

Yeast

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Yeast Functional Analysis Report

Functional analysis of yeast gene families involved in metabolism of vitamins B₁ and B₆

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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 B₆, *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 B₆ is essential for growth, copies 2 and 3 seem more related with B₁ biosynthesis during the exponential phase. Copyright © 2002 John Wiley & Sons, Ltd.

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

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1 Introduction

2
3 Paralogous gene families, which cover the three
4 domains of life (Galperin, 2001; Goffeau *et al.*,
5 1996; Henikoff *et al.*, 1997; Rubin *et al.*, 2000)
6 represent an important part of all genomes
7 sequenced so far. They comprise ca. 40%
8 of the yeast genome (Blandin *et al.*, 2000).
9 This gene redundancy can be associated with
10 an exact functional redundancy (Brookfield,
11 1997). Examples are the rDNA genes, the
12 histone genes in species having a rapid early
13 embryonic development, such as the sea urchins
14 (Tartof, 1975) and the *CUP1* gene from
15 *Saccharomyces cerevisiae*. However, in many
16 cases, paralogous genes have undergone functional
17 specializations and are only partially redundant
18 or even functionally non-redundant. The *HSP70*
19 paralogous gene family of *S. cerevisiae* is an
20 interesting example (Boorstein *et al.*, 1994). On the
21 one hand, some of its members have overlapping
22 functions, such as *SSA1*, *SSA2* and *SSA3*. None
23 of them is essential, but the triple deletion mutant
24 exhibits a synthetic phenotype and is not viable.
25 On the other hand, other members have acquired
26 essential functions, such as *KAR2* and *SSC1*,

27 although they encode proteins very similar to the
28 *Ssa* proteins. To obtain an exhaustive overview
29 of these relationships in *S. cerevisiae*, we and
30 others have undertaken a systematic functional
31 characterization of 35 gene families containing
32 two to four members with uncharacterized or
33 poorly characterized functions (Dujon *et al.*, in
34 preparation).

35 Here we report a study of the *SNZ* and *SNO* gene
36 families of *S. cerevisiae*, each consisting of three
37 members (called 1, 2 and 3), located adjacently
38 in chromosomes XIII, XIV and VI, respectively
39 (Figure 1). *SNO2* and *SNO3* nucleotide sequences
40 are almost identical (99%), as well as *SNZ2* and
41 *SNZ3* sequences. *SNO1* and *SNZ1* sequences are
42 more divergent from their respective counterparts
43 (around 81% identical). Copies 2 and 3 are located
44 within large subtelomeric duplicated regions that
45 encompass other genes, including two members
46 of a family of thiamine (vitamin B₁) putative
47 biosynthetic enzymes: *THI5* and *THI2*. Homo-
48 logues of *SNZ* and *SNO* genes have been found
49 in a wide range of microorganisms and plants
50 (Galperin, 2001; Mittenhuber, 2001), thereby mak-
51 ing their functional analysis of general interest. The
52

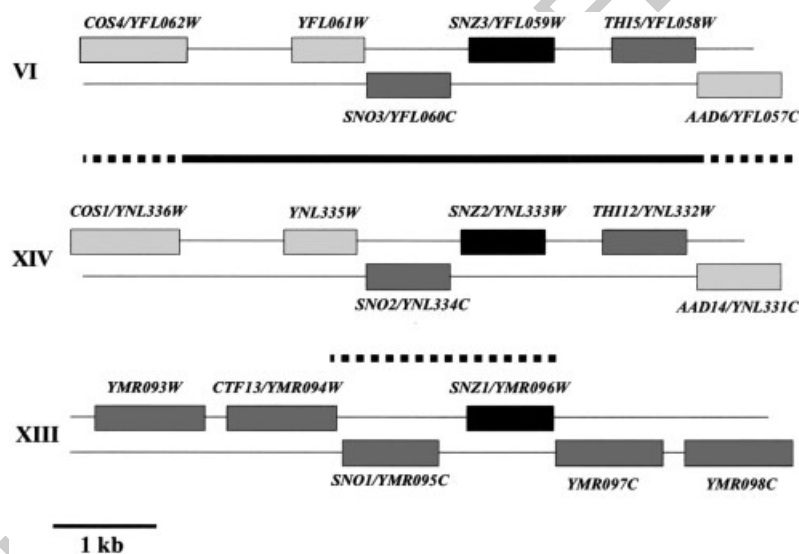


Figure 1. Chromosomal organization of *SNZ*, *SNO* and *THI5/12* families. Chromosomal regions from chromosomes VI, XIV (both subtelomeric) and XIII, including the gene families, are shown. Genes transcribed in 'Watson' orientation are shown in the upper line and those transcribed from the 'Crick' strand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

1 *SNZ* genes in yeast were originally discovered as
 2 expressed in stationary phase (Braun *et al.*, 1996),
 3 and the *SNO* genes were found as proximal and
 4 coordinately regulated with the *SNZ* genes (Padilla
 5 *et al.*, 1998). Three different studies have revealed
 6 that homologues of the *SNZ* and *SNO* genes from
 7 *Aspergillus nidulans* (Osmani *et al.*, 1999), *Neu-*
 8 *rospora crassa* (Bean *et al.*, 2001) and *Cercospora*
 9 *nicotinae* (Ehrenshaft *et al.*, 1999; Ehrenshaft and
 10 Daub, 2001) were related to the biosynthesis of
 11 pyridoxal (vitamin B₆).

12 In this study we demonstrate that *SNO1* and
 13 *SNZ1* are required for growth of yeast in the

presence of low level of intracellular vitamin B₆. 14
 We also show that transcripts of *SNO2*, *SNO3*, 15
SNZ2 and *SNZ3* are accumulated in the absence 16
 of external thiamine, as well as *THI5* and *THI11* 17
 transcripts, and that Snz proteins can interact with 18
 Thi5 and Thi12 proteins. 19

Materials and Methods 21

Yeast strains 22

The yeast strains used or constructed in this work 23
 are listed in Table 1 BY4741, BY4742, CML235 24
 25
 26

Table 1. Yeast strains used and constructed

Strain	Genotype	Source or reference
FY1679	<i>MATa</i> α , <i>ura3-52/ura3-52</i> , <i>leu2-Δ1/LEU2</i> , <i>trp1-63/TRP1</i> , <i>his3-Δ200/HIS3</i>	Thierry and Dujon, 1992
BY4741	<i>MATa</i> , <i>leu2-Δ0</i> , <i>his3-Δ1</i> , <i>met15-Δ0</i> , <i>ura3-Δ0</i>	Brachmann <i>et al.</i> , 1998
BY4742	<i>MATa</i> , <i>leu2-Δ0</i> , <i>his3-Δ1</i> , <i>lys2-Δ0</i> , <i>ura3-Δ0</i>	Brachmann <i>et al.</i> , 1998
CML235	<i>MATa</i> , <i>ura3-52</i> , <i>leu2-Δ1</i> , <i>his3-Δ200</i>	Spore from FY1679
CML236	<i>MATa</i> , <i>ura3-52</i> , <i>leu2-Δ1</i> , <i>his3-Δ200</i>	Spore from FY1679
W303-1A	<i>MATa</i> , <i>ade2-1</i> , <i>leu2-2</i> , <i>122</i> , <i>ura3-1</i> , <i>his3-11</i> , <i>trp1-1a</i> , <i>can100</i>	H. Ronne
PJ69-4a	<i>MATa</i> , <i>ade2</i> , <i>trp1-109</i> , <i>leu2-3</i> , <i>112</i> , <i>ura3-52</i> , <i>his3-200</i> , <i>gal4 Δ</i> , <i>gal80Δ</i> , <i>GAL2:ADE2</i> , <i>LYS2::GAL1:HIS3</i> , <i>met2::GAL7:lacZ</i>	James <i>et al.</i> , 1996
PJ69-4 α	<i>MATa</i> , <i>ade2</i> , <i>trp1-109</i> , <i>leu2-3</i> , <i>112</i> , <i>ura3-52</i> , <i>his3-200</i> , <i>gal4Δ</i> , <i>gal80Δ</i> , <i>GAL2::ADE2</i> , <i>LYS2::GAL1:HIS3</i> , <i>met2::GAL7:lacZ</i>	James <i>et al.</i> , 1996
BQS1029	(BY4742) <i>snz1-Δ0::LEU2</i>	This work
BQS1037	(BY4741) <i>snz1-Δ0::MET15</i>	This work
BQS1067	(BY4741) <i>snz3-Δ0::LEU2</i>	This work
BQS1068	(BY4742) <i>snz2-Δ0::URA3</i>	This work
BQS1148	(BY4742) <i>snz1-Δ0::KanMX4</i> , <i>snz2-Δ0::URA3</i>	This work
BQS1149	(BY4741) <i>snz1-Δ0::KanMX4</i> , <i>snz3-Δ0::LEU2</i>	This work
BQS1060	(BY4742) <i>snz3-Δ0::LEU2</i> , <i>snz2-Δ0::URA3</i>	This work
BQS1073	(BY4742) <i>snz3-Δ0::LEU2</i> , <i>snz2-Δ0::URA3</i> , <i>snz1-Δ0::KanMX4</i>	This work
FYBL1-8B	<i>MATa</i> , <i>ura3-Δ851</i> , <i>leu2-Δ1</i> , <i>his3-Δ200</i> , <i>lys2-Δ202</i>	Fairhead <i>et al.</i> , 1996
FYBL119-5B	<i>MATa</i> , <i>ura3-Δ851</i> , <i>leu2-Δ1</i> , <i>his3-Δ200</i> , <i>yol055c-Δ::KANMX2</i> , <i>yp1258c-Δ::KANMX2</i> , <i>yp121w-Δ::KANMX2</i>	Llorente <i>et al.</i> , 1999
FYBL1-8B/BL138	<i>MATa</i> , <i>ura3-Δ851</i> , <i>leu2-Δ1</i> , <i>his3-Δ200</i> , <i>lys2-Δ202</i> , <i>thi2-Δ::HIS3</i>	Llorente <i>et al.</i> , 1999
FYBL1-8B/BL142	<i>MATa</i> , <i>ura3-Δ851</i> , <i>leu2-Δ1</i> , <i>his3-Δ200</i> , <i>lys2-Δ202</i> , <i>thi3-Δ::HIS3</i>	Llorente <i>et al.</i> , 1999
MML21	(CML235) <i>sno1-Δ0::KanMX4</i>	This work
MML23	(CML235) <i>sno2-Δ0::KanMX4</i>	This work
MML25	(CML235) <i>sno3-Δ0::KanMX4</i>	This work
MML27	(CML235) <i>sno1-Δ0::KanMX4</i> , <i>sno2-Δ0::KanMX4</i> , <i>sno3-Δ0::KanMX4</i>	This work
MML49	(CML236) <i>sno1-Δ0::KanMX4</i> , <i>sno2-Δ0::KanMX4</i>	This work
MML50	(CML236) <i>sno2-Δ0::KanMX4</i> , <i>sno3-Δ0::KanMX4</i>	This work
MML259	(CML235) <i>sno1-Δ0::KanMX4</i> , <i>sno3-Δ0::KanMX4</i>	This work

1 and CML236 are the wild-type strains from which
2 the *snz* and *sno* mutants were obtained. All
3 strains, except for W303-1A, are from the S288c
4 genetic background.

6 Generation of multiple mutants

8 Single null mutations in the genes were gener-
9 ated by disruption with the cassettes described
10 by Wach *et al.* (1994) or Brachman *et al.* (1998).
11 Multiple mutants were generated by successive
12 transformations with different markers or by con-
13 ventional crosses. Disruptions were tested by ana-
14 lytical PCR using the adequate oligonucleotide
15 sets (Rodríguez-Navarro *et al.*, 1999; Wach *et al.*,
16 1994).

17 In the cases of *SNZ2* (YNL333W) and *SNZ3*
18 (YFL059W), their almost identical sequences pre-
19 cluded targeted disruption of each copy. *SNZ2*
20 and *SNZ3* are located in different chromosomes
21 (see Figure 1). We designed a 'blind' disruption
22 and crossing strategy using two different mark-
23 ers, *URA3* and *LEU2*, in both *MAT^a* and *MAT^α*.
24 Crosses between randomly selected *a* and *α* clones
25 with different marker were made and diploids were
26 sporulated to obtain, in some cases, *Leu⁺Ura⁺*
27 spores that, therefore, corresponded to a *snz2 snz3*
28 double mutant. Thus, the parental strains that had
29 been used for that particular cross should corre-
30 spond to single *snz2* and *snz3* mutants. The identity
31 of the single and double mutants was corroborated
32 by Southern blot after pulsed-field electrophoresis
33 (not shown). Double and triple mutants, including
34 $\Delta snz1$ deletion, were made from the single or the
35 *snz2 snz3* double mutants by disrupting *SNZ1* with
36 the *kanMX4* cassette (Wach *et al.*, 1994).

37 In the case of *SNO2* and *SNO3*, the *kanMX4*
38 cassette was used to disrupt both genes. Individual
39 mutants in each of them were distinguished by
40 Southern analysis after *ScaI-XhoI* digestion of
41 genomic DNA (a *XhoI* site is present upstream
42 of *SNO3* that is absent in the corresponding
43 *SNO2* region).

46 Growth conditions

47 The *S. cerevisiae* strains were routinely grown on
48 YPD (1% yeast extract, 2% peptone and 2% glu-
49 cose), minimal SD medium [0.67% yeast nitro-
50 gen base (YNB without amino acids, DIFCO), 2%
51

glucose, supplemented with auxotrophic require- 52
ments]; or minimal SC medium [0.67% yeast nitro- 53
gen base (YNB without amino acids, DIFCO), 54
2% glucose, supplemented with Drop-out mix 55
(DIFCO)]. Vitamin B₆-deficient medium (SC-B6) 56
was prepared by substituting the pre-mixed YNB 57
for a mixture of the same components except 58
from vitamin B₆ [biotin, pantothenic acid, nico- 59
tinic acid, thiamine, inositol, H₃BO₃, CuSO₄, KI, 60
MnSO₄, NaMoO₃, ZnSO₄, H₂KPO₄, (NH)₂SO₄, 61
MgSO₄, CaCl₂, FeCl₃, Na₂MoO₄] at the same con- 62
centrations as the DIFCO medium in 0.5 M, pH 63
6, 2-[N-morpholine]ethanesulphonic acid buffer. 64
For control experiments this medium was supple- 65
mented with vitamin B₆ to 2 µg/ml. For comple- 66
mentation analysis with pCM plasmids, SC- 67
B6 in the absence (derepressing conditions) or in 68
the presence (repressing conditions) of doxycycline 69
was used. 70

71 Thiamine-deficient medium was prepared as 71
described (Llorente *et al.*, 1999). It is identical 72
to the B₆-deficient medium but without thiamine 73
and plus vitamin B₆ to 2 µg/ml. Geneticin-resistant 74
strains were grown on YPD plates containing 75
200 mg/l geneticin (Gibco BRL). Meiosis induc- 76
tion was carried out by growing cells in YPD 77
to saturation and then in YPA (1% yeast extract, 78
2% peptone and 2% acetate) to 2 × 10⁷ cells/ml. 79
After washing cells twice with water they were 80
resuspended in sporulation medium (0.5% potas- 81
sium acetate). 82

83 For phenotypic analysis, the growth of haploid 83
mutants was checked on YP 2% glycerol and in 84
YPD containing 1.2 M NaCl, 0.8 M KCl, or 1.8 M 85
sorbitol. Cells were grown at 15 °C, 28 °C and 86
37 °C for 2–3 days or longer when necessary. 87

88 Effects of overexpression of *SNZ1* and *SNZ2/3* 88
genes on growth curves were made after trans- 89
formation of strain W303-1A to uracil prototro- 90
phy with plasmids pCM262SNZ1, pCM262SNZ2 91
or empty pCM262 vector. At all times before the 92
actual experiment, expression from the *tetO₇* pro- 93
moter was turned off by the presence of 2 µg/ml 94
doxycycline in the culture medium. To measure 95
growth curves, transformed cells were initially cul- 96
tivated in liquid SC medium lacking uracil and 97
containing doxycycline. The culture was then split 98
in two aliquots, one of which was rinsed free of 99
doxycycline and then cultured without doxycycline. 100
Growth of cells at 30 °C in 400 µl microchambers 101
with continuous shaking was measured as turbidity 102

1 in a Labsystems Bioscreen C Microbiology Work-
2 station, using a wide-band visible light filter.

3 4 5 Analysis of sensitivity to menadione

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

14 15 Northern analysis

16 Isolation of total RNA, electrophoresis, radioactive
17 or non-radioactive probe labelling, hybridization
18 and signal detection were all done as previously
19 described (Llorente *et al.*, 1999; Gallego *et al.*,
20 1997). Probes were generated by PCR amplifica-
21 tion from genomic DNA, using oligonucleotides
22 designed to amplify the entire ORF without adja-
23 cent sequences (Table 2).

Two-hybrid

25
26 A library of genomic *S. cerevisiae* DNA from the
27 *his3 ade2 gal4* strain JB974 in the Gal4 activation
28 domain (Gal4-AD) fusion vector pACT2 (13) was
29 used. The construction of this library will be
30 described elsewhere (Ramne A, Sunnerhagen P
31 *et al.*, in preparation).

32 Bait clones encoding fusions of Gal4 DNA-
33 binding domains (Gal4-DB) and proteins of inter-
34 est were constructed using homologous recom-
35 bination *in vivo* (Muhlrad *et al.*, 1992). Briefly,
36 full-length coding sequences of genes were PCR
37 amplified from total genomic *S. cerevisiae* FY1679
38 DNA, using the Roche Expand High Fidelity™
39 system and hybrid primers with 30 nucleotides of
40 homology to the gene and 17–21 nucleotides of
41 homology to sequences flanking the cloning site
42 (Table 2) of the Gal4-DB vector pGBT9 (Bartel
43 *et al.*, 1993). PCR products were co-transformed
44 with pGBT9 restricted with *Bam*HI and *Eco*R1
45 into *S. cerevisiae* PJ69-4α (James *et al.*, 1996). In
46 our hands, >90% of plasmids from tryptophan pro-
47 totrophs obtained in this manner contained an insert
48 of the correct size.

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

1 Transformants (50–100, picked at random from
2 each transformation) were pooled and used for
3 subsequent mass mating with PJ69-4a transformed
4 with the *S. cerevisiae* genomic DNA Gal4-AD
5 fusion library. Diploid cells with a functional two-
6 hybrid interaction were selected on medium lacking
7 tryptophan, leucine and histidine, and containing
8 3 mM 3-aminotriazole (3-AT) and 2 mg/l adenine.
9 The identity and reading frame of genes in prey
10 plasmids was verified by partial sequencing.

11

12 Plasmid construction

13

14 Recombinant clones containing *SNZ1* or *SNZ2*
15 open reading frames in the pCM262 plasmid vec-
16 tor were constructed by gap repair (see Table 2)
17 in *S. cerevisiae* similarly to the construction of
18 two-hybrid bait clones. pCM262 is an episomal
19 plasmid derived from pCM190 (Garí *et al.*, 1997),
20 designed to overexpress genes tagged at the C-
21 terminus with three haemagglutinin (HA) epitopes
22 and six histidine residues in tandem, under the
23 control of the *tetO₇* promoter. The synthetic 3HA-
24 6His cassette was introduced as a *PstI*-*AscI* frag-
25 ment in the polylinker of pCM190 (Rodríguez-
26 Manzanque MT, Herrero E, to be described else-
27 where). After co-transformation into FY1679 of
28 pCM262 (restricted with *PstI* and *NotI*) and PCR
29 products containing the respective ORFs, uracil
30 prototrophs were picked and checked for expres-
31 sion of full-length protein product by Western anal-
32 ysis, using anti-HA antibodies. Plasmids were then
33 recovered into *E. coli* from such yeast transfor-
34 mants and the correctness of their restriction pat-
35 terns verified.

36

37 Macroarray analysis

38

39 We used the hybridization membranes produced by
40 J. Hoheisel (Hauser *et al.*, 1998) and followed his
41 recommendations for use. Briefly, single-stranded
42 $\alpha^{33}\text{P}$ dCTP-radiolabelled complex cDNA samples
43 were synthesized by reverse transcription of the
44 same RNA extracts as for Northern blots. An
45 equimolar mix of the 12 anchored 17-mer oligonu-
46 cleotides dT₁₅(A,C,G)N was used to prime for the
47 reverse transcription reaction. 1/20th of the sam-
48 ple was run on a 5% denaturing polyacrylamide
49 gel and then exposed for 30 min onto X-ray film
50 (Kodak) to check the efficiency of labelling and the
51 extent of the reverse transcription reaction. Samples

that gave good results displayed a smear rang- 52
ing approximately from 80 to >600 nucleotides. 53
The samples were used to hybridize the mem- 54
branes in the same conditions as for Northern blots. 55
Hybridization signals were revealed with a Phos- 56
phorimager (Molecular Dynamics 445SI) after 24 h 57
exposure. Images were analysed using the XDot- 58
sReader program commercialized by COSE. For 59
each hybridization, the intensities were normalized 60
by the mean intensity of the membrane, for compar- 61
ative purposes. Only genes that showed more than 62
a three-fold increase ratio in the absence of thi- 63
amine vs. its presence, and that were differentially 64
expressed in several experiments, were considered. 65

66 Results

67 Requirement for vitamin B₆ of the different 68 mutants 69

70 The *SNZ* and *SNO* homologues *SOR1* and *PDX2*, 71
respectively, from *C. nicotinae* (Ehrenshaft *et al.*, 72
1999; Ehrenshaft and Daub 2001) and *pyroA* 73
from *A. nidulans* (Osmani *et al.*, 1999) have been 74
described as required for vitamin B₆ biosynthesis 75
in those organisms, suggesting that the *SNZ* and 76
SNO genes could be related to the same pathway 77
in yeast. To check this hypothesis, we constructed 78
all the combinations of single, double and triple 79
deletion mutants for each family. All these mutants 80
grew as well as the control strain on YP or com- 81
plete synthetic media at either 15 °C, 28 °C or 37 °C 82
and with glucose or glycerol as sole carbon sources 83
(not shown). Because vitamin B₆ is a common 84
compound of standard complete and minimum cul- 85
ture media for yeast, we assayed the growth in a 86
synthetic medium without it. We did not see any 87
major growth defect when the inoculum was pre- 88
cultured in YPD or SC. However, when those cells 89
were pre-grown in SC-B6 medium, single *snz1*, 90
sno1 mutants and *snz* triple mutants showed a 91
strong growth defect (Figure 2A, B, C). This defect 92
was more acute in *snz1* than in the *sno1* mutant. 93
Neither single *snz2*, *snz3*, *sno2* or *sno3* mutants 94
(not shown) or double *snz2 snz3* mutants showed 95
any growth defects under these conditions. Triple 96
sno mutant behaved similarly to *snz* triple mutant 97
(not shown). 98

99 Defects of the *snz1* and *sno1* mutants were 100
further analysed with regard to the growth phase 101
of the pre-culture. When the culture time was 102

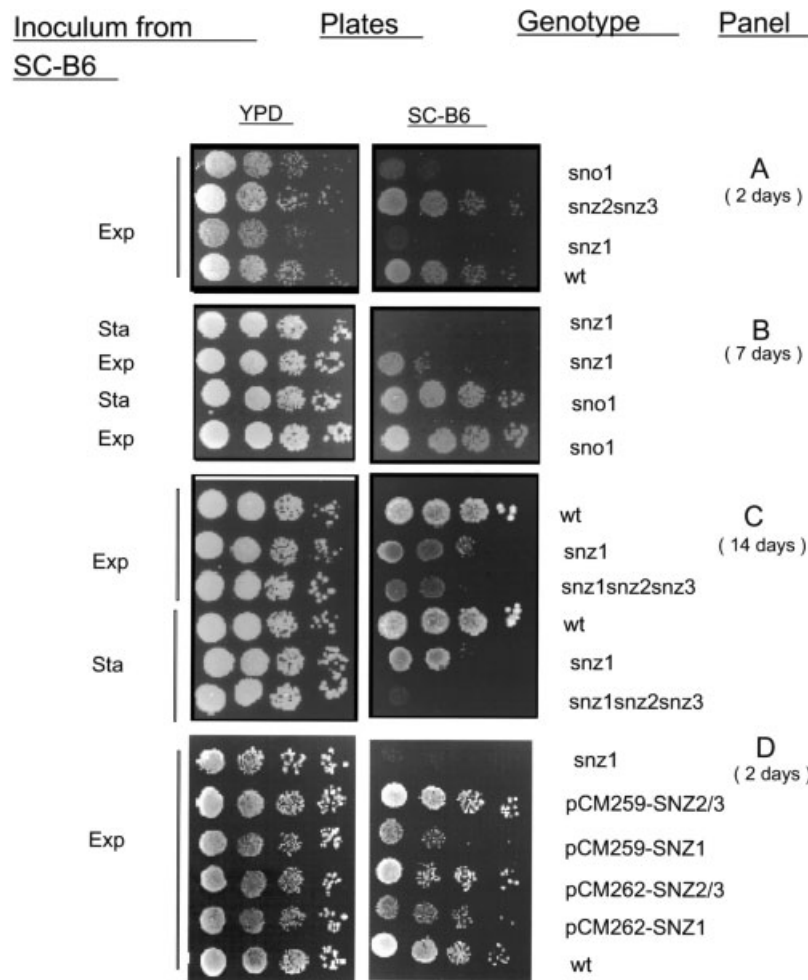


Figure 2. Growth of *snz* and *sno* mutant strains in a vitamin B₆-lacking medium. Serial four-fold dilutions of exponentially (Exp) or stationary (Sta) phase cultures (inoculum) were spotted on to YPD (control) and SC-B6 plates. Plates were incubated at 28 °C for 24–36 h (all YPD plates), 2 days [SC plates (A, D)], 7 days [SC plates (B)] or 14 days [SC plates (C)]. Complementation of the auxotrophy for vitamin B₆ 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

1 extended to 7 days, the *snz1* mutant was more
 2 severely defective than the *sno1* mutant and the
 3 defect worsened if the preculture proceeded from
 4 stationary phase (Figure 2B). Although, at first
 5 sight, the triple mutant *snz1 snz2 snz3* behaved
 6 identically to the single *snz1* mutant (not shown),
 7 very long incubation times (2 weeks, Figure 2C)
 8 revealed a stronger growth defect for the triple
 9 mutant. It is worth noting that the growth level seen
 10 in YPD control plates is similar for the wild-type
 11 and for all the mutants tested, suggesting that the
 12 viability of the cells is unaffected by the absence
 13 of those gene products during stationary phase.

The growth defect of *snz1* mutant can be
 14 complemented by the overexpression of both *SNZ1*
 15 and *SNZ2* (see Figure 2D). This result suggests that
 16 *SNZ2* and *SNZ3* code for a protein with a sim-
 17 ilar activity to Snz1p. As expected, the addition
 18 of vitamin B₆ alone also restores the growth (data
 19 not shown).
 20

In conclusion, *SNZ1* and *SNO1* are both required
 21 for growth when cells are depleted in vitamin
 22 B₆. The residual growth in SC-B6 observed for
 23 *snz1 snz2 snz3* triple mutants pre-cultured until
 24 the exponential phase probably reflects traces of
 25 vitamin B₆ in the cells. On the other hand, *SNZ2*
 26

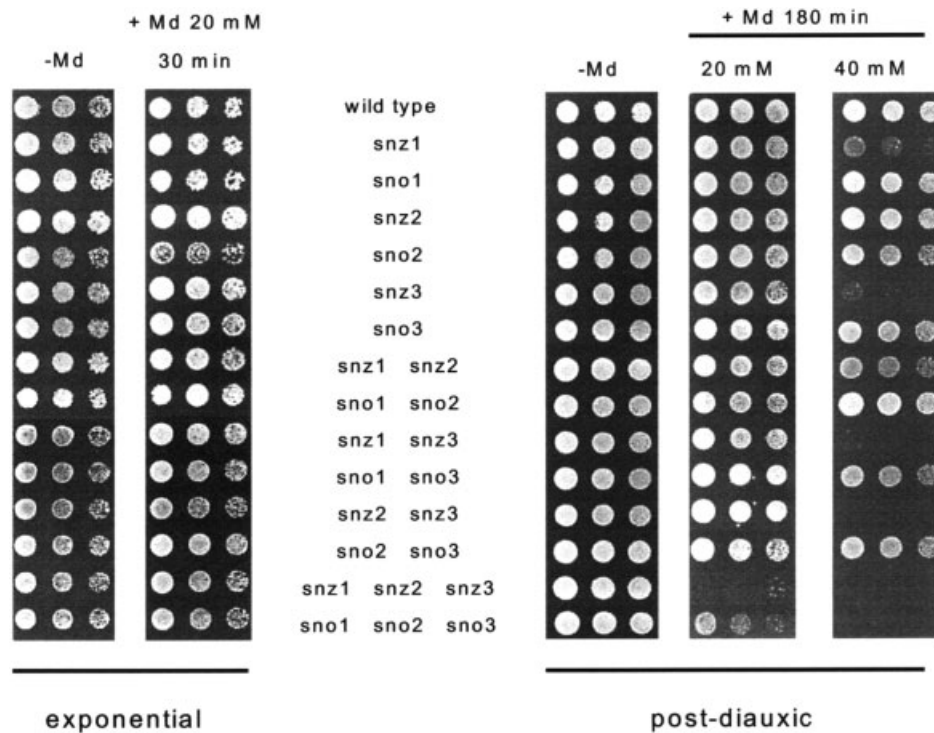


Figure 3. Menadione sensitivity of *snz* and *sno* mutant strains. Sensitivity of *snz* and *sno* mutants to menadione treatment. Cells were grown in YPD medium at 30 °C, to either exponential phase (2×10^7 cells/ml) or 20 h later (post-diauxic phase) and were treated with the indicated menadione concentrations for 30 min (exponential cells) or 180 min (post-diauxic plates). After treatments, cells were diluted in fresh YPD medium (1 : 5 serial dilutions) and 2 μ l 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

1 and *SNZ3* have no complete functional redundancy
2 with *SNZ1*, despite similar biochemical properties
3 of their products.

5 Menadione sensitivity

6
7 It has been shown previously that some of the
8 *snz* and *sno* mutants are sensitive to methylene
9 blue, a generator of singlet oxygen (Padilla *et al.*,
10 1998), one of the most active ROS (reactive oxygen
11 species). Similarly, the *SNZ*-homologous genes
12 *pyrA* and *SOR1* genes are known to protect *A.*
13 *nidulans* and *C. nicotinae*, respectively, against
14 singlet oxygen (Ehrenshaft *et al.*, 1999; Osmani
15 *et al.*, 1999).

16 We tested the sensitivity of *snz* and *sno* mutants
17 to the superoxide generator, menadione. Mena-
18 dione sensitivities of all the mutants were similar
19 to that of the control strain when treated during the
20 exponential phase for 30 min (Figure 3) or longer

(not shown). However, when treated during the
21 post-diauxic phase for 180 min with 20 mM men-
22 adione, the triple *sno1 sno2 sno3* and, especially,
23 *snz1 snz2 snz3* mutants, were extremely sensitive to
24 it. By using a higher drug concentration (40 mM) it
25 was possible to observe that the single *snz1* or *snz3*
26 and, not surprisingly, the double *snz1 snz3* and
27 *snz2 snz3* mutants were more sensitive than other
28 single or double mutants. All mutants, as well as
29 wild-type cells, were highly resistant to both drug
30 concentrations when treated during the stationary
31 phase (not shown).
32

33 These results confirm that *SNO*, and especially
34 *SNZ* genes, confer resistance to ROS to the cells.
35 These results also show that the protection effect
36 is more dependent on *SNZ1* and, surprisingly,
37 *SNZ3* than on *SNZ2*, and that this effect is masked
38 by the intrinsic resistance of advanced stationary
39 cells to environmental stresses (Werner-Washburne
40 *et al.*, 1996).

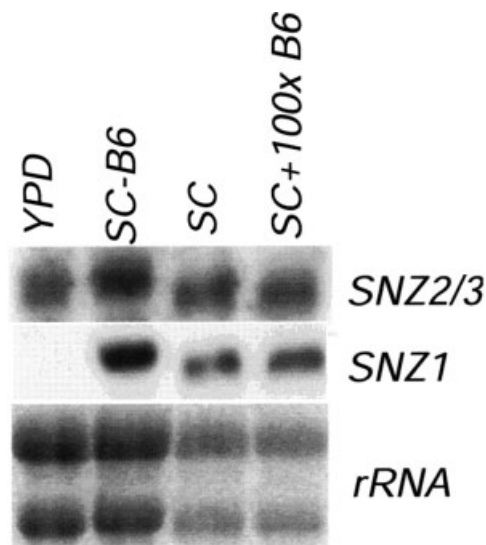


Figure 4. Influence of vitamin B₆ in SNZ expression. Total RNA from log phase cultures in YPD, SC (vitamin B₆ is 2 µg/ml), SC – B₆ and SC + B₆ (vitamin B₆ is 0.2 mg/ml) was analysed in a wild-type strain with a Northern experiment using SNZ1 and SNZ2/3 probes (made with oligonucleotides SNZ2A1 and SNZ2A4). Total rRNA is included as loading control

1 Vitamin B₆ effects on expression of SNZ genes

2 The role of SNZ and SNO genes in vitamin
3 B₆ biosynthesis suggested possible regulation of
4 their expression by this vitamin. Figure 4 shows
5 that SNZ1 expression in exponential phase is not
6 dependent on either the absence or the presence
7 of a high vitamin B₆ concentration (0.2 mg/ml),
8 although it is repressed in YPD. SNZ2–3, however,
9 are expressed approximately at the same rate in all
10 the four conditions.

13 Overexpression

14 In the course of these experiments, we noted
15 that overexpression (from the doxycycline-regu-
16 lated *tetO₇* promoter) of Snz1–3HA–6His or
17 Snz2–3HA–6His in *S. cerevisiae* strain W303-1A
18 caused a marked delay in recovery from station-
19 ary phase (Figure 5). A similar response was seen
20 in strain FY1679 (not shown). When cells overex-
21 pressing these proteins were diluted 1 : 100 from
22 saturated overnight cultures into fresh medium,
23 resumption of logarithmic growth occurred up
24 to 16 h later than for cells not overexpressing
25 either protein. This effect was clearly stronger
26

for the Snz2 fusion protein (Figure 5C) than for
52 Snz1p (not shown). Once logarithmic growth had
53 resumed, only a minor effect on growth rate was
54 seen after the longest periods in stationary phase.
55 Density at saturation was also largely unaffected.
56 As can be seen (Figure 5D), this effect is clearly
57 dependent on gene expression: at a high con-
58 centration of doxycycline, 2 µg/ml (transcription
59 repressed), no delay was observed. At interme-
60 diate concentrations (0.05 or 0.5 µg/ml), the delay
61 was less pronounced (not shown). The addition of
62 vitamin B₆ at any stage of the experiment did not
63 change the results (not shown).
64

Two-hybrid screens

65
66
67 In order to find more clues to the specific roles of
68 SNZ genes, we conducted two-hybrid screens. In
69 the first one, with the SNZ1 bait, out of 10 positive
70 clones obtained that encoded *bona fide* in-frame
71 proteins, three contained sequences of the YHR198c
72 ORF. These represented two independent clones
73 with a common overlapping segment, encoding a
74 short central portion of the Yhr198c protein (amino
75 acids 167–180).
76

77 When SNZ2 was used as the bait, YJR156c
78 (THI11) was found as reactive prey in the correct
79 reading frame. The THI11 gene product is proba-
80 bly involved in the biosynthesis of the pyrimidine
81 precursor of thiamine (Hohmann and Meacock,
82 1998), and it is homologous to the *Schizosaccha-*
83 *romyces pombe* Nmt1 protein (Van Dyck *et al.*,
84 1995). Thi11p has three paralogues in the yeast
85 genome, with almost identical sequences, Thi5p,
86 Thi12p and Thi13p. In similar screens using SNO1
87 or SNO2/3 as baits, no preys were found that cor-
88 responded to proteins involved in vitamin biosyn-
89 thesis (not shown).
90

91 Vitamin B₁ effects on gene expression

92
93 The close proximity on chromosomes VI and XIV
94 of the putative thiamine biosynthetic genes THI5
95 and THI12, respectively, to the SNZ3/SNO3 and
96 SNZ2/SNO2 loci (see Figure 1) together with the
97 results of our two-hybrid experiments (see above)
98 may suggest a possible functional link between
99 these two classes of genes. Therefore, we inves-
100 tigated the effects of vitamin B₁ depletion on the
101 transcription of the SNZ and SNO families, and on
102 the prototrophy of the corresponding null mutants.

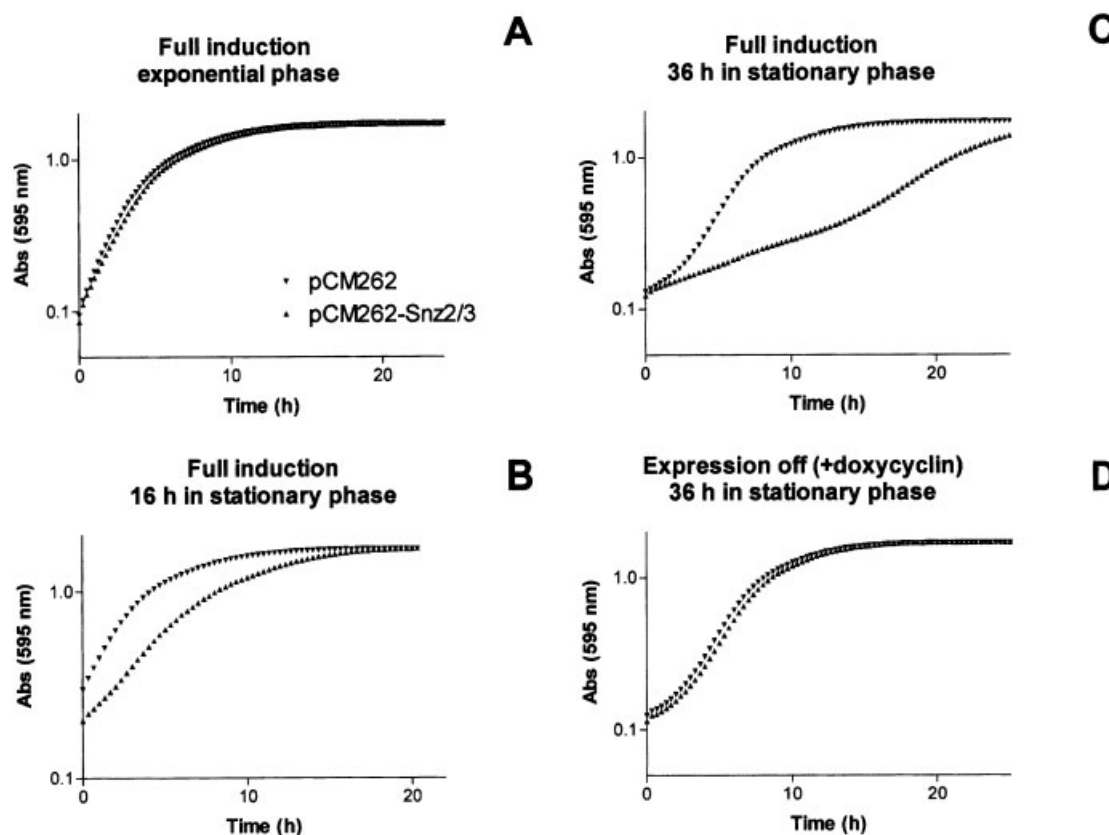


Figure 5. Growth of SNZ2 overexpressing strains after dilution in fresh medium. W303-1A cells transformed with empty vector pCM262 (inverted triangles), pCM262-SNZ1 (not shown), or pCM262-SNZ2 (triangles) were grown in liquid SC — ura medium in the presence or absence of 2 $\mu\text{g/ml}$ doxycycline, 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

1 It is known that the concentration of exogenous
 2 thiamine influences the transcript amounts of
 3 several genes involved in its metabolism, such
 4 as *THI4*, 5, 6, 10, 11, 12, 13, 20, 21, 22
 5 and *PHO3*. Moreover, this regulation is under
 6 the positive control of one or both of the
 7 two regulators Thi2p and Thi3p (Hohmann and
 8 Meacock, 1998). In order to have an exhaustive
 9 list of genes whose transcripts are regulated by
 10 the extracellular concentration of thiamine, we used
 11 macroarrays of genes produced by the J. Hoheisel
 12 laboratory (Hauser et al., 1998). Hybridizations
 13 were performed using complex cDNA samples
 14 synthesized from RNAs of the wild-type strain
 15 FYBL1-8B, grown in the presence of high
 16 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

Table 3. Yeast transcripts regulated by extracellular thiamine

Genes		Induction	$\Delta thi2$	$\Delta thi3$	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 $\Delta thi2$ and $\Delta thi3$ 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.

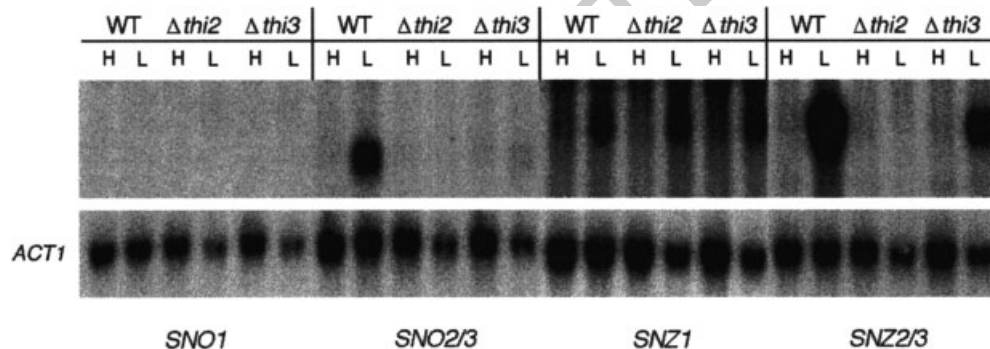


Figure 6. Gene expression analysis in thiamine-limiting conditions. 10 μ g total RNA extracted from cells grown in SC to exponential phase in the presence of high (H) or low (L) extracellular thiamine concentration were loaded and run on a 1.5% agarose gel containing 0.6% formaldehyde, transferred to 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 μ M extracellular thiamine; L, no thiamine for the wild-type strain (WT) FYBL1-8B, and 10^{-2} μ M for the auxotrophic strains $\Delta thi2$ (FYBL1-8B/BL138) and $\Delta thi3$ (FYBL1-8B/BL142). *ACT1* was used as loading control for all the samples

1 other transcripts is dependent on the presence of
 2 either Thi2p or Thi3p or both. These results were
 3 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

4
 5
 6

1 here because of the very stringent criteria used for
2 significance levels (see Materials and methods) but
3 were detected as induced by Northern blot. It is
4 impossible to conclude whether both the transcripts
5 of *SNO2* and *SNO3* are regulated in the same
6 way because their nucleotide sequences are nearly
7 identical, which must generate cross-hybridization.
8 This is also the case for *SNZ2* and *SNZ3*, as well
9 as for the *PHO* gene family, of which only the
10 transcripts of *PHO3* have been described as being
11 regulated by extracellular thiamine concentration
12 (Nishimura *et al.*, 1992).

13 The Northern blot experiments shown in Figure 6
14 illustrate that the *SNO1* transcripts are unde-
15 tectable with this approach during exponential
16 growth phase, and remain unaffected by the extra-
17 cellular concentration of thiamine. *SNO2–3* tran-
18 scripts are detectable during the exponential growth
19 phase only in the absence of extracellular thiamine,
20 in a *THI2–3*-dependent manner. *SNZ1* transcripts
21 are slightly more abundant during the exponential
22 growth phase in the presence of low concentra-
23 tion of extracellular thiamine with respect to high
24 concentration, but this accumulation is not depen-
25 dent on either Thi2p or Thi3p. *SNZ2–3* transcripts
26 accumulation occurs clearly in the absence of extra-
27 cellular thiamine, and is completely dependent on
28 Thi2p and only partially dependent on Thi3p.

29

30

31 Discussion

32

33 Role of *SNZ* and *SNO* genes in vitamin B₆ 34 biosynthesis

35 Most microorganisms and plants possess at least
36 one pathway leading to pyridoxine and pyridoxal
37 5'-phosphate synthesis. The *Escherichia coli* path-
38 way has been extensively studied (see Drewke and
39 Leistner, 2001) but the corresponding pathway in
40 yeast is not well defined. Nevertheless, the fact that
41 the L-[amide-¹⁵N]glutamine label is incorporated
42 efficiently into pyridoxine in *S. cerevisiae* but not
43 in *E. coli* indicates that vitamin B₆ biosynthesis
44 must be significantly different in either organism
45 (Tazuya *et al.*, 1995).

46 Other eukaryotic organisms, such as *A. nidu-*
47 *lans*, *Mucor racemosus* and *N. crassa* and prokary-
48 otes, such as *Staphylococcus aureus* and *Bacillus*
49 *subtilis*, may have biosynthetic pathways for pyri-
50 doxine similar to that of *S. cerevisiae* (Tanaka
51 *et al.*, 2000). It has recently been shown that the

pyroA gene from *A. nidulans* (Osmani *et al.*, 1999) 52
and the *PDX1* (*SOR1*) gene from *C. nicotinae* 53
(Ehrenshaft *et al.*, 1999) are involved in *de novo* 54
biosynthesis of vitamin B₆ in those fungi. It has 55
been suggested that mutations in *pdx-1* and *pdx-* 56
2 (*SNZ* and *SNO* homologues, respectively) cause 57
pyridoxine auxotrophy in *N. crassa* (Bean *et al.*, 58
2001). A more recent study shows an involve- 59
ment of *PDX2* of *C. nicotiana* in the pyridoxine 60
biosynthesis pathway (Ehrenshaft and Daub, 2001). 61
The fungal genes are 58–67% (*SNZ*) and 36–38% 62
(*SNO*) identical to the yeast genes. The *SNZ* and 63
SNO families are widely represented in eubacte- 64
ria, archaea and eukaryotes (Braun *et al.*, 1996; 65
Ehrenshaft *et al.*, 1999; Galperin, 2001; Mittenhu- 66
ber, 2001). Our results indicate that the *SNZ* and 67
SNO gene families are also involved in the vita- 68
min B₆ biosynthesis in *S. cerevisiae*. We identified 69
a functional specialization within these two gene 70
families, since the absence of copy 1 leads to a 71
more severe growth phenotype than the absence 72
of copy 2 and 3 when cells are grown in SC-B6. 73
Although the *SNZ2–3* genes seem to be dispens- 74
able for any condition tested, they should code for 75
proteins with a similar activity to Snz1p because 76
they can complement, at least as efficiently as *SNZ1* 77
itself (Figure 2D), the *snz1* null phenotype when 78
overexpressed. This suggests that all the three *SNZ* 79
genes code for enzymes involved in vitamin B₆ 80
biosynthesis, probably at the last step, the ring 81
closure, as suggested by Ehrenshaft *et al.* (1999). 82
Vitamin B₆, however, seems not to be a regula- 83
tor of *SNZ* gene transcription (Figure 4), although 84
the high transcription levels already present in syn- 85
thetic medium might mask the effect of the absence 86
of the vitamin. 87

88 The phenotypes of *sno* mutants are less 89
pronounced than that of the corresponding *snz* 90
mutants. It has been suggested that *SNO* genes are 91
involved in the first step of pyridoxal biosynthesis 92
(Ehrenshaft and Daub, 2001; Osmani *et al.*, 1999). 93
Thus, it is conceivable that, if the *SNZ* genes code 94
for pyridoxine biosynthetic enzymes, the substrate 95
for Snz proteins could be produced in the absence 96
of Sno proteins, although in very minor amounts, 97
by alternative pathways.

98 *SNZ* and *SNO* genes have an interesting effect 99
on sensitivity to oxidative radicals. Padilla *et al.* 100
(1998) have shown that *sno1* or *snz1* mutations 101
are very sensitive to the singlet oxygen generator 102
methylene blue. Furthermore, it has been shown

1 that B₆ vitamers are efficient quenchers of sin-
2 glet oxygen *in vitro* (Bilski *et al.*, 2000; Ehrenshaft
3 and Daub, 2001) and that externally added vitamin
4 B₆ effectively suppresses the toxicity of methy-
5 lene blue in *A. nidulans* (Osmani *et al.*, 1999). We
6 have shown here that a different kind of ROS, the
7 superoxide radical, produced by menadione, has
8 a similar effect on *snz/sno* mutants. It has been
9 argued that B₆ can act in active oxygen resistance
10 and that such as a protective effect is more neces-
11 sary in stationary phase, when cells are subjected to
12 increased oxidative stress (Ehrenshaft *et al.*, 1999).
13 Vitamin B₆ may be just an antioxidant or, per-
14 haps, its destruction by ROS causes deficiency of
15 B₆ vitamers that are necessary for other metabolic
16 uses (Osmani *et al.*, 1999). This last hypothesis
17 is supported by the fact that neither H₂O₂ nor
18 menadione induce the transcription of any of these
19 genes (Gasch *et al.*, 2000). Furthermore, prelim-
20 inary data from the groups of Joaquim Ros and
21 Enrique Herrero (University of Lleida, Spain) indi-
22 cate that addition of vitamin B₆ reduces the level
23 of protein carbonylation (a parameter measuring
24 protein oxidation) induced by addition of oxidants
25 such as menadione or hydrogen peroxide. Finally,
26 the striking difference between menadione sensitiv-
27 ity of *snz2* and *snz3* (see Figure 3) suggests that
28 the two genes have somewhat different roles, in
29 spite of their high sequence similarity.

31 Integration of biosynthetic pathways for 32 vitamins B₁ and B₆

33 It is known that in some prokaryotes (e.g. *E.*
34 *coli*), some precursors and enzymes (e.g. the *pdxK*
35 gene product) are shared by biosynthetic path-
36 ways for the vitamins B₁ and B₆ (Begley *et al.*,
37 1999; Mittenhuber, 2001). In *S. cerevisiae* the fact
38 that the transcription of the *SNZ2-3* and *SNO2-3*
39 genes is induced by the absence of thiamine in a
40 Thi2p/Thi3p-dependent manner, suggests that those
41 genes have a function related with the biosynthesis
42 of vitamin B₁ as well. Two additional facts support
43 this view.

44 First, the finding of interactions between Thi and
45 Snz proteins by two-hybrid analysis (see below)
46 provides an independent suggestion for a common
47 functional pathway for Snz and Thi proteins, i.e.
48 a putative role for the Snz, and also Sno, proteins
49 in thiamine biosynthesis, and corroborates the idea
50 of Hohmann and Meacock (1998). Our results sug-
51 gest, however, that Snz2-3 and Sno2-3 proteins,

but not Snz1p and Sno1p, are related to vitamin
B₁ biosynthesis: *SNZ1* and *SNO1* transcription is
not induced by thiamine depletion. Although all the
members of Snz and Sno protein families can reason-
ably be considered as putative enzymes acting
on the same reactions, it cannot be dismissed that
they may act at different times or cellular com-
partments, similarly to other cases of isoenzymes
(Matthews *et al.*, 2000). Several pathways for thi-
amine 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, Pérez-Ortín, not shown).
This is consistent with the fact that under anaero-
bic 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 pro-
tein 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 thi-
amine molecule (Hohmann and Meacock, 1998).
It is therefore interesting that three consecutive
genes repeated twice in the genome have a com-
mon transcriptional response. The three couples
SNO/SNZ share divergently transcribed promoters,
something that supports the notion of common reg-
ulatory sequences (Padilla *et al.*, 1998). This head-
to-head arrangement has been also found in the
yeasts *Candida albicans* and *S. kluyveri* (Llorente
B, unpublished), in the fungus *N. crassa* (Bean
et al., 2001), and in the sponge *Suberites domuncu-*
lata (Seack *et al.*, 2001) that suggests an ancient
origin for *SNO-SNZ* co-regulation. The close asso-
ciation 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 syn-
tenic 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

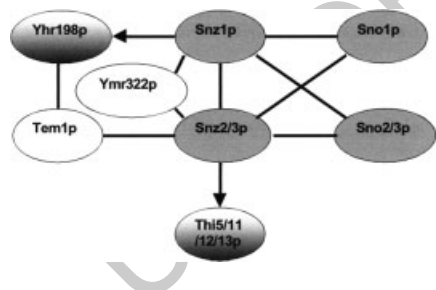
1 duplication, since they are not detected in some *S.*
2 *cerevisiae* strains (Padilla *et al.*, 1998). The func-
3 tional specialization of copies 2 and 3 and their
4 association with *THI5* locus may thus have devel-
5 oped recently.

7 A protein complex of Snz, Sno and Thi proteins

8 As discussed above, our two-hybrid results show
9 that Snz1p interacts with Yhr198p and Snz2/3p
10 with Thi11p. Previous two-hybrid analysis showed
11 interactions between the Snz and Sno proteins
12 themselves and with other proteins (DIP database,
13 2001; Ito *et al.*, 2000; Padilla *et al.*, 1998; Uetz
14 *et al.*, 2000). A scheme of the putative interactions
15 is shown in Figure 7.

16 Examples of multi-enzymatic complexes comprising
17 enzymes that catalyse successive steps in a
18 metabolic pathway are well known (Matthews
19 *et al.*, 2000). It seems that every one of the Snz
20 and Sno proteins can interact with itself and with
21 the other members. Because the transcription pro-
22 files of copies 2 and 3, on one hand, and copy 1,
23 on the other hand, are so different that it is possi-
24 ble that the protein complex changes depending on
25 the physiological circumstances of the cell. In fact,
26 Padilla *et al.* (1998) described a 230 kDa complex
27 dependent on Snz1p and which only appears during
28 stationary phase. Because the predicted molecu-
29 lar weight for these proteins is between 25 kDa
30 (Sno) and 32 kDa (Snz), the complex found should
31 include several copies of them and/or additional
32 proteins. The two-hybrid experiments show candi-
33 dates for those interacting proteins.

34 The first candidate is Yhr198p. No definiti-
35 ve function has yet been assigned to *YHR198c*,
36 but it carries a purine/pyrimidine phosphoribosyl
37



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

transferase signature, indicative of a role in the
purine/pyrimidine salvage pathway. It has been
described to interact with Tem1p 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 over-
expressed as 3HA-6His-tagged fusions in a non-
regulated way, caused a delay in the recovery
from the stationary phase. The most direct expla-
nation for the delay is that some metabolite(s) that
are exhausted during this phase but necessary to
resume growth are, in this condition, more dif-
ficult 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 pro-
teins 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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References

- 1
2
3 Bartel PL, Chien C, Sternglanz R, Fields S. 1993. Using the two-
4 hybrid system to detect protein–protein interactions. In *Cellular*
5 *Interactions in Development: a Practical Approach*, Hartley D
6 (ed.). Oxford University Press: Oxford; 153–179.
7 Bean LE, Dvorachek WH Jr, Braun EL, *et al.* 2001. Analysis
8 of the *pdx-1 (snz-1/sno-1)* region of the *Neurospora crassa*
9 genome: correlation of pyridoxine-requiring phenotypes with
10 mutations in two structural genes. *Genetics* **157**: 1067–1075.
11 Begley TP, Downs DM, Ealick SE, *et al.* 1999. Thiamine
12 biosynthesis in prokaryotes. *Arch Microbiol* **171**: 293–300.
13 Bilski P, Li MY, Ehrenshaft M, Daub ME, Chignell CF. 2000.
14 Vitamin B₆ (pyridoxine) and its derivatives are efficient singlet
15 oxygen quenchers and potential fungal antioxidants. *Photochem*
16 *Photobiol* **71**: 129–300.
17 Blandin G, *et al.* 2000. Genomic exploration of the *Hemias-*
18 *comycetes* yeasts: 4. The genome of *Saccharomyces cerevisiae*
19 revisited. *FEBS Lett* **487**: 31–36.
20 Boorstein WR, Ziegelhoffer T, Craig EA. 1994. Molecular
21 evolution of the *HSP70* multigene family. *J Mol Evol* **38**: 1–17.
22 Brachmann CB, Davies A, Cost GJ, *et al.* 1998. Designer deletion
23 strain derived from *Saccharomyces cerevisiae* S288C: a useful
24 set of strains and plasmids for PCR-mediated gene disruption
25 and other applications. *Yeast* **14**: 115–132.
26 Braun EL, Fuge EK, Padilla PA, Werner-Washburne M. 1996. A
27 stationary-phase gene in *Saccharomyces cerevisiae* is a member
28 of a novel, highly conserved gene family. *J Bacteriol* **178**:
29 6865–6872.
30 Brookfield JF. 1997. Genetic redundancy. *Adv Genet* **36**: 137–155.
31 Burke D, Dawson D, Stearns T. 2000. *Methods in Yeast Genetics*.
32 Cold Spring Harbor Laboratory Press: New York.
33 DIP database 2001. <http://dip.doe-mbi.ucla.edu/>
34 Drewke C, Leistner E. 2001. Biosynthesis of vitamin B₆ and
35 structurally related derivatives. *Vit Horm* **61**: 121–155.
36 Durfee T, Becherer K, Chen P-L, *et al.* 1993. The retinoblastoma
37 protein associates with the protein phosphatase type 1 catalytic
38 subunit. *Genes Dev* **7**: 555–569.
39 Ehrenshaft M, Daub ME. 2001. Isolation of *PDX2*, a second novel
40 gene in the pyridoxine biosynthesis pathway of eukaryotes,
41 archaeobacteria and a subset of eubacteria. *J Bacteriol* **183**:
42 3383–3390.
43 Ehrenshaft M, Bilski P, Li MY, Chignell CF, Daub ME. 1999.
44 A highly conserved sequence is a novel gene involved in *de*
45 *novo* vitamin B₆ biosynthesis. *Proc Natl Acad Sci USA* **96**:
46 9374–9378.
47 Fairhead C, Llorente B, Denis F, Soler M, Dujon B. 1996. New
48 vectors for combinatorial deletions in yeast chromosomes and
49 for gap-repair cloning using ‘split-marker’ recombination. *Yeast*
50 **12**: 1439–1457.
51 Gallego C, Garí E, Colomina N, Herrero E, Aldea M. 1997. The
Cln3 cyclin is downregulated by translational repression and
degradation during G₁ arrest caused by nitrogen deprivation in
budding yeast. *EMBO J* **16**: 7196–7206.
Galperin MY. 2001. Conserved ‘hypothetical’ proteins: new hints
and new puzzles. *Comp Funct Genom* **2**: 14–18.
Garí E, Piedrafita L, Aldea M, Herrero E. 1997. A set of
vectors with a tetracycline-regulatable promoter system for
modulated gene expression in *Saccharomyces cerevisiae*. *Yeast*
13: 837–848.
Gasch AP, Spellman PT, Kao CM, *et al.* 2000. Genomic
expression programs in the response of yeast cells to
environmental changes. *Mol Biol Cell* **11**: 4241–4257. See also
<http://genome-www.stanford.edu/yeast.stress/>.
Goffeau A, Barrell BG, Bussey H. 1996. Life with 6000 genes.
Science **274**: 563–567.
Hauser NC, Vingron M, Scheideler M, *et al.* 1998. Transcriptional
profiling on all open reading frames of *Saccharomyces*
cerevisiae. *Yeast* **14**: 1209–1221.
Henikoff S, Greene E, Pietrokovski S, Bork P, Attwood TK,
Hood L. 1997. Gene families: the taxonomy of protein paralogs
and chimeras. *Science* **278**: 609–614.
Hohmann S, Meacock PA. 1998. Thiamine metabolism and thi-
amine diphosphate-dependent enzymes in the yeast *Saccha-*
romyces cerevisiae: genetic regulation. *Biochim Biophys Acta*
1385: 201–209.
Ito T, Tashiro K, Muta S, *et al.* 2000. Toward a protein–protein
interaction map of the budding yeast: a comprehensive system
to examine two-hybrid interactions in all possible combinations
between the yeast proteins. *Proc Natl Acad Sci USA* **97**:
1143–1147.
James P, Halladay J, Craig EA. 1996. Genomic libraries and a host
strain designed for highly efficient two-hybrid selection in yeast.
Genetics **144**: 1425–1436.
Llorente B, Fairhead C, Dujon B. 1999. Genetic redundancy and
gene fusion in the genome of baker’s yeast, *Saccharomyces*
cerevisiae: functional characterization of a three-member gene
family involved in the thiamine biosynthetic pathway. *Mol*
Microbiol **32**: 1140–1152.
Matthews CK, Van Holde K, Ahern KG. 2000. *Biochemistry*, 3rd
edn. Addison-Wesley: San Francisco, CA.
Mittenhuber G. 2001. Phylogenetic analyses and comparative
genomics of vitamin B₆ (pyridoxine) and pyridoxal phosphate
biosynthesis pathways. *J Mol Microbiol Biotechnol* **3**: 1–20.
Muhlrad D, Hunter R, Parker R. 1992. A rapid method for
localized mutagenesis of yeast genes. *Yeast* **8**: 79–82.
Muller EH, Richards EJ, Norbeck J, *et al.* 1999. Thiamine repres-
sion and pyruvate decarboxylase autoregulation independently
control the expression of the *Saccharomyces cerevisiae* *PDC5*
gene. *FEBS Lett* **449**: 245–250.
Nishimura H, Kawasaki Y, Kaneko Y, Nosaka K, Iwashima A.
1992. Cloning and characteristics of a positive regulatory
gene, *THI2 (PHO6)*, of thiamine biosynthesis in *Saccharomyces*
cerevisiae. *FEBS Lett* **297**: 155–158.
Osmani AH, May GS, Osmani SA. 1999. The extremely con-
served *pyroA* gene of *Aspergillus nidulans* is required for pyri-
doxine synthesis and is required indirectly for resistance to
photosensitizers. *J Biol Chem* **274**: 23 565–23 569.
Padilla PA, Fuge EK, Crawford ME, Errett A, Werner-
Washburne M. 1998. The highly conserved, co-regulated *SNO*
and *SNZ* gene families in *Saccharomyces cerevisiae* respond to
nutrient limitation. *J Bacteriol* **180**: 5718–5726.
Rodríguez-Navarro S, Estruch F, Pérez-Ortín JE. 1999. Functional
analysis of 12 ORFs from *Saccharomyces cerevisiae* chromo-
some II. *Yeast* **15**: 913–919.
Rubin GM, *et al.* 2000. Comparative genomics of the eukaryotes.
Science **287**: 2204–2215.
Seack J, *et al.* 2001. Identification of highly conserved genes:
SNZ and *SNO* in the marine sponge *Suberites domunculata*:
their gene structure and promoter activity in mammalian cells.
Biochim Biophys Acta **1520**: 21–24.

1	Souciet J-L, et al. 2000. Genolevures. <i>FEBS Lett</i> 487 : 1–149.	52
2	Tanaka K, Tazuya K, Yamada K, Kumaoka H. 2000. Biosynthesis	53
3	of pyridoxine: origin of the nitrogen atom of pyridoxine in	54
4	microorganisms. <i>J Nutr Sci Vitaminol</i> 46 : 55–57.	55
5	Tanaka K, Tazuya K, Yamada K, Kumaoka H. 2000. Biosynthesis	56
6	of thiamine under anaerobic conditions in <i>Saccharomyces</i>	57
7	<i>cerevisiae</i> . <i>Biol Pharm Bull</i> 23 : 108–111.	58
8	Tartof KD. 1975. Redundant genes. <i>Ann Rev Genet</i> 9 : 355–385.	59
9	Tazuya K, Adachi Y, Masuda K, Yamada K, Kumaoka H. 1995.	60
10	Origin of the nitrogen atom of pyridoxine in <i>Saccharomyces</i>	61
11	<i>cerevisiae</i> . <i>Biochim Biophys Acta</i> 1244 : 113–116.	62
12	Thierry A, Dujon B. 1992. Nested chromosomal fragmentation	63
13	in yeast using the meganuclease I-Sce I: a new method for	64
14	physical mapping of eukaryotic genomes. <i>Nucleic Acids Res</i> 21 :	65
15	5625–5631.	66
16	Uetz P, et al. 2000. A comprehensive analysis of protein–protein	67
17	interaction in <i>Saccharomyces cerevisiae</i> . <i>Nature</i> 403 : 623–627.	68
18		69
19		70
20		71
21		72
22		73
23		74
24		75
25		76
26		77
27		78
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