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Disruption of Six Novel *Saccharomyces cerevisiae* Genes Reveals that *YGL129c* is Necessary for Growth in Non-Fermentable Carbon Sources, *YGL128c* for Growth at Low or High Temperatures and *YGL125w* is Implicated in the Biosynthesis of Methionine

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Six open reading frames (ORFs) from chromosome VII, *YGL131c*, *YGL129c*, *YGL128c*, *YGL125w*, *YGL124c* and *YGL121c*, were disrupted by deletion cassettes with short flanking regions homologous to the target locus (SFH). *YGL129c* is necessary for growth in non-fermentable carbon sources, *YGL128c* for growth at low or high temperatures and *YGL125w* is implicated in the biosynthesis of methionine. With regard to the other ORFs, basic phenotypic analyses did not reveal any significant clues about their function. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — gene disruption; functional analysis; *Saccharomyces cerevisiae*; DnaJ; methylenetetrahydrofolate reductase

INTRODUCTION

The genome of the yeast *Saccharomyces cerevisiae* was systematically sequenced through an international effort (Goffeau *et al*., 1996). The complete sequence was carried out public in 1996 and its primary analysis revealed the existence of 6000 protein-coding genes. The data obtained confirmed several characteristics of the genome that were already known, and shed light on others that were totally unknown. The most interesting and striking feature was the evidence of the high number of open reading frames (ORFs) which had not been previously characterized by conventional methods; these orphan genes represent 30–35% of the yeast genome and the majority of them are probably actual genes whose functions remain to be determined experimentally.

The European Union has set up the EUROFAN network for systematic functional analysis of the novel yeast genes (Oliver, 1996). As part of the EUROFAN six-pack programme, we report here the generation of specific deletant strains of six novel genes located on chromosome VII and the results obtained in the basic phenotypic analysis. Five of the six ORFs analysed (*YGL131c*, *YGL129c*, *YGL128c*, *YGL125w* and *YGL124c*) have been sequenced in our laboratory and their main characteristics previously reported (Rodríguez-Belmonte *et al*., 1996; Tizón *et al*., 1996). The sixth ORF (*YGL121c*) was sequenced at the laboratory of G. Lauquin.

Sporulation of heterozygous deletants and tetrad analysis have been carried out. Homozygous deletants have also been constructed. The ORFs have been cloned in order to facilitate the future

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work of researchers, and the disruption cassettes have also been cloned for eventual deletion in different genetic backgrounds.

MATERIALS AND METHODS

Strains and media

The *Escherichia coli* strain DH5 served as the plasmid host. For selective growth, the bacteria were grown on 2 × YT or LB containing 40 mg/l ampicillin or kanamycin.

As *S. cervisiae* strains, we used the EUROFAN reference strain FY1679 (**a**/α, *ura3-52*/*ura3-52*, *leu2Δ1*/*LEU2*, *trp1Δ63*/*TRIP*, *his3Δ200*/*HIS3*, *GAL2*+/*GAL2*+), and CEN.PK2 (**a**/α, *ura3-52*/*ura3-52*, *leu2-3*,*112*/*leu2-3*,*112*, *trp1-289*/*trp1-289*, *his3Δ1*/*his3Δ1*). As the control strain for functional analysis, the haploid strain FY73 (α progenitor of the strain FY1679) was generally used. For routine culture, *S. cerevisiae* was grown on 1% yeast extract, 1% peptone and 2% glucose (YPD). The media used for functional analysis were: glucose-complete medium (CM-D) containing 2% glucose and 0·67% yeast nitrogen base and 40 mg/ml of amino acids, glycerol-complete medium (CM-G) containing 2% glycerol instead of glucose and glucose minimal medium (SD), which contained 0·67% yeast nitrogen base supplemented only with the required amino acids in accordance with the auxotrophies, and 2% glucose.

Routine DNA manipulations, including plasmid preparation, subcloning, agarose electrophoresis and transformation followed standard protocols (Sambrook *et al*., 1989).

Construction of gene-specific deletion cassettes

The dominant resistance module, *kan*MX4, containing the *kan*^{r} gene of the *E. coli* transposon *Tn*903 and included in the vector pFA6a-kanMX4, was used to replace the yeast coding sequence and to select for *S. cerevisiae* transformants (Wach *et al*., 1994). The method employed to create the deletion cassettes is based on the polymerase chain reaction (PCR)-targeting strategy (Baudin *et al*., 1993). The deletion cassette contained *kan*MX4 flanked by two 40 bp regions homologous to the target sequence (SFH: short flanking homology) from the promotor and the terminator of the yeast gene to be disrupted.

The cassettes were generated by PCR with *Taq* DNA polymerase (Boehringer–Mannheim) and *Vent* DNA polymerase (New England Biolabs) using pFA6a-kanMX4 as template. For each ORF, a pair of 60-mer primers was designed to contain 40 nucleotides at the 5′ end homologous to the target sequence followed by 20 nucleotides at the 3′ end homologous to pFA6a-kanMX4 MCS (Table 1). The deletion cassettes were designed to replace at least 80% of the target ORF. To avoid changing the expression of adjacent ORFs, the end-points chosen for the deletion were positioned at least 350 bp upstream from the start codons and 250 bp downstream from the stop codons of adjacent ORFs. The SFH-PCR products were directly used to transform yeast.

Transformation of yeast cells

Transformations of yeast cells were performed by a procedure modified from Ito *et al*. (1983). Transformed cells were grown at 30°C in YPD for 12 h and spread on YPD plates containing 200 mg/l geneticin (G418 from Gibco BRL). To purify transformants from background, each large colony was streaked out on fresh YPD–geneticin plates. Only those clones which grew after the double selection were analysed further.

Verification of yeast transformants

Correct replacement of the targeted gene at the genomic locus was verified by analytical PCR. Genomic DNA of the disrupted strain was used as template and appropriate primers to check the two junctions corresponding to the replacement are summarized in Table 1 and positioned in Figure 1. Four independent PCRs were carried out, with two of them using genomic DNA of the strain FY1679 as a control and the other two using genomic DNA of the strain bearing the disrupted ORF (a representative example is shown in Figure 1). PCR reactions with oligonucleotides A1+A2, A3+A4, A1+K2 and A4+K3 yielded PCR products of predictable and different sizes. Thus, the wild-type and the disrupted strains were able to be differentiated.

Tetrad analysis of the heterozygous deletions in FY1679

The heterozygous deletants were first induced to sporulate on a sporulation medium containing (10 g/l potassium acetate, 2·5 g/l yeast extract, 1 g/l glucose, 2% agar). After 6–8 days of incubation at 30°C, sporulation was checked under a light microscope. Ten tetrads were dissected and analysed (see

Table 1. Oligonucleotides used in the study. The sequence of the multiple cloning site from pFA6a-kanMX4 appears separated from the rest of the sequence with an upright bar.

ORF	Name	Sequence	Position
YGL131c	S1	5′GTGCTAAGACCAAGAGTAGCAATACGGAAACTCCCAGGAAT/CGTACGCTGCAGGTCGAC3′	265742–265701
	S2	5′ATATCGATATCAAACATAGCTGGCTCAATTCCTTCCATCAA/ATCGATGAATTCGAGCTCG3′	261697–261737
	A1	5′CAACACCGCAGACCATCTG3′	266378–266359
	A2	5′GCGTGACCAGCTAAAGGC3′	265864–265882
	A3	5′GGTCCAATCTTGCGCGGC3′	261623–261606
	A4	5′AGTTGGAGGTGATTGCAGACGA3′	261074–261095
YGL129c	S1	5′ATTGGGCAAAGGCTGTTCACTACGGCACGATCTCTCCAGG/CGTACGCTGCAGGTCGAC3′	269052–269012
	S2	5′CGATTAGTTCGTTAACTTCCTGTTTGTTCAGTTTAGGAACC/ATCGATGAATTCGAGCTCG3′	267869–267909
	A1	5′CGCGTCGACCATCCTGACAAACACCCGGAC3′	270001–269981
	A2	5′CCACGTGGCTTGTACTTGTTC3′	269096–269116
	A3	5′AGTGGTAACGGTAACCCAAGAG3′	267782–267761
	A4	5′CGCGAGCTCCCACATCTTTCTGATGGTCTG3′	266808–266828
YGL128c	S1	5′AAGTCCATCGCCGTACTACAAGCCTCGCCACGACTATGCCA/CGTACGCTGCAGGTCGAC3′	270183–270143
	S2	5′GAGACCAGTCTTGCAATATCGTCATTCAGTTCCGTCTCC/ATCGATGAATTCGAGCTCG3′	269322–269360
	A1	5′GCACCAGCGACACCTTCCT3′	270758–270740
	A2	5′CGAGGCTTGTAGTACGGCG3′	270157–270175
	A3	5′GGAGACGGAACTGAATGACGA3′	269360–260340
	A4	5′CGTAGTGAACAGCCTTTGCCC3′	269028–269048
YGL125w	S1	5′CACAACCACCACAGTTACTACTACAACCACATCGCAATATG/CGTACGCTGCAGGTCGAC3′	272486–272526
	S2	5′CAATTCTGTTAAAGAACTGTTATCGTTGTCTGTGTTATTA/ATCGATGAATTCGAGCTCG3′	274360–274321
	A1	5′CGCGTCGACGGGCGTCGTGCACTGGC3′	271760–271776
	A2	5′CGGGACGAAGTACTCGAATG3′	272601–272582
	A3	5′TCCATTCCATCCTACTAAGCC3′	274298–274318
	A4	5′GGTAGGAATGGCGTGGGTG3′	274940–274922
YGL124c	S1	5′CTATCAAAGTACACAAACGTAGAATCAGTACATCGGAACTATG/CGTACGCTGCAGGTCGAC3′	276755–276713
	S2	5′CCTCCCTGTCACAAGTTAAAACACGGCCCCATCACTTAT/ATCGATGAATTCGAGCTCG3′	274766–274804
	A1	5′CGTGTAAACCTTCTTCGCGTC3′	277105–277085
	A2	5′CCCTTCGGTATCTCAGCATCT3′	276672–276692
	A3	5′GGTAGGAATGGCGTGGGTG3′	274940–274922
	A4	5′CGATAAACCGCATAGTGCCC3′	274200–274219
YGL121c	S1	5′GAACTACAGAGAAGATAAGAAATCTTTTAAGTAGCAATCATG/CGTACGCTGCAGGTCGAC3′	281197–281156
	S2	5′TCTTGTTAGCTCTTCTTGTAACTGTGTGAACAAATCGTCG/ATCGATGAATTCGAGCTCG3′	280826–280865
	A1	5′-GGTTCTTGGCCACGACGC-3′	281884–281866
	A2	5′-GGTTCAGCCCCAGCCGAG-3′	281109–281126
	A3	5′-CTGGACTGGGACATCGACG-3′	280879–280861
	A4	5′CGCGAGCTCGGGGGATGGAGTTGCAGGAGC3′	279982–280002
*kan*MX4	K2	5′-CCGGGGATCGCAGTGGTGAG-3′	817–798
	K3	5′-ACGGTTTGGTTGATGCGAGTG-3′	922–942

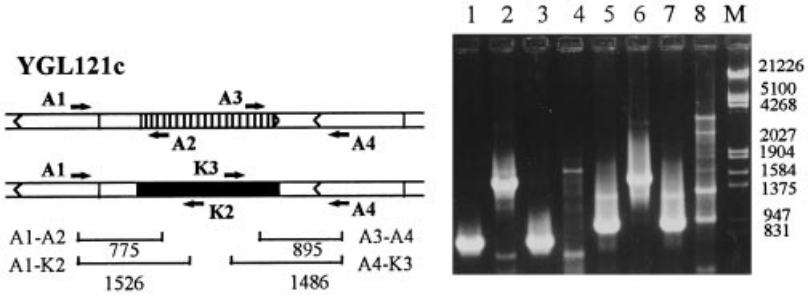

Figure 1. Verification of gene disruption by analytical PCR. Left: schematic representation of normal and disrupted alleles, dashed box represents the ORF to be disrupted, boxes in white the adjacent ORFs and solid boxes the kanamycin cassette. Oligonucleotides used for verification of gene disruption are indicated by arrows, and their sequences are shown in the Table 1. Bars indicate the expected sizes of PCR products. Right: electrophoresis of the PCR products. W, wild type (FY1679); Δ, disrupted strain. 1, Δ (A1–A2); 2, Δ (A1–K2); 3, W (A1–A2); 4, W (Al–K2); 5, Δ (A3–A4); 6, Δ (A4–K3); 7, W (A3–A4); 8, W (A4–K3). M represents Marker III (Boehringer–Mannheim).

representative examples in Figures 2–4). Spores were then checked for germination and their genotype was determined.

Standard phenotypic test

Growth of two independent Kan^r spores was tested for each viable disruption. Wild-type and mutant strains were first grown overnight in liquid YPD and then the cultures were diluted to an A_{600}=0·05. When the cultures reached an A_{600}=0·1–0·2, they were diluted to 1:10, 1:100, 1:1000 and 1:10,000. To analyse growth characteristics, 5 μl of each cell suspension was spotted on solid CM-D, CM-G and SD media. Plates were incubated at 15°C, 30°C and 37°C for various periods of time and then photographed.

Construction of homozygous deletants

Homozygous deletants for the non-lethal disruptions were obtained by crossing between **a** and α deleted haploids chosen by their suitable genotypes for diploid selection.

Sporulation efficiency assay

Wild-type FY1679 and homozygous deleted diploids were allowed to grow overnight in liquid YPD followed by 10 days in a solid minimal sporulation medium (1% potassium acetate, supplemented with the required amino acids). Cells were removed and observed under the light microscope to determine their sporulation frequencies.

Cloning of ORF replacement cassettes and construction of cognate gene clones

Cloning of ORF replacement cassettes were performed as described by Huang *et al.* (1997). To obtain a disruption cassette consisting of the *kan*MX4 module flanked by long sequences homologous to the target gene (LFH) we performed PCR on DNA from the haploid disruptants obtained by SFH in FY1679 and sporulation. The oligonucleotide pairs used in this PCR were chosen at ∼350 bp from the start and ∼250 bp from the stop codons. Each LFH replacement cassette was cloned into the multicloning site of pUG7 plasmid at the *Eco*RV site (Güldener *et al.*, 1996). The EUROFAN reference strain CEN.PK2 was disrupted with the excised cassettes in order to demonstrate their functionality.

Cognate gene clones for ORFs *YGL128c*, *YGL124c* and *YGL121c* were obtained through traditional cloning methods, while in order to clone wild-type ORFs *YGL131c*, *YGL129c* and *YGL125w* we resorted to the gap-repair technique (Rothstein, 1991). Gene clones were verified by sequencing and those deleted strains showing characteristic phenotypes were transformed with the corresponding gene clone for complementation.

RESULTS AND DISCUSSION

The six ORFs were disrupted in strain FY1679 using SFH deletion cassettes. Correct gene replacement was verified by analytical PCR as described in Materials and Methods. The six genes were also disrupted in the EUROFAN reference strain CEN.PK2 using the excised ORF replacement cassettes. Correct replacement of the targeted gene at the genomic locus was verified by PCR using the same procedures described for strain FY1679.

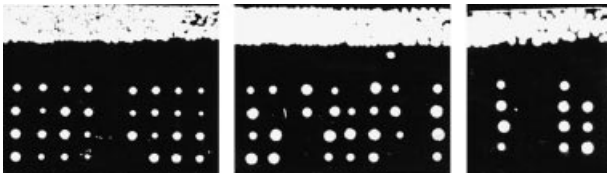

Figure 2. Tetrad analysis of the ORF *YGL131c*.

After gene disruption in the diploid strain FY1679, these cells were sporulated and at least 10 tetrads were dissected on YPD medium. This analysis revealed whether or not the genes were essential, and if the disruption resulted in different colony morphology. None of the genes was essential for growth in complete media with glucose as a carbon source at 30°C. Each spore was tested for mating type and G-418 resistance. The genotypes of the haploid strains were determined by checking auxotrophic requirements. Homozygous diploid strain formation was normal for all of the ORFs except for ORF *YGL128c*, in which the growth of the homozygous diploid strain was slow. We analysed sporulation efficiencies in the homozygous diploid deletants using strain FY1679 as a control. We found very low (6–8%) sporulation efficiencies in strain FY1679, similar levels of sporulation in homozygous diploids bearing the ORFs *YGL131c* and *YGL121c* disrupted, and lower than 1% sporulation efficiencies in homozygous diploid strains with ORFs *YGL129c*, *YGL128c*, *YGL125w* and *YGL124c* disrupted. In the case of *YGL128c*, it is advisable to relate this result to the poor growth of the homozygous diploid.

Growth characteristics of **a** and α haploid deletants as well as strain FY73 were recorded. The latter, used as a control, presented low growth at 15°C and on CM-G. Phenotypes resulting from a particular gene disruption as well as other analyses carried out to support functional analysis are discussed below for each individual ORF.

Disruption of ORF YGL131c

The sequence analysis of the protein predicted by this ORF reveals the existence of cysteine residues in strategic positions to form zinc fingers (Cys_{320}-Cys_{323}-N_{14}-Cys_{338}-Cys_{341}; Cys_{1041}-Cys_{1044}-N_{13}-Cys_{1058}-Cys_{1061}; Cys_{1091}-Cys_{1094}-N_{13}-Cys_{1108}-Cys_{1111}; Cys_{1179}-Cys_{1182}-N_{9}-Cys_{1192}-Cys_{1195}; Cys_{1324}-Cys_{1327}-N_{13}-Cys_{1341}-Cys_{1344}). This could be related to a putative DNA-binding capacity, and indirectly to a regulatory role in gene expression. It also contains, at position 1158, a GQSMQPAL motif characteristic of signal-peptidases I.

Tetrad analysis of 10 asci revealed that two spore colonies were sensitive and two resistant to G418 and that YGL131c is not an essential gene. The two geneticin-resistant spores resulted in slightly smaller colonies than those formed by wild-type spores (Figure 2). However, in spite of this we could not find any differences in CM media between the growth of deleted strains and that of wild-type cells (Table 2). The deletion does not produce any anomaly in the mating process, neither is sporulation in the homozygous deletant affected.

Disruption of ORF YGL129c

An analysis of 10 tetrads revealed 2:2 segregation, with the two G418-resistant colonies being smaller than the two G418 sensitive ones (Figure 3A). This indicates that it is a non-essential gene. However, haploid deletants (**a** and α) are unable to grow on glycerol, while the control strain grows normally (Figure 3B). This phenotype suggests that this gene could be related to a mitochondrial function (Nelson *et al.*, 1993). Sequence analysis revealed the presence of a characteristic motif of mitochondrial transporters at the 3′ end of the protein (positions 476–484). The absence of growth on glycerol is a common phenotype when genes related to mitochondrial transport are oblated; for example, genes *AAC2* (Lawson and Douglas, 1988), *FLX1* (Wu *et al.*, 1995) or *MIR1* (Murakami *et al.*, 1990). The capacity of growth on glycerol of the null-strain is not restored by complementation with the wild-type gene clone. This situation has been described in the literature for the gene *MDJ1* and it was attributed to the irreversible loss of mitochondrial DNA (Rowley *et al.*, 1994).

Disruption of YGL128c

A tetrad analysis of 20 asci revealed a 2:2 segregation in which G418-resistant spores arose

Table 2. Quantitative growth characteristics of the ORFs *YGL131c*, *YGL1124c* and *YGL121c*. Quantitative growth was recorded as follows: 0, none; +, poor; ++, reduced; +++, normal; d: days. The growth of wild-type cells at 30°C on CM-D was defined as normal (+++). WT, wild-type cells.

		CM-D			CM-G			SD		
		15°C (6 d)	30°C (2 d)	37°C (2 d)	15°C (14 d)	30°C (4 d)	37°C (4 d)	15°C (14 d)	30°C (2 d)	37°C (2 d)
WT	*MATa*	+++	+++	+++	+	++	++	+++	+++	+++
YGL131c	*MAT***a**	++	+++	+++	+	++	++	+++	+++	+++
	MATa	++	+++	+++	+	++	++	+++	+++	+++
YGL124c	*MAT***a**	+++	+++	+++	+	++	++	+++	+++	+++
	MATa	+++	+++	+++	+	++	++	+++	+++	+++
YGL121c	*MAT***a**	+++	+++	+++	+	++	++	+++	+++	+++
	MATa	+++	+++	+++	+	++	++	+++	+++	+++

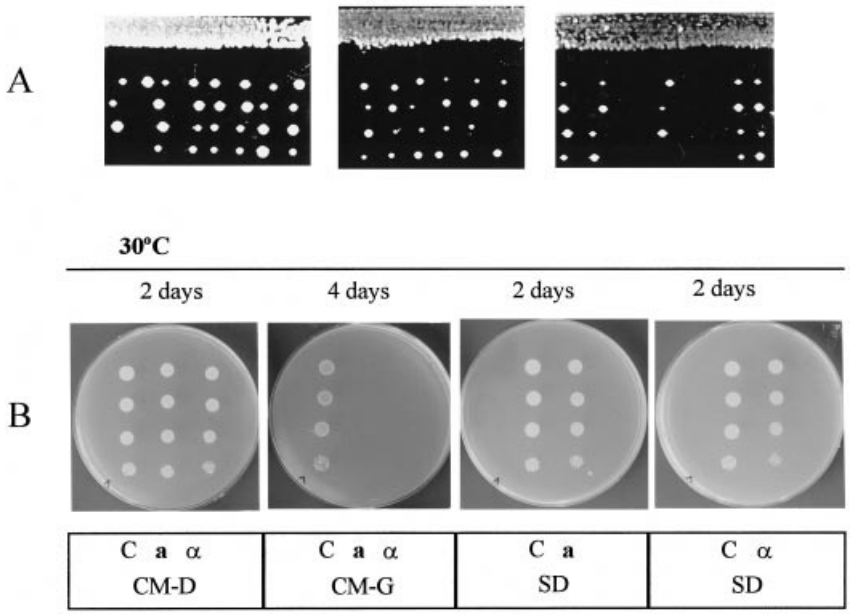

Figure 3. Phenotype of the haploid deletants of the ORF *YGL129c*. (A) Tetrad analysis. (B) Growth assays at 30°C: C, wild-type cells (FY73); **a**, α, haploid deletants with mating a or α.

in minute colonies only after long-term growth (Figure 4A). Colonies bearing the *YGL128c* deletion germinate poorly, which indicates that this gene, if not essential, is of great importance to yeast cells. Functional analysis demonstrated the inability of haploid deletants to grow at non-permissive temperatures (15°C and 37°C) (Figure 4B). This ability was recovered by the transformation of mutant haploid with the cognate gene clone. Moreover, we found an abnormal cell morphology for disrupted cells with the light microscope, characterized by a high frequency of cells with two buds (Figure 4C). This indicates a defect, probably caused during the final stages of cell division. Sequence analysis revealed a characteristic motif of DnaJ proteins at the N-terminal end of the protein. It is the J domain, which is conserved in all DnaJ *E. coli* homologous proteins (Sunshine, 1977; Saito and Uchida, 1978; Silver and Way, 1993; Kelley and Landry, 1994) and

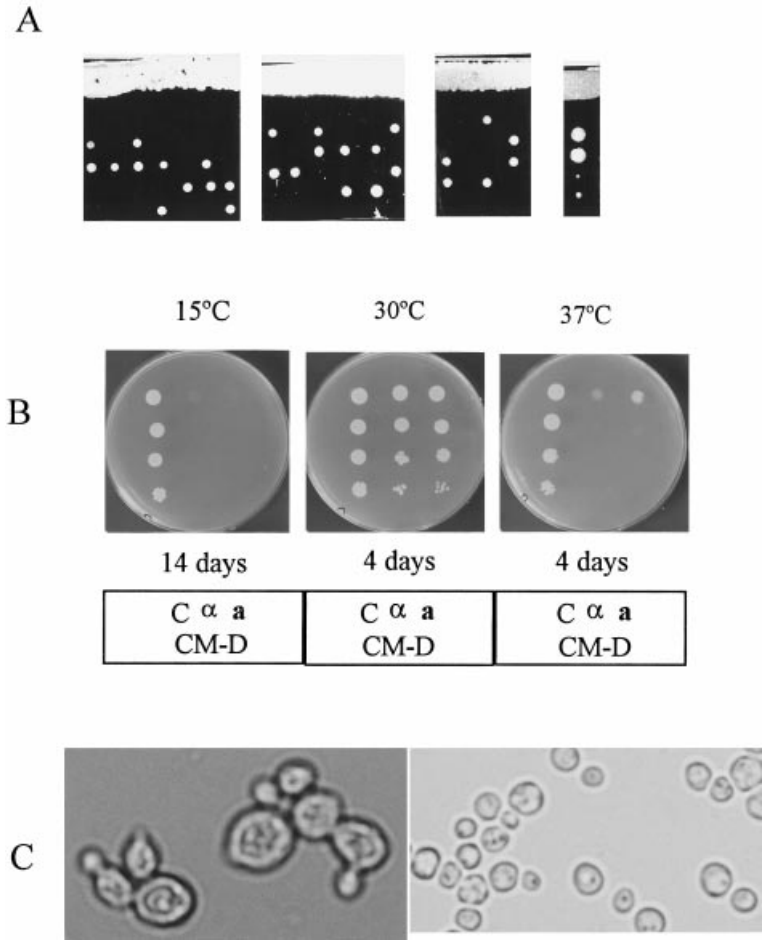

Figure 4. Phenotype of haploid deletants of the ORF *YGL128c*. (A) Tetrad analysis; the rightmost tetrad corresponds to a long-term germination. (B) Growth assays on CM-D: C, wild-type cells (FY73); **a**, α, haploid deletants with mating a or α. (C) Differences in morphology between deletant cells (left, magnification × 2500) and wild-type cells (right, magnification × 1600).

other yeast homologues (Rodríguez-Belmonte *et al.*, 1996). Minute colony formation on germination plates or the absence of growth associated to gene deletion is common among the DnaJ protein family, such as in Sis1 and Ydj1 proteins (Luke *et al.*, 1991; Caplan and Douglas, 1991); the absence of growth of haploid deletants at non-permissive temperatures is also frequent, for example, in Sec63 and Mdj1 proteins (Sadler *et al.*, 1989; Rowley *et al.*, 1994). Other characteristics related to *YGL128c* deletion, such as the slow growth of homozygous diploids or the changes in cell morphology, are related to the DnaJ family as well. DnaJ proteins are heat shock proteins (Hartl, 1996); we have found that *YGL128c* transcription is induced by heat-shock during a systematic study of 200 ORFs of unknown function carried out in our laboratory as part of the EUROFAN I B2 consortium (data to be published elsewhere). Other data supporting this result include the promotor analysis, in which consensus motifs for HSF binding are present at positions –515 and –360, as well as a STRE element at position –241.

Disruption of YGL125w

Sporulation and dissection of 10 tetrads revealed that all four meiotic segregants were viable and

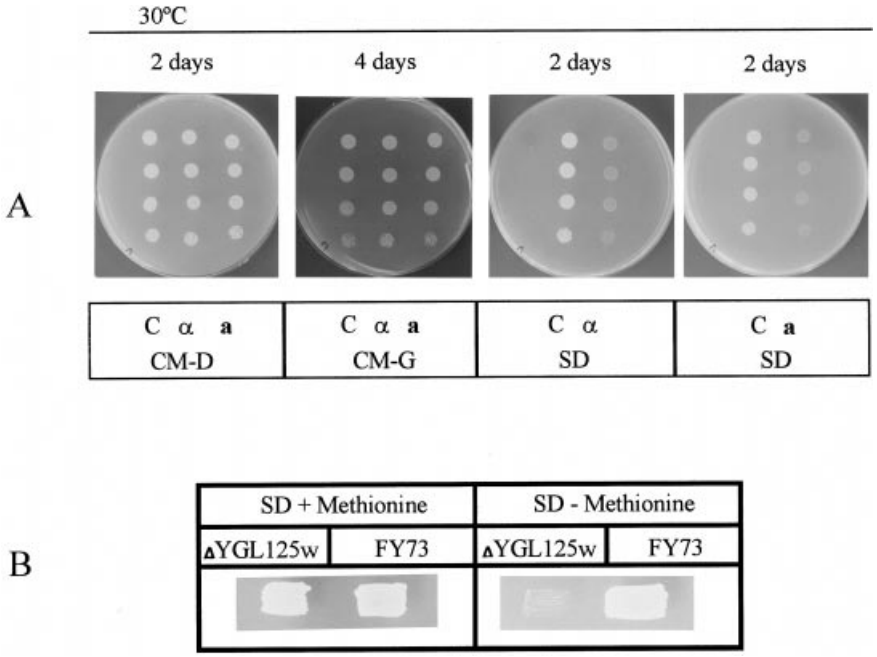

Figure 5. Phenotype of haploid deletants of the ORF *YGL125w*. (A) Growth assays at 30°C: C, wild-type cells (FY73); **a**, α, haploid deletants with mating a or α. (B) Growth of the haploid deletant on SD supplemented with methionine and SD without the amino acid. FY73 strain was used as a control.

resulted in colonies with similar morphology, indicating that *YGL125w* is a non-essential gene. However, haploid deletants are unable to grow on SD medium (Figure 5A) and the growth is restored by complementation with the gene clone. FASTA analysis revealed that the nucleotidic sequence shows a strong homology to human and *E. coli* methylenetetrahydrofolate reductase (MTHFR) genes. MTHFR is an enzyme necessary for methionine biosynthesis. MTHFR deficiency in humans have serious consequences, such as a delay in development, motor abnormalities or psychiatric disorders (Goyette *et al.*, 1994, 1995, 1996). To confirm that the *YGL125w* ORF was related to amino acid biosynthesis, we placed the deletant on the SD medium supplemented with methionine. Growth was found to be perfectly normal (Figure 5B). A plasmid containing the wild-type gene (cognate clone) complements the *met13* mutation (R. Borts, personal communication). We may therefore conclude that the YGL128c protein takes part in methionine biosynthesis. The *S. cerevisiae* genome contains another ORF which is homologous to human and *E. coli* MTHFR, ORF *YPL023c*. We think that this ORF carries out a totally different function from ORF *YGL125w* or that it is highly limited in function, since when we disrupted ORF *YGL125w*, the deletant showed very little growth on the SD medium, indicating that *YPL023c* is either not functional or expressed at low levels.

Disruption of YGL124c

Tetrad analysis revealed that this is a non-essential gene. The growth of deletant strains was as good as in the isogenic wild-type cells (Table 2). The only phenotype associated with the *YGL124c* deletion was a small decay in the sporulation frequency. So we could not relate this gene to any previously known function.

Disruption of YGL121c

An analysis of 10 tetrads revealed 2:2 segregation with two spore colonies which are resistant and two which are sensitive to G418; all four are viable and have a normal morphology. No differences in growth characteristics were observed in CM-D, CM-G and SD medium between haploid

deletants and wild-type cells (Table 2). Sporulation efficiency and mating efficiency were also normal.

The absence of a phenotype in some of the deletant strains is not surprising; many of the ORFs analysed during the EUROFAN-I project resulted in this conclusion. It has been previously reported that a high proportion of non-essential genes do not show any phenotype under standard laboratory conditions (Burns *et al.*, 1994). However, we do know that ORFs *YGL124c* and *YGL121c*, which do not have any phenotypes associated with their mutations and basic functional analysis, are expressed as has been shown by our B2 consortium mentioned above. Further experiments through specific functional analysis are necessary before the functionality of these novel genes can be assigned to specific areas of the biological activity of yeast.

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