantes que las glutaredoxinas en la protección de las células de la levadura contra hidroperóxidos (Garrido y Grant, 2002).

La familia de glutaredoxinas (Grx3, Grx4 y Grx5) de función inicialmente desconocida estudiada en esta memoria presenta similaridad de secuencia con glutaredoxinas de otros organismos. Estas tres glutaredoxinas se diferencian de Grx1 y Grx2 en que poseen una única cisteína en su centro activo. El análisis de las bases de datos muestra que existen glutaredoxinas monotiólicas desde bacterias hasta humanos, aunque no se ha caracterizado su función (Figuras 1 y 2, artículo 1). Una hipótesis sobre la manera que tienen estas enzimas de mantener un estatus reducido de los grupos tiol de las proteínas implicaría la única cisteína que poseen y el glutatión (Bushweller *et al*., 1992). Grx3 y Grx4 presentan un 71% de identidad de secuencia entre ellas, mientras que ambas tienen con Grx5 una identidad de secuencia del 29%.

Los mutantes simples de los genes *GRX3* y *GRX4* no muestran deficiencias de crecimiento destacables (Rodríguez-Manzaneque *et al*., 1999), y tampoco se han descrito para los mutantes *grx1* y *grx2* (Luikenhuis *et al*., 1998). En cambio, la ausencia de *GRX5* provoca un retardo en el crecimiento en medio YPD respecto de la cepa salvaje un crecimiento deficiente en medio SD y una ausencia de crecimiento en medio rico con glicerol (metabolismo respiratorio). Además, se ha observado un mayor contenido de proteínas carboniladas en células mutantes *grx5*, respecto de células salvajes y de mutantes simples de glutaredoxinas (Tabla 2, artículo 1). Este aumento en la carbonilación de proteínas se observa tanto en condiciones normales como bajo estrés

oxidativo provocado por H_2O_2 o menadiona. El contenido de proteínas carboniladas es una medida del grado de oxidación de las proteínas, por lo que estos resultados indicarían que Grx5 está implicado en respuesta a estrés oxidativo, tanto en condiciones normales como en presencia de agentes oxidantes externos. Ensayos realizados mediante la técnica de Western blot revelan que mutantes que carecen de Grx5 presentan una proteína marcadamente más oxidada que el resto (Figura 3B, artículo 1). Dicha proteína es la transcetolasa, implicada en la vía de las pentosas fosfato. Es importante remarcar que en *E. coli* la transcetolasa es específicamente inhibida en mutantes carentes de superóxido dismutasa y en condiciones de hiperoxia (Benov y Fridovich, 1999), lo que confirma la sensibilidad de esta enzima a las condiciones oxidantes.

La sensibilidad mostrada por un mutante *grx5* frente a agentes oxidantes no discrimina H_2O_2 del anión superóxido (Figura 4, artículo 1), como es el caso de las glutaredoxinas ditiólicas, en que el mutante *grx1* es sensible a estrés provocado por el anión superóxido, mientras que el mutante *grx2* es sensible a estrés provocado por H_2O_2 (Luikenhuis *et al*., 1998).

El análisis de las secuencias de esta nueva familia de glutaredoxinas indica que presentan una elevada homología con glutaredoxinas ya caracterizadas. De todas maneras, era imprescindible comprobar que la similitud estructural se correlacionaba con una similitud funcional, esto es, que Grx3, Grx4 y Grx5 tenían también actividad glutaredoxina. Para ello se realizó un ensayo de las actividades glutaredoxina en la cepa salvaje y en las diferentes cepas mutantes (Tabla 2, artículo 1). La ausencia de las glutaredoxinas monotiólicas de la levadura produce una disminución en la actividad glutaredoxina celular total, pero la disminución es más drástica cuando falta *GRX5*. Ello indica que los genes *GRX3*, *GRX4* y *GRX5* codifican para oxidoreductasas de tipo glutaredoxina en *S. cerevisiae*. Cuantitativamente, el descenso más drástico de actividad se produce cuando falta *GRX2* (Tabla 2, artículo 1). La presencia de al menos una glutaredoxina monotiólica es necesaria, ya que el triple mutante *grx3 grx4 gxr5* es inviable.

La falta de Grx5 tiene un efecto aditivo sobre la ausencia de otras enzimas protectoras frente a estrés oxidativo. La ausencia del gen *SOD1* (que codifica para una superóxido dismutasa, en principio citosólica, pero que también podría hallarse en la mitocondria) causa defectos en el crecimiento aeróbico, auxotrofías para lisina, metionina y cisteína y crecimiento muy pobre en glicerol (Gralla y Kosman, 1992;

Strain *et al*., 1998), de manera bastante similar a las deficiencias observadas en el mutante *grx5*. La doble mutación *grx5 sod1* tiene efectos aditivos en la tasa de crecimiento y en la sensibilidad a estrés oxidativo por menadiona o H_2O_2 (resultados no mostrados). Ello corroboraría el papel de Grx5 en la respuesta frente a estrés oxidativo en la misma línea que la proteína Sod1.

Se ha descrito que ciertos mutantes de levadura sensibles a estrés oxidativo lo son también a estrés osmótico, aunque se desconozcan las causas a nivel molecular (Krems *et al*., 1995). En el caso de mutantes *grx5*, se ha observado que presentan una mayor sensibilidad a KCl (Figura 5, artículo 1), y se ha comprobado que estas células no tienen defectos de pared. En levaduras, algunos de los genes afectados por estrés osmótico y oxidativo están regulados por factores de transcripción comunes, entre ellos Skn7 y Yap1 (Lee *et al*., 1999; Morgan *et al*., 1997). De todas maneras, en la región promotora de *GRX5* no se observan sitios de unión para estos factores de transcripción. En estos momentos se desconoce el significado de estos resultados, de modo que tendrán que realizarse más estudios para clarificarlo.

En cuanto a la regulación de la expresión de estos genes, *GRX3* y *GRX4* se expresan en fase exponencial (hasta bien entrada la fase post-diáuxica en el caso de *GRX4*) mientras que la expresión de *GRX5* desaparece durante el shift diáxico. Además, la expresión de estos genes no se induce bajo situaciones de estrés oxidativo, sino que parece ser constitutiva (Figura 7, artículo 1). En cambio, los niveles de proteína Grx5 en presencia de menadiona a concentraciones subletales parece aumentar unas dos veces (Figura 2B, artículo 2), lo que sugiere algún tipo de regulación post-transcripcional sobre Grx5. Nos planteamos si el estrés oxidativo endógeno generado en un mutante *grx5* era capaz de inducir la transcripción de un gen como *DDR2*, que contiene un elemento STRE en su promotor y es activado por agentes oxidantes (Marchler *et al*., 1993). Sin embargo, el resultado fue negativo. Tampoco se vio una inducción de la expresión de *GRX5* en los diferentes mutantes simples y múltiples de glutaredoxinas (resultados no mostrados), por lo que no existe un efecto compensatorio por la falta de este gen sobre la expresión de los genes de otras glutaredoxinas. De todo lo anterior podemos concluir que *GRX5* es un gen de expresión constitutiva y que su producto es activo tanto en condiciones de crecimiento normal como en presencia de agentes oxidantes.

Grx5 se encuentra en la matriz mitocondrial y está implicada en la biosíntesis de centros Fe/S

En este trabajo se ha demostrado que Grx5 está localizada en la mitocondria (Figura 2, artículo 2; Figura 4, artículo 3). Además, se ha visto que esta localización es indispensable para la correcta función de la enzima, ya que deleciones de diferentes longitudes en el extremo N-terminal de la proteína (portador de la secuencia de compartimentalización mitocondrial), que provocan la no internalización de Grx5 en la mitocondria, causan un fenotipo similar a un mutante nulo *grx5* (Figura 2, artículo 2).

Se ha identificado en *S. cerevisiae* una tioredoxina en mitocondria, Trx3, cuya función sería proteger del estrés oxidativo generado durante el metabolismo respiratorio (Pedrajas *et al*., 1999). Células carentes de *TRX3* no presentan alteraciones en la tasa de crecimiento ni en la sensibilidad a oxidantes. El doble mutante *grx5 trx3* no muestra una mayor sensibilidad a oxidantes que el mutante simple *grx5* (resultados no mostrados), lo que está a favor de una separación de funciones entre Grx5 y la Trx3 mitocondrial.

La evidencia inicial de que Grx5 está implicada en la síntesis mitocondrial de centros Fe/S derivó del hecho que la sobreexpresión de dos genes que participan en la biosíntesis de los centros Fe/S (*SSQ1* e *ISA2*) suprime los defectos del mutante *grx5* respecto del crecimiento en medio mínimo y la sensibilidad a menadiona (Figura 1, artículo 2). El proceso de síntesis de centros Fe/S en *S. cerevisiae* tiene lugar en la mitocondria (Craig *et al*., 1999; Lill y Kispal, 2000), en concordancia con la localización también mitocondrial de Grx5. Una vez sintetizados los centros Fe/S, éstos pasan a incorporarse a las apoproteínas mitocondriales, citoplasmáticas y nucleares. Los componentes de esta biosíntesis son un conjunto de proteínas, llamadas conjuntamente ISC, de algunas de las cuales se desconoce la función exacta.

De los dos genes que suprimen los defectos del mutante *grx5*, la supresión es más eficiente en el caso de *SSQ1*. Su producto es una chaperona que actuaría en la síntesis de centros Fe/S junto con otra chaperona, Jac1, y de hecho se ha visto una interacción genética entre ambos. Mutaciones con pérdida parcial de función en cualquiera de estas dos chaperonas suprimen la sensibilidad a oxígeno del mutante *sod1* (Strain *et al*., 1998). Aunque se desconoce la función exacta de ambas proteínas en la síntesis de los centros, podrían proteger los residuos de cisteína de las proteínas de la maquinaria ISC mitocondrial durante la síntesis de aquellos. Por su parte, Isa2 es homóloga de la proteína Isa1 y ambas presentan similaridad de secuencia con las

proteínas bacterianas IscA (Kaut *et al*., 2000). Isa1 e Isa2 poseen tres residuos de cisteína altamente conservados que podrían formar un motivo de unión a hierro, aunque se desconoce de qué manera actuarían. Se ha postulado que las dos proteínas pudieran formar dímeros debido a la similaridad fenotípica mostrada por los mutantes simples *isa1* e *isa2* (Mühlenhoff y Lill, 2000). Nuestros resultados, sin embargo, apoyan el hecho que Isa1 e Isa2 desempeñarían funciones no exactamente idénticas. Así, (i) sólo la sobreexpresión de *ISA2*, no de *ISA1*, suprime los defectos del mutante *grx5* (resultados no mostrados), y (ii) Grx5 interacciona físicamente, de acuerdo con la técnica del doble-híbrido, con Isa1, no con Isa2 (Figura 1 y 2, artículo 3).

Una segunda evidencia del papel de Grx5 en la síntesis de centros Fe/S es que la ausencia de aquella causa efectos fenotípicos similares a la de otras proteínas involucradas en la síntesis de los centros: acumulación de hierro en la mitocondria y disminución de las actividades enzimáticas de proteínas con centros Fe/S, tanto mitocondriales como citoplasmáticas (Figura 5, artículo 2). En la mitocondria, el exceso de hierro produce (vía reacción de Fenton) daño en el DNA mitocondrial y en las proteínas que intervienen en la fosforilación oxidativa. Ello explica que diferentes mutantes en la biosíntesis de centros Fe/S se comporten como mutantes "petite" incapaces de crecer en fuentes de carbono respirables.

Otra observación que apoya la intervención de Grx5 en la biosíntesis de centros Fe/S es que el mutante *grx5* presenta auxotrofías para los aminoácidos leucina, lisina y ácido glutámico (Figura 3C, artículo 2). Esto se explica porque dichos aminoácidos requieren enzimas con centros Fe/S en algún paso de su biosíntesis. El glutamato requiere la aconitasa (Gangloff *et al*., 1990), mientras que la síntesis de lisina necesita la enzima mitocondrial homoaconitasa (De Freitas *et al*., 2000). Auxotrofías para glutamato y lisina han sido también descritas para los mutantes *isa1* e *isa2* (Jensen y Culotta, 2000). La biosíntesis de la leucina requiere la isopropil malato isomerasa (Leu1) y se han visto deficiencias en este enzima en mutantes en muchos de los componentes de la biosíntesis de centros Fe/S (Lange *et al*., 2000). De este modo se explica la incapacidad de los mutantes *grx5* para crecer en medio definido no suplementado con los citados aminoácidos.

La acumulación de hierro en ausencia de Grx5 cabe relacionarla con la oxidación de macromoléculas celulares. Un mutante *grx5* no sólo acumula hierro en la mitocondria, sino también en el citosol, a diferencia del resto de mutantes en la biosíntesis de centros Fe/S (Figura 5C, artículo 2). Así, la disminución de las

actividades de enzimas con centros Fe/S podría ser una consecuencia de la sensibilidad de los centros Fe/S a la alta concentración de ROS acumulada en la célula, y no debida directamente a la ausencia de Grx5. Sin embargo, se ha demostrado que la reducción de los niveles de hierro libre intracelular en el mutante *grx5* hasta alcanzar niveles propios de la cepa salvaje (bien utilizando un quelante de hierro o bien reduciendo la entrada de hierro), no revierte las deficiencias en enzimas con centros Fe/S, lo que apoya que es la ausencia de Grx5 lo que produce una disminución de las actividades de estas enzimas (Figura 6, artículo 2). Este hecho, junto con la imposibilidad de suprimir los defectos de crecimiento de un mutante *grx5* en condiciones anaeróbicas o por la adición de reductores externos, confirma que Grx5 participa directamente en la síntesis de centros Fe/S (Figura 7, artículo 2).

Independientemente de que la acumulación de hierro libre intracelular en ausencia de Grx5 sea secundaria a la disfunción en la síntesis de centros Fe/S, aquella muestra diferencias con otros mutantes en dicha síntesis y con mutantes en el gen de la frataxina (*YFH1*), en los cuales la acumulación del metal parece ser exclusivamente mitocondrial. Se ha comprobado que la acumulación no es el resultado de la inducción del sistema de transporte de hierro de alta afinidad en el mutante *grx5*. Este sistema depende del activador transcripcional Aft1 (Casas *et al*., 1997). Mutantes *yfh1* presentan una acumulación de hierro mitocondrial, una disminución en citosol y una inducción elevada de los genes *FET3* y *FTR1* (Babcock *et al*., 1997). Por el contrario, la inducción de la expresión de los genes *FET3* y *FTR1* (dependiente de Aft1) es muy baja en células *grx5* (resultados no mostrados). No se conoce el mecanismo del transporte de hierro a través de la mitocondria, pero se cree que Yfh1 estaría implicada en este proceso. Quizás una proteína con centros Fe/S podría también participar en el mismo, siendo sensora de los niveles de hierro. La incapacidad para sintetizar los centros tendría, así, un efecto drástico sobre la homeostasis de hierro.

La función desempeñada por Grx5 en la biosíntesis de centros Fe/S es diferente a la propuesta para la proteína PICOT humana (homóloga de Grx5), que interviene en la señalización a través de la vía de la proteín quinasa C϶ (Isakov *et al*., 2000; Witte *et al*., 2000). Grx5 contiene un dominio PICOT-HD, aunque le falta la región N-terminal de la proteína humana, cuya homóloga sí está presente en Grx3 y Grx4. Parece claro que PICOT-HD podría ser compartido por diferentes oxidoreductasas a lo largo de la escala evolutiva, pero éstas desarrollarían diferentes funciones biológicas en la célula.

¿Cómo asociar, pues, la función primaria de Grx5 en la biosíntesis de centros Fe/S con la alta sensibilidad de un mutante *grx5* a estrés oxidativo? La hipótesis que proponemos es que la ausencia de Grx5 afectaría negativamente la síntesis de centros Fe/S, lo que viene reflejado por la disminución de la actividad de las proteínas con centros Fe/S. A continuación se podrían dar dos alternativas o incluso las dos a la vez: (i) la concentración de hierro libre aumentaría al no poder incorporarse como parte de los centros Fe/S; o (ii) la deficiencia en las actividades de las enzimas mitocondriales con centros Fe/S afectaría a un regulador de la entrada de hierro en la mitocondria que, por tanto, sería incapaz de reconocer variaciones en la concentración de hierro, con lo que la entrada de este metal quedaría desregulada. En cualquier caso, el hierro libre acumulado, vía reacción de Fenton, favorecería la formación de ROS, produciéndose el daño celular antes mencionado. Queda por explicar el porqué en células carentes de Grx5 el hierro se acumula también en el citosol.

Grx3 y Grx4 son glutaredoxinas monotiólicas con funciones claramente diferenciadas a Grx5

Varias son las evidencias que indican que Grx3 y Grx4 podrían realizar funciones diferentes a Grx5. En primer lugar, existen diferencias fenotípicas muy marcadas entre los mutantes simples, tanto en el crecimiento como en el grado de oxidación de los mutantes en condiciones normales y bajo estrés oxidativo (Figura 4, artículo 1). El marcaje de las proteínas con GFP muestra que tanto Grx3 como Grx4 se encuentran localizadas en el núcleo (Figura 4, artículo 3). Sin embargo, no se ha reconocido ningún marcador típicamente nuclear en la secuencia de estas dos proteínas. La presencia de glutaredoxinas en el núcleo no es un caso aislado, ya que se ha descrito en humanos una glutaredoxina localizada en este orgánulo (Lundberg *et al*., 2001). Ni la sobreexpresión de *GRX3* ni la de *GRX4* suprimen los defectos de un mutante *grx5* como lo hace *SSQ1* o *ISA2* (Figura 1B, artículo 2), por lo que de nuevo se hace patente que Grx5 realiza diferentes funciones en la célula respecto de Grx3 y Grx4.

La técnica del doble-híbrido nos ha mostrado una serie de proteínas que podrían tener alguna relación con la posible función de Grx4 (Tabla 1, artículo 3). Msh6 y Rtg3 se ha visto que interaccionan con Grx4 y que, al igual que Grx4, se encuentran localizadas en el núcleo. Msh6 está implicada en la reparación de bases desapareadas en el núcleo e interacciona con Msh2 para realizar esta función (Marsischky *et al*., 1996).

Realizando esta misma función está el complejo Msh2-Msh3. La posible relación de Grx4 con reparación de bases es aun desconocida y se han de realizar más estudios en este sentido. Rtg3 es un factor de transcripción con un dominio cremallera básico de leucina que se une a regiones específicas del promotor de diversos genes como *ACO1* y *CIT2*, regulando su expresión (Liu y Butow, 1999). En células que presentan una disfuncionalidad en la mitocondria este factor de transcripción se acumula en el núcleo (Sekito *et al*., 2000). Grx4 también parece interaccionar con Nth1. Aunque se trata de una proteína citosólica, la inducción de *NTH1* se produce bajo diferentes condiciones de estrés, entre ellas el oxidativo (Zähringer *et al*., 1998). Nth1 es una trehalasa que cataliza la hidrólisis de la trehalosa. La trehalosa se acumula en situaciones de estrés protegiendo del daño producido a las membranas y a proteínas (Nwaka and Holzer, 1998). El promotor de *NTH1* contiene elementos STRE de respuesta a estrés y la inducción de este gen está regulada vía Msn2/Msn4 (Zähringer *et al*., 2000). Aunque la relación propuesta entre Grx4 y Nth1 se basa en datos muy preliminares, cabría pensar en cambios en la localización de Grx4 en función del estado redox celular, de modo que en condiciones oxidantes Grx4 quizá se localizase en el citosol modulando la actividad de Nth1.

Todo lo anterior confirma que las glutaredoxinas monotiólicas podrían ser reguladoras de funciones celulares específicas que sucederían en diferentes compartimentos, las cuales dependerían del estado redox de grupos sulfidrilo de proteínas concretas.

A partir de los resultados obtenidos en este trabajo se pueden extraer las siguientes conclusiones:

Primera: *GRX3*, *GRX4* y *GRX5* forman una familia de genes que codifican para glutaredoxinas monotiólicas en la levadura *Saccharomyces cerevisiae*, las cuales poseen homólogos estructurales desde bacterias a humanos.

Segunda: Grx5 es la glutaredoxina que juega el papel más importante en *S. cerevisiae* en relación con la proliferación celular y en situaciones de estrés oxidativo y osmótico, ya que su ausencia provoca defectos fenotípicos y bioquímicos destacables.

Tercera: Al menos una de las tres glutaredoxinas monotiólicas es necesaria para la viabilidad celular de *S. cerevisiae*.

Cuarta: Un mutante *grx5* es considerablemente más sensible a estrés oxidativo por menadiona o peróxido de hidrógeno que los mutantes en las otras dos glutaredoxinas monotiólicas, al tiempo que posee un alto grado de carbonilación de proteínas celulares tanto en condiciones constitutivas como por la acción de oxidantes externos.

Quinta: La enzima transcetolasa es particularmente sensible a las condiciones oxidantes generadas en el mutante carente de Grx5.

Sexta: Grx5 se localiza en la matriz mitocondrial y es funcional sólo cuando se encuentra en el interior de este orgánulo. Grx3 y Grx4 son, en cambio, nucleares.

Séptima: Grx5 está implicada en la síntesis de centros Fe/S, proceso que, en levaduras, tiene lugar en la mitocondria.

Octava: La ausencia de Grx5 produce una acumulación de hierro tanto en el citosol como en la matriz mitocondrial, así como una disminución de la actividad de enzimas con centros Fe/S.

Novena: La sobreexpresión de *SSQ1* suprime casi totalmente los defectos del mutante *grx5*, mientras que la de *ISA2* lo hace parcialmente. El fenotipo del mutante *grx5* no se ve afectado por la sobreexpresión de las otras dos glutaredoxinas monotiólicas.

Décima: Grx5 interacciona físicamente con Isa1, aunque no con Isa2 o con otros componentes de la maquinaria de biosíntesis de los centros Fe/S.

Décimoprimera: Isa1 e Isa2, a pesar de presentar una alta homología de secuencias entre ellas, podrían llevar a cabo funciones al menos parcialmente diferentes entre ellas en relación con la síntesis de centros Fe/S.

Decimosegunda: Las auxotrofías de un mutante *grx5* para los aminoácidos leucina, lisina y glutámico son explicables en base a que algunas de las enzimas que intervienen en su síntesis poseen centros Fe/S y por tanto son inactivas en el mutante.

Decimotercera: La función primera de Grx5 es participar en la biosíntesis de centros Fe/S. El daño oxidativo acumulado en la célula cuando no está presente es consecuencia de la deficiencia en la biosíntesis de centros Fe/S y de la consiguiente acumulación de hierro libre, que genera especies reactivas de oxígeno.

Decimocuarta: Grx4 podría interaccionar físicamente con proteínas relacionadas con estrés oxidativo así como con otras proteínas nucleares.

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