### Yeast Functional Analysis Report

# Disruption and phenotypic analysis of six open reading frames from chromosome VII of *Saccharomyces cerevisiae* reveals one essential gene[†]

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## Abstract

Six open reading frames (ORFs) located on chromosome VII of *Saccharomyces cerevisiae* (YGR205w, YGR210c, YGR211w, YGR241c, YGR243w and YGR244c) were disrupted in two different genetic backgrounds using short-flanking homology (SFH) gene replacement. Sporulation and tetrad analysis showed that YGR211w, recently identified as the yeast ZPR1 gene, is an essential gene. The other five genes are non-essential, and no phenotypes could be associated to their inactivation. Two of these genes have recently been further characterized: YGR241c (YAP1802) encodes a yeast adaptor protein and YGR244c (LSC2) encodes the *β*-subunit of the succinyl-CoA ligase. For each ORF, a replacement cassette with long flanking regions homologous to the target locus was cloned in pUG7, and the cognate wild-type gene was cloned in pRS416. Copyright © 2001 John Wiley & Sons, Ltd.

**Keywords:** *Saccharomyces cerevisiae*; EUROFAN; gene disruption; ZPR1; YAP1802; LSC2; YHR162w

## Introduction

Analysis of the *Saccharomyces cerevisiae* genome sequence has revealed that, of the about 6000 open reading frames (ORFs) found, a fraction of about one-third potentially encodes for proteins of unknown function (Goffeau *et al.*, 1996). In order to investigate the functions of these novel genes, the European Functional Analysis Network (EUROFAN) was launched (Oliver, 1996). This project aims to generate deletion mutants for each ORF in two yeast strains, followed by functional analysis of the disruptants, in order to elucidate the function of at least 1000 genes.

Within this project, we disrupted six ORFs, YGR205w, YGR210c, YGR211w, YGR241c, YGR243w and YGR244c, which were identified in the course of the sequencing of the chromosome VII (Guerreiro *et al.*, 1996, 1997). General information about these ORFs is shown in Table 1. Only two ORFs showed significant homology with known proteins: YGR211w with the mammalian zinc-finger protein ZPR1 (Galcheva-Gargova *et al.*, 1996) and YGR244c with the *Neocallimastix frontalis* succinyl-CoA synthetase *β*-subunit.

In addition to gene disruptions and basic functional analysis, we constructed cloned cassettes useful for gene replacement in different strains and also cloned the cognate ORFs for gene complementation experiments.

[†]This paper is dedicated to the memory of Professor Rudi Planta, who passed away on 1 June 2000. His bright intelligence and generosity have contributed to the development of our research on the yeast *Saccharomyces cerevisiae* and it is thanks to him that we have embarked in the venture of participating in the sequencing of the yeast genome. Over the years of our acquaintance we have entertained with him very enlightening and stimulating exchange of points of view from which very important and consequent lessons were shared with us.

## Materials and methods

### Strains, media and plasmids

The *S. cerevisiae* strains used were the EUROFAN reference strain FY1679 (MAT**a**/α *ura3-52/ura3-52 leu2Δ1/+ trp1Δ63/+ his3Δ220/+*) and CEN.PK2 (MAT**a**/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-289/trp1-289 his3Δ1/his3Δ1*).

Yeast cells were grown in YPD medium (1% yeast extract, 2% Bacto-peptone, 2% glucose). For the selection of the geneticin-resistant transformants, cells were grown on YPD plates containing 200 mg/l geneticin (G-418, Gibco-BRL). Phenotypic analyses were performed in synthetic complete medium [0.667% Yeast Nitrogen Base (YNG) without amino acids (Difco), supplemented with appropriate amino acids and uracil] with 2% glucose (CM) or 3% glycerol (CMGly), and in minimal medium (MM; 0.67% YNG without amino acids supplemented with uracil, histidine, leucine and tryptophan, 2% glucose).

Plasmids pRS416 (Sikorski and Hieter, 1989), pFA6–*kan*MX4 (Wach, 1994) and pUG7 (Güldener *et al.*, 1996) were obtained from the European *Saccharomyces cerevisiae* Archives for Functional Analysis (EUROSCARF). Plasmids pUG7 and pRS416 were used to clone the disruption cassette and the cognate gene, respectively, and pFA6–*kan*MX4 was used as a source of the *kan*MX4 module.

### Construction of short flanking homology (SFH) cassettes and ORF disruption

The disruptions in the FY1679 strain were performed using SFH deletion cassettes, according to Wach *et al.* (1994). For all the ORFs, two chimeric primers (S1 and S2; Table 2) were designed containing, at the 5′ end, 45–55 nucleotides complementary to the target sequence and, at the 3′ end, 19/22 nucleotides homologous to the terminal regions of the *kan*MX4 module. These primers and the *Not*I-digested plasmid pFA6–*kan*MX4 as template were used to amplify, by PCR, DNA fragments containing the *kan*MX4 selectable marker flanked by short sequences homologous to the target locus. PCR conditions were as follows: 5 ng/μl *Not*I-digested pFA6–*kan*MX4, 0.4 pmol/μl each of S1 and S2 primers, 0.2 mM dNTPs, 1.5 mM MgCl$_2$ and 0.04 U/μl *Taq* polymerase (Pharmacia) in 1 × reaction buffer (Pharmacia). PCR programme: 2 min at 94°C followed by 5 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C, and 20 cycles at 94°C, 30 s at 60°C and 90 s at 72°C, with a final incubation of 2 min at 72°C.

About 3 μg of the resulting DNA fragment was used to transform yeast by the lithium acetate method (Gietz *et al.*, 1995). Transformed cells were allowed to recover in 10 ml YPD for 12 h, and 200 μl were spread on plates containing G-418. After an incubation of 48 h, the large colonies were streaked on new YPD-G-418 plates, and single cell-derived colonies were used to check the correct integration of the selection marker.

### Verification of gene replacement by PCR

The correct integration of the *kan*MX4 marker into the genomic locus was verified by PCR using whole *S. cerevisiae* cells (Huxley *et al.*, 1990). Primers were designed that were specific to the upstream (A1), downstream (A4) and internal (A2 and A3) regions of each ORF, and to the internal (K2 and K3)

**Table 1.** Characteristics of the ORFs

| Systematic name | Coordinates in chromosome VII | No. of amino acids | CAI[a] | Function/similarity |
|---|---|---|---|---|
| YGR205w | 909207–910076 | 290 | 0.17 | Similar to *S. pombe* hypothetical protein SPAC630.09c |
| YGR210c | 914732–913500 | 411 | 0.13 | Similar to GTP-binding protein *S. pombe* SPBC428.15 |
| YGR211w | 915235–916692 | 486 | 0.24 | *ZPR1*: zinc-finger protein |
| YGR241c | 976576–974873 | 568 | 0.16 | *YAP1802*: yeast adaptor protein |
| YGR243w | 977331–977768 | 146 | 0.10 | Similar to superfamily hypothetical protein YHR162w |
| YGR244w | 979314–978084 | 427 | 0.19 | *LSC2*: succinyl-CoA ligase β-subunit |

[a]CAI, codon adaptation index following Sharp and Li (1987).

Table 2. Oligonucleotides used in this study

| Name | Sequence[a] |
|---|---|
| S1-YGR205w | 5'-gcgttccattgcatctctgaagtgaataatataaaaagtagtaaagctagaaatg **cgtacgctgcaggtcgac**-3' |
| S2-YGR205w | 5'-ggtcataacatcactattcaatacatctcgttttcgtagaatatacattcctgttggagtcg **atcgatgaattcgagctcg**-3' |
| A1-YGR205w | 5'-ctggtttggctacctatgc-3' |
| A2-YGR205w | 5'-ctatcgtgtagtccaatacc-3' |
| A3-YGR205w | 5'-gagactacaacaggagcatg-3' |
| A4-YGR205w | 5'-ctcccattgctattttcc-3' |
| S1-YGR210c | 5'-gtataagctgtctgtaagaataaaacggaaatagagccagtatg**cgtacgctgcaggtcgac**-3' |
| S2-YGR210c | 5'-catatactttgatgattacttaatattcactggcactcaagtta**atcgatgaattcgagctcg**-3' |
| A1-YGR210c | 5'-gaacctacccgataacgc-3' |
| A2-YGR210c | 5'-gagcaagcacactcaacttg-3' |
| A3-YGR210c | 5'-ggttctgcaagcagcaacgg-3' |
| A4-YGR210c | 5'-gactctcgtgtatgtgtgcg-3' |
| S1-YGR211w | 5'-gcatatatacacagagatacatattatacctataccgttaagaaataggatagaaaataatg **cgtacgctgcaggtcgac**-3' |
| S2-YGR211w | 5'-ggatatatgtaaagaggaagaggaaaggggggaggtgataccgaggccaacgatcgtta **atcgatgaattcgagctcg**-3' |
| A1-YGR211w | 5'-caagttagttgcagaaaagc-3' |
| A2-YGR211w | 5'-ggtttgctgagccaactttg-3' |
| A3-YGR211w | 5'-ccaacccgcttctcaaattc-3' |
| A4-YGR211w | 5'-cttgcgcttttttccttttc-3' |
| S1-YGR241c | 5'-cggctaccccctataatagatatagaggcgtgtttgtgaaacaatg**cgtacgctgcaggtcgac**-3' |
| S2-YGR241c | 5'-gacggcctgttgagaacaaaaaactgtagtatctatcgctcacta**atcgatgaattcgagctcg**-3' |
| A1-YGR241c | 5'-cctttcggttttgcgtgacc-3' |
| A2-YGR241c | 5'-cttgcaatcctcttgcgctc-3' |
| A3-YGR241c | 5'-gatgcagggtcagcaaacag-3' |
| A4-YGR241c | 5'-gtgtgctcgatattagagag-3' |
| S1-YGR243w | 5'-ctttaagactatacgcataagcattcaagacacatagaaacacaaacctatatttttaatg **cgtacgctgcaggtcgac**-3' |
| S2-YGR243w | 5'-ggtcataaaaaatcattaaaaaaatgcgagttcaggaacatattatcgtttacgtaatca **atcgatgaattcgagctcg**-3' |
| A1-YGR243w | 5'-cttgcaatcctcttgcgctc-3' |
| A2-YGR243w | 5'-ctgataccttctcaacaggc-3' |
| A3-YGR243w | 5'cagcaaagcaaactgcatcc-3' |
| A4-YGR243w | 5'-gacattatcaataagtcaagg-3' |
| S1-YGR244c | 5'-aatacaatcgaaaaaaatactgaagcattgcaactgaacaaaatg**cgtacgctgcaggtcgac-**3' |
| S2-YGR244c | 5'-gcatatatactttattattaactctttgtttttctcgagaagctta**atcgatgaattcgagctcg**-3' |
| A1-YGR244c | 5'-cacagatacattcatctctc-3' |
| A2-YGR244c | 5'-cgtttagcctgcttaattgc-3' |
| A3-YGR244c | 5'-gacattatcaataagtcagg-3' |
| A4-YGR244c | 5'-cgtaaacgataatatgttcc-3' |
| K2 | 5'-ctgaccatctcatctgtaac-3' |
| K3 | 5'-ggttgtattgatgttggacg-3' |

[a]The bases homologous to pFA6a multiple cloning site are in **bold**.

region of the _kan_MX4 cassette (Table 2). Colony PCR was carried out by picking yeast cells with a toothpick from single colonies to a PCR tube and heating them for 1 min in a microwave, followed by the addition of 25 μl of the PCR reaction mix (2.5 μl 10 × reaction buffer (Pharmacia), 10 pmol each of the internal primers A2/K2 or A3/K3, 20 pmol external primer A1 or A4, 0.2 mM dNTPs and 1 U _Taq_ polymerase (Pharmacia), 2.5 mM MgCl₂). PCR programme: 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 45°C and 90 s at 72°C, with a final incubation of 5 min at 72°C.

## Tetrad analysis

The heterozygous deletants were induced to sporulate on 1% potassium acetate solid medium for 3–5 days at 25°C. Once the cells had sporulated, the yeast cells were picked with a toothpick and resuspended in 50 μl 1 M sorbitol containing 0.5 mg/ml Zymolyase-100T (Seikagaku Kogyo Co). After 15 min incubation at 30°C, the reaction was stopped by the addition of 800 μl sterile water. A sample was then spread on a thin layer of YPD and the spores of each tetrad were separated using a

micromanipulator. About 10 tetrads per ORF were dissected and the correct segregation of the geneticin resistance, auxotrophic markers and mating type was analysed by replica-plating on selective media.

Homozygous disruptants were obtained by crossing G418 resistance haploids of opposite mating type with complementary auxotrophies, in YPD plates. After 1 day, the crossing was replica-plated to minimal media with uracil and streaked in the same media.

## Plasmid construction

ORF replacement cassettes containing long flanking regions were obtained by PCR amplification with the A1–A4 pair of primers, using the heterozygous diploid strains as templates. PCR was carried out with the whole cells, as described above, but with 20 pmol of each primer. The fragments had been cloned in the unique *Eco*RV site of the pUG7 vector, and the recombinant *E. coli* were selected on the basis of kanamycin resistance. The resulting plasmids were analysed by digestion with different restriction enzymes and checked by DNA sequencing.

The wild-type sequences corresponding to the YGR210c, YGR241c, YGR243w and YGR244c ORFs were cloned in the pRS416 centromeric plasmid as restriction fragments isolated from different plasmid subclones of cosmids pEGH301 and pEGH620, used for the sequencing project (Guerreiro *et al.*, 1996, 1997). The ORF fragments were the following: a 2021 bp *Dra*I fragment for YGR210c; a 3120 bp *Pst*I–*Kpn*I fragment for YGR241c; a 1249 bp *Sal*I–*Xho*I fragment for YGR243w; and a 1874 bp *Cla*I fragment for YGR244c. In the case of YGR205w and YGR211w, a PCR fragment using the A1–A4 pair of primers were generated as described for the construction of the replacement cassettes. The correct insertion of the fragments in the resulting plasmids was analysed by digestion with appropriate restriction enzymes and, in the case of pYCG-YGR205w and pYCG-YGR211w, checked by sequencing.

## Phenotypic tests

The diploid heterozygous and homozygous and the MAT**a** and MATα haploid mutants for each ORF, together with FY1679 diploid and MAT**a** and

MATα haploid strains, were grown until stationary phase on liquid YPD medium. Serial dilutions were made in order to spot 10, $10^2$, $10^3$, $10^4$ and $10^5$ cells with an $8 \times 6$ Replica Plater (Sigma) on YPG, YPG and minimal media at 15°C, 30°C and 37°C.

Complementation of the phenotype was performed in the case of deletion of the ORF YGR211w. The mutant was transformed with pYCG–YGR211w, sporulated and tetrads dissected. The resulting haploids were scored for resistance to geneticin.

## Results and discussion

### Deletion of the six ORFs in strain FY1679

The ORFs YGR205w, YGR210c, YGR211w, YGR241c, YGR243w and YGR244c were deleted in the strain FY1679 by the SFH method (Wach *et al.*, 1994), using a *kan*MX4 marker flanked by 45–65 nt homologous to the 5′ and 3′ regions of the target gene. The disruptions were verified by PCR analysis with the primers listed in Table 2. All the PCR diagnostics gave two fragments, one corresponding to the deletant copy and the other to the wild-type copy (Figure 1). The six heterozygous diploids were plated onto 1% acetate potassium solid medium, and sporulation was allowed to proceed at 25°C for 3–5 days. About 10 tetrads per ORF were then dissected to a YPD plate. The strain disrupted for YGR211w yielded only two geneticin-sensitive viable spores per tetrad, whereas all the other five disrupted strains exhibited four spores with a 2:2 segregation of the G418-resistance to sensitivity. For each non-lethal deletion, homozygous disruptants were obtained by crossing geneticin-resistant haploids of opposite mating type carrying complementary auxotrophies. All the obtained haploids and homozygous diploids were checked for correct disruption by PCR analysis (Figure 1). Homozygous disruptants were also allowed to sporulate, each one yielding four geneticin-resistant viable spores per tetrad.

All the G418-resistant strains carrying deletions for each of the five ORFs were assessed for their ability to grow on minimal media, on glucose or glycerol-rich media, and at three temperatures: 15°C, 30°C and 37°C. No significant growth difference was identified under any of the conditions tested.
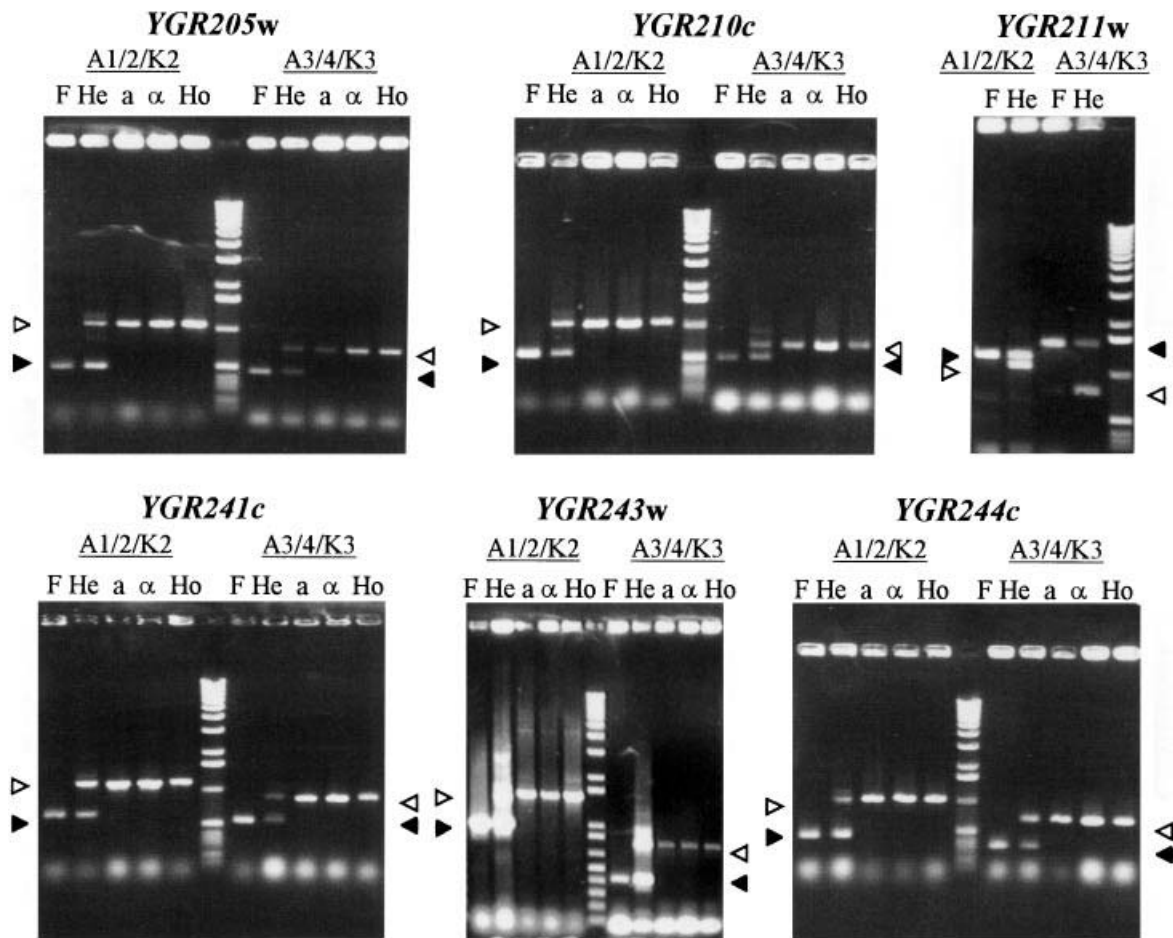
**Figure I.** Verification of *kan*MX gene replacements by PCR. Reactions were carried out using genomic DNA from FY1679 strain (F) and from FY1679 heterozygous (He), a and α haploids, and homozygous (Ho) disruptants of the six genes as templates. Primer A1 + A2 + K2 and A3 + A4 + K3 were used in combination. A molecular weight 1 kb DNA ladder was used as standard except for YGR243w analysis, where a 1 kb plus DNA ladder was used instead. 1 kb DNA ladder fragment sizes (in kb): 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.6, 1.0, 0.5, 0.4, 0.35, 0.3, 0.22, 0.2, 0.15, 0.13, 0.08; 1 kb plus DNA ladder fragment sizes (in kb): 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.6, 1.0, 0.85, 0.65, 0.5, 0.4, 0.3, 0.2, 0.1. Open arrowheads represent the expected sizes of PCR products for disrupted genes (A1–K2 or K3–A4), whereas closed arrowheads represent the expected sizes for non-disrupted genes (A1–A2 or A3–A4). Expected sizes: YGR205w A1–A2 (552 bp), A1–K2 (1154 bp), A3–A4 (544 bp), K3–A4 (822 bp); YGR210c A1–A2 (585 bp), A1–K2 (1073 bp), A3–A4 (527 bp), K3–A4 (693 bp); YGR211w A1–A2 (1273 bp), A1–K2 (1101 bp), A3–A4 (1521 bp), K3–A4 (784 bp); YGR241c A1–A2 (656 bp), A1–K2 (1180 bp), A3–A4 (551 bp), K3–A4 (882 bp); YGR243w A1–A2 (1023 bp), A1–K2 (1514 bp), A3–A4 (420 bp), K3–A4 (795 bp); YGR244c A1–A2 (469 bp), A1–K2 (1047 bp), A3–A4 (346 bp), K3–A4 (702 bp)

## Construction of plasmids carrying the replacement cassettes (pYORC) and the cognate wild-type genes (pYCG)

SFH–PCR is an easy method for constructing deletant strains, but has low transformation efficiency. In order to delete these ORFs easily in a different strain, long flanking homology cassettes for each ORF were constructed by PCR amplification of the heterozygous diploid DNA, using the external primers A1 and A4. The PCR fragments were cloned into the pUG7 vector, and the resulting pYORC (<u>y</u>east <u>OR</u>F <u>r</u>eplacement <u>c</u>assette) plasmids were selected based on their kanamycin-conferring resistance to *E. coli*. These constructs were tested on strain CEN.PK2, which has a different genetic background to FY1679. The cassettes were excised from pYORC plasmids with *Not*I and used to

transform CEN.PK2 cells. The deletants were selected on G418 plates and the disruptions in the correct loci were verified by PCR.

Wild-type genes were directly subcloned into pRS416 plasmid (Sikorski and Hieter, 1989), either as restriction fragments isolated from part of the genomic cosmids pEGH301 and pEGH620 or by PCR products. One of the resulting pYCG (yeast cognate genes) plasmids, pYCG–YGR211w, was used to transform the cognate heterozygous diploid, in order to check the recovery of the non-viability phenotype observed.

### YGR205w

Disruption of this ORF did not result in any detectable phenotype with respect to the tests that were employed. The YGR205w ORF encodes a hypothetical protein of 290 amino acids with a strong homology with the *Schizosaccharomyces pombe* hypothetical protein SPAC630.09c.

### YGR210c

No detectable phenotype regarding the tests used was found in the case of disruption of this ORF. The YGR210c ORF encodes a protein of 411 amino acids with a strong homology with the *Sz. pombe* protein SPBC428.15. Both proteins belong to the *Methanococcus jannaschii* GTP-binding protein superfamily.

### YGR211w

Disruption of the YGR211w is lethal, since the resulting diploid strain produced tetrads that showed a 2:2 segregation of geneticin-sensitive viable to non-viable spores (data not shown). The transformation of the heterozygous diploid with the wild-type gene carried on a replicative plasmid (pYCG–YGR211w) allowed the production of four viable spores showing a 2:2 segregation of geneticin-sensitive to geneticin-resistant spores on synthetic complete media minus uracil, confirming that the disruption of YGR211w is responsible for the observed lethality. YGR211 encodes a protein of 486 amino acids that presented a strong homology to the mouse Zpr1 protein, a zinc-finger protein that binds to the epidermal growth factor receptor (Galcheva-Gargova *et al.*, 1996). Recently, Zpr1 has been shown to interact with the elongation factor eEF-1α in both mammalian and yeast cells

through a small region present in its N-terminus (Gangwani *et al.*, 1998). In proliferating cells, Zpr1p and a fraction of the eEF-1α distributes to the nucleus, whereas in starved cells, redistribution between nucleus and the cytoplasm was observed (Galcheva-Gargova *et al.*, 1998; Gangwani *et al.*, 1998). However, although the binding of Zpr1p to eEF-1α is required for normal growth of yeast cells, this interaction is not required for viability, which is dependent on the C-terminus of Zpr1p (Gangwani *et al.*, 1998).

### YGR241c

Disruption of this ORF does not result in any detectable phenotype with respect to the tests that were used. The YGR241c ORF encodes a protein of 568 amino acids that has been recently identified as yAP180B (Wendland and Emr, 1998), a member of the non-heterotetrameric clathrin assembly protein AP180 family, which was first characterized in neuronal cells (Murphy *et al.*, 1991; Zhou *et al.*, 1993). yAP180B shares 43% identity with yAP180A, which is encoded by YHR161c, and the two genes were renamed as YAP1801 and YAP1802. Both yAP180 proteins were found to interact with Pan1p and clathrin *in vitro*. However, strains that lack one or both yAP180 proteins are viable in several conditions, including high temperature and 3% glycerol, which supports our phenotypic analysis (Wendland and Emr, 1998). Moreover, deletion of both YAP180 genes alone or together with other genes coding for subunits of the yeast heterotetrameic AP complexes do not display the phenotypes of clathrin-deficient cells (e.g. poor growth, mislocalization of Golgi-resident proteins and slowed endocytosis), suggesting that APs are not required for clathrin recruitment onto membranes (Huang *et al.*, 1999).

### YGR243w

Disruption of this ORF does not result in any detectable phenotype regarding the tests that were employed. The YGR243w ORF encodes a hypothetical protein of 146 amino acids with 73.6% identity with Yhr162w, so it is possible that the lack of phenotype observed for the Δygr243w strains was due to a rescue from YHR162w gene. The YHR162w superfamily has been found through the genome sequencing projects of several organisms (e.g. *Sz. pombe* SPAC24B11.09, *A. thaliana*

T10I14.140, *C. elegans* F53F10.3, *D. melanogaster* CG9396 and CG9399 and *H. sapiens* DKFZP564B167) but its function is still unknown. However, the rat member of the family, the O44 gene, was found to be transcribed into nearly 100 different mRNAs due to alternative transcription initiation and polyadenylation sites, and a alternative splicing site that produces mRNAs coding for proteins with different C-termini was also detected (Tsou *et al.*, 1986).

## YGR244c

Disruption of this ORF does not result in any detectable phenotype with respect to the tests that were employed. The YGR244c ORF encodes Sclβ, the succinyl-CoA ligase β-subunit, and was renamed as *LSC2*. The succinyl-CoA ligase is composed of α and β subunits, being the α subunit encoded by *LSC1* (YOR142w). Recently, Δ*lsc1*, Δ*lsc2* and Δ*lsc1*Δ*lsc2* deletion mutants were phenotypically characterized for growth on various non-fermentable carbon sources (Przybyla-Zawislak *et al.*, 1998). As described by these authors, none of these mutants showed deficiency for growth on semi-synthetic media containing acetate, in contrast with other TCA-cycle mutant strains, but presented discernible growth defects on pyruvate or glycerol-containing media. We have not observed any growth defect on glycerol medium, but that could be due to the different media we used (complete synthetic instead of semi-synthetic). This hypothesis is supported by the lack of growth defect observed by Przybyla-Zawislak *et al.* (1998) when they used glycerol-containing rich medium (YP) instead of semi-synthetic medium.

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