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Yeast Functional Analysis Report

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

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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

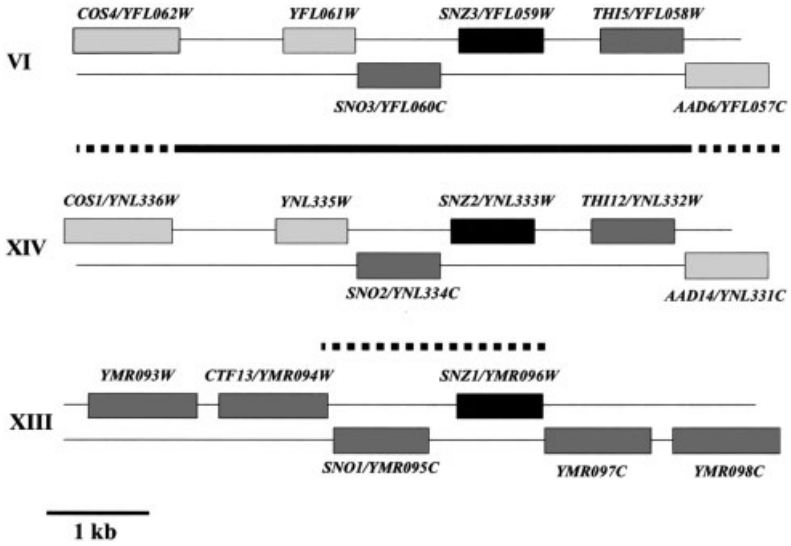

Figure 1. Chromosomal organization of *SNZ, SNO* and *THI5/12* families. Chromosomal regions from chromosomes VI, XIV (both subtelomeric) and XIII, including the gene families, are shown. Genes transcribed in 'Watson' orientation are shown in the upper line and those transcribed from the 'Crick' strand 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

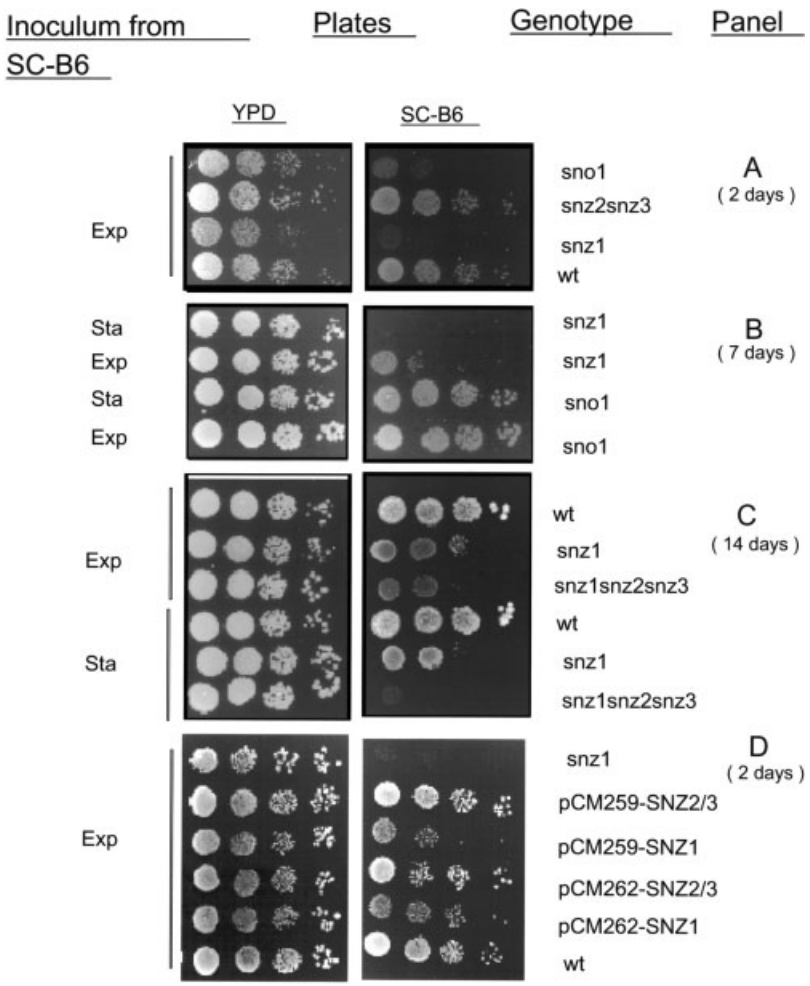

Figure 2. Growth of *snz* and *sno* mutant strains in a vitamin B_6-lacking medium. Serial four-fold dilutions of exponentially (Exp) or stationary (Sta) phase cultures (inoculum) were spotted on to YPD (control) and SC-B6 plates. Plates were incubated at 28 °C for 24–36 h (all YPD plates), 2 days [SC 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.

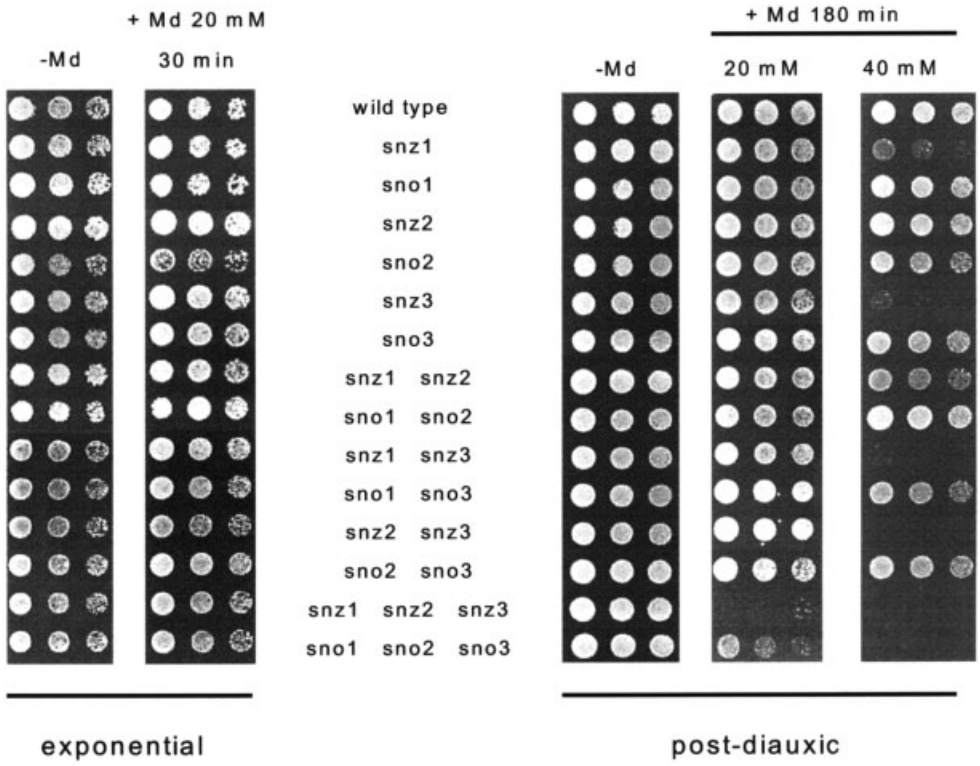

Figure 3. Menadione sensitivity of *snz* and *sno* mutant strains. Sensitivity of *snz* and *sno* mutants to menadione treatment. Cells were grown in YPD medium at 30 °C, to either exponential phase (2×10^7 cells/ml) or 20 h later (post-diauxic phase) and were treated with the indicated menadione concentrations 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.

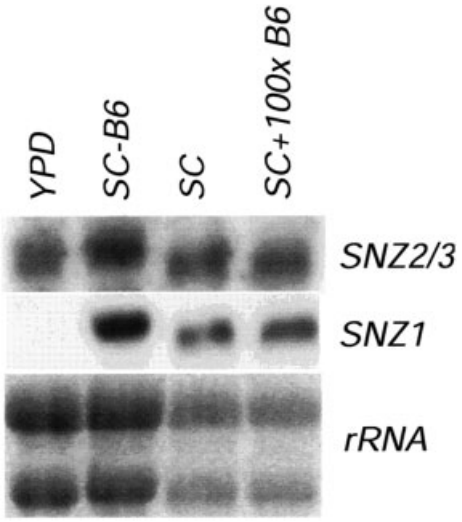

Figure 4. Influence of vitamin B_6 in *SNZ* expression. Total RNA from log phase cultures in YPD, SC (vitamin B_6 is 2 μg/ml), SC − B_6 and SC + B_6 (vitamin B_6 is 0.2 mg/ml) was analysed in a wild-type strain with a Northern experiment using *SNZ1* and *SNZ2/3* probes (made with oligonucleotides SNZ2A1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

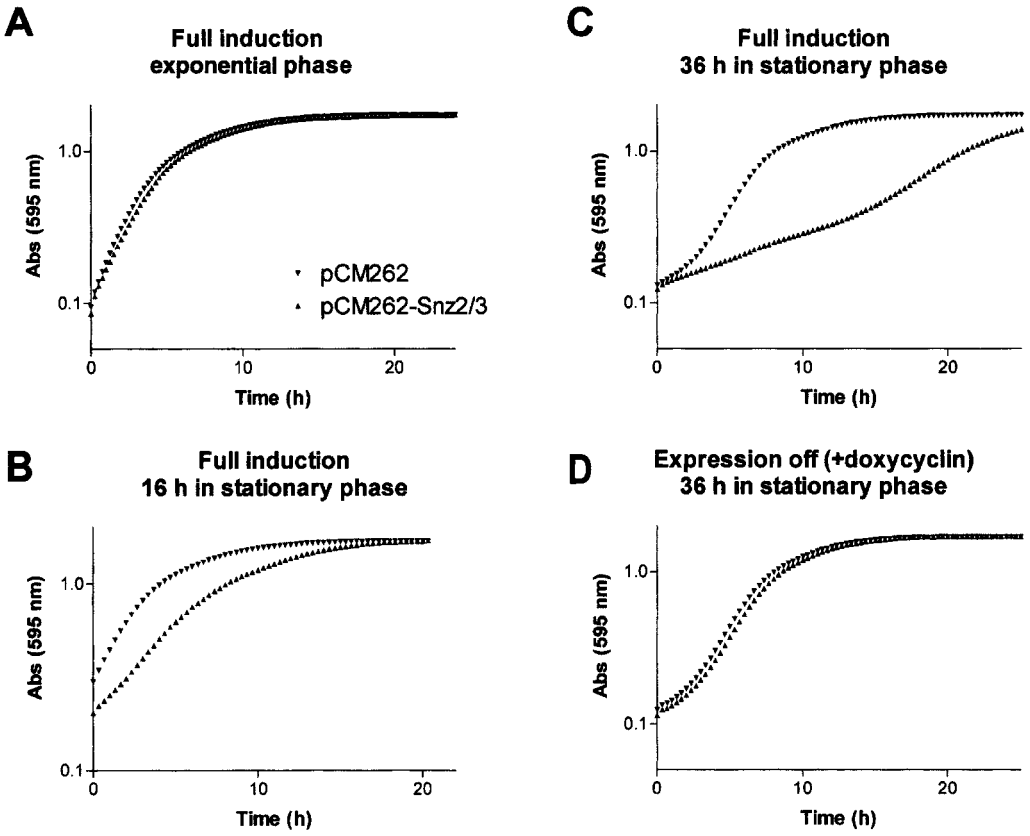

Figure 5. Growth of *SNZ2* overexpressing strains after dilution in fresh medium. W303-1A cells transformed with empty vector pCM262 (inverted triangles), pCM262-SNZ1 (not shown), or pCM262-SNZ2 (triangles) were grown in liquid SC − ura medium in the presence or absence of 2 μg/ml doxycycline, as indicated 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.

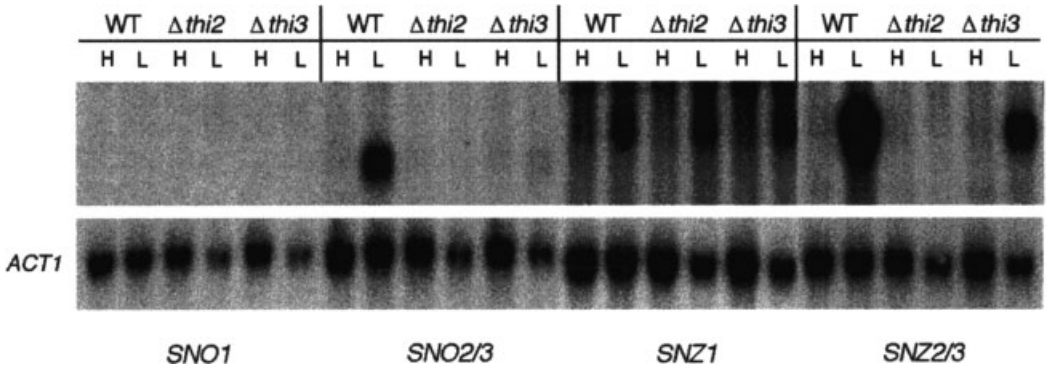

Figure 6. Gene expression analysis in thiamine-limiting conditions. 10 μg total RNA extracted from cells grown in SC to exponential phase in the presence of high (H) or low (L) extracellular thiamine concentration were loaded and run on a 1.5% agarose gel containing 0.6% formaldehyde, transferred to 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

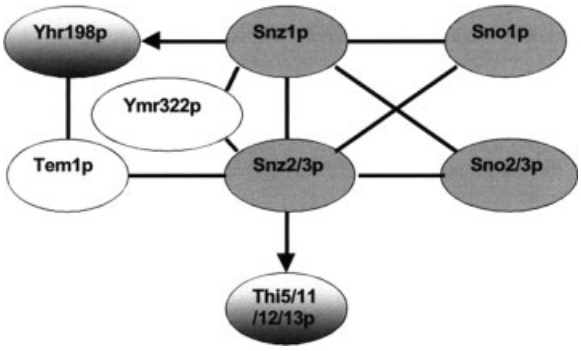

Figure 7. Summary of two-hybrid interactions of Snz, Sno and Thi families. Protein preys detected in this work are represented in degraded grey. Interacting proteins a linked by bars and arrows. Sno and Snz proteins are highlighted. See text for further discussion

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).

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