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Yeast Functional Analysis Report

Functional analysis of the *Saccharomyces cerevisiae* YFR021w/YGR223c/YPL100w ORF family suggests relations to mitochondrial/ peroxisomal functions and amino acid signalling pathways

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Abstract

***Saccharomyces cerevisiae* YFR021w, YGR223c and YPL100w are paralogous ORFs of unknown function. Phenotypic analysis of overexpression, single-, double- and triple-ORF deletion strains under various growth conditions indicated mitochondria-related functions for all three ORFs. Two-hybrid screens of a yeast genomic library identified potentially interacting proteins for the three ORFs. Among these, the transcriptional activator Rtg3p interacted with both Yfr021wp and Ypl100wp and both ORF single deletions reduced the constitutive expression of the *RTG*-regulated *CIT2* and *DLD3* genes and caused typical retrograde response of *CIT2* and *DLD3* under growth conditions requiring functional mitochondria, indicating that YFR021w and YPL100w are also involved in unidentified mitochondrial functions. Ptr3p, a component of the amino acid sensor Ssy1p/Ptr3p, was also found as a two-hybrid interactant of Yfr021wp. Of the three single-ORF deletions, *ypl100wΔ* exhibited *ptr3Δ*-similar phenotypes. These findings, combined with the fact that *RTG*-dependent expression is modulated by specific amino acids, suggested possible relations of Yfr021wp and Ypl100wp to amino acid signalling pathways. Under most conditions examined, the effects of the single- and double-ORF deletions indicated that *YFR021w*, *YPL100w* and *YGR223c* are not parts of the same pathway. We found no unique phenotype attributed to the deletion of *YGR223c*. However, its function interferes with the function of the other two ORFs, as revealed by the effects of double- and triple-ORF deletions.**

Keywords: EUROFAN; gene family; *YFR021w*; *YPL100w*; *YGR223c*; two-hybrid; Rtg3p; Ptr3p; *CIT2*; *DLD3*

Introduction

The *Saccharomyces cerevisiae* genome was the first to be sequenced among eukaryotic organisms, uncovering a plethora of ORFs of unknown function (Goffeau *et al*., 1996, 1997). In spite of its relatively small size compared to multicellular eukaryotes, it contains a significant number of gene families encoding paralogous genes. Approximately 800 families of 2–24 members of a variable percentage of similarity have been so far identified by bioinformatic analysis among the nearly 6250 yeast ORFs (Llorente *et al*., 1999). Gene duplications allow functional specialization and improved fitness of the evolving organisms. In many instances, single-member gene deletions do not reveal any distinct phenotype, indicating that paralogous genes may have redundant (back-up) functions. Undetectable phenotypes may also be due to the restricted range of applied phenotypic tests, and

this is one of the reasons for not having identified all genes of the extensively studied yeast prior to genomic sequencing. Other reasons could derive from evidence indicating that undetectable phenotypes are due to interactions between unrelated genes and/or use of alternative pathways (Wagner, 2000). It is obvious that combinations of individual ORF(gene)-directed assays, such as the identification of protein interacting partners, cellular localization or whole genome microarray-based growth tests, can identify distinct roles. In the framework of the EUROFAN program for the characterization of unknown function yeast genes, we participated in the functional analysis of gene families by using a number of basic and more specialized assays for each ORF (Dujon, 1998). Our goal was to find distinct biological roles for each family member. Here we report our results for the three-member ORF family YFR021w, YPL100w and YGR223c.

Materials and methods

Strains and media

BY4741 and BY4742 strains (Brachmann *et al.*, 1998) were used to generate ORF deletions. PJ69-4A and PJ69-4α (gifts from D. Lockshon and J.-C. Jauniaux; James *et al.*, 1996; Uetz *et al.*, 2000) were used for two-hybrid assays and FYBL3 (Fairhead *et al.*, 1998) for PCR-derived gene isolations (Table 1).

E. coli XL-1 Blue *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1*lac*$^-$ F′[*proA*$^+$*B*$^+$ *laq*I^q *lacZ*Δ*M15 Tn10(tet*^{r})] was used for plasmid propagation work and *E. coli* KC8 (*hsdR, leuB600, trpC9830, pyrF::Tn5, hisB463, lacDX74, strA, galU,K*), to select the pACTII (*LEU2*) plasmids rescued from yeast (Ausubel *et al.*, 2000; Struhl *et al.*, 1979).

Standard bacterial and yeast growth media LB, YPD (1% yeast extract, 2% peptone and 3% dextrose), YPG (2% glycerol), YPOl (0.2% oleic acid and 0.25% Tween 40), SC (synthetic complete YNBD, containing all amino acids), YNBD (0.67% yeast nitrogen base and 3% dextrose), YNBAc (0.5% yeast extract, 0.67% yeast nitrogen base and 2% potassium acetate, pH 5.5), YNBGly (0.67% yeast nitrogen base and 2% glycerol) and culturing procedures were used as previously described (Ausubel *et al.*, 2000; Guthrie and Fink, 1991; Kaiser *et al.*, 1994). FOA plates (YNBD, 0.1% 5-fluoro-orotic acid and uracil) were used for the isolation of *ura3* mutants. For sporulation, diploid cells grown on YPD plates for 1 day were transferred on minimal sporulation medium (MSM) plates and incubated for 5–7 days. Tetrads were digested with Lyticase (Boehringer-Mannheim) and dissected with a manual Singer Micromanipulator (Singer Instruments).

Plasmid constructions: molecular biology techniques

For ORF overexpression, PCR-synthesized fragments (Table 2) were subcloned into pCM190 (*URA3*) vector (Gari *et al.*, 1997), in which transcription is driven by the tet-Vp16 transcriptional activator that binds to multiple *tet*O binding sites located in front of the *CYC1* TATA box. Addition of tetracycline or its derivatives (e.g. 0.5–2.0 μg/ml doxycycline) to the growth medium lowers the expression of the pCM190-cloned ORF.

Vent (New England Biolabs) and Pwo polymerases (Boehringer-Mannheim) were used for all PCR reactions. Plasmid DNA was purified from bacteria using Qiagen Kits or precipitation by PEG (Ausubel *et al.*, 2000). DNA sequencing analysis was performed on ALF sequencer (Pharmacia). RNA blot hybridization was performed using 50 μg total RNA samples, analysed on 1.5% agarose gels and transferred onto nylon membranes (Georgatsou and Alexandraki, 1999).

Construction of ORF-specific deletion cassettes

ORF deletions were performed by gene replacement via homologous recombination of PCR-generated DNA fragments (Muhlrad *et al.*, 1992) by the 'short flanking homology' approach (Wach *et al.*, 1994). Specific auxotrophic selection markers were amplified using primers consisting of 40 ORF-specific nucleotides, followed by 20 vector-specific nucleotides (Table 2) (pRS400 series; Brachmann *et al.*, 1998). To avoid interference with adjacent ORF expression, the marker integration sites for YGR223c were chosen to map 259 nucleotides upstream of its stop codon, and for YPL100w 156 nucleotides upstream of its stop codon. At least 2 μg PCR-generated product were used to transform yeast cells (TRAFO protocol: **http://www.umanitoba.ca/medicine/biochem/gietz/method.html**). Transformants grown on appropriate selective

Table 1. *S. cerevisiae* strains used in this study

Name	Genotype	Source or reference
PJ69-4A	*MAT***a** *trp1-901 leu2-3,112, ura3-52 his3-200 gal4*Δ *gal80*Δ UAS*GAL2-ADE2LYS2*::UAS*GAL1*-HIS3 met2::UAS*GAL7-lacZ*	James *et al.* (1996) Lockshon D, Fields S.
PJ69-4α	*MAT*α *trp1-901 leu2-3,112, ura3-52 his3-200 gal4*Δ *gal80*Δ UAS*GAL2-ADE2LYS2*::UAS*GAL1*-HIS3 met2::UAS*GAL7-lacZ*	James *et al.* (1996) Lockshon D, Fields S.
BY4741	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	Brachmann *et al.* (1998)
BY4742	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	Brachmann *et al.* (1998)
BY41-*yfr021w*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 yfr021w*Δ::*URA3*	This work
BY41-*yfr021w*Δ*ura3*	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 yfr021w*Δ::*ura3*(NcoI/Klenow)	This work
BY42-*yfr021w*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 yfr021w*Δ::*URA3*	This work
BY41-*ygr223c*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 ygr223c*Δ::*HIS3*	This work
BY42-*ygr223c*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 ygr223c*Δ::*HIS3*	This work
BY41-*ypl100w*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0* met15Δ*0 ura3*Δ*0 ypl100w*Δ::*LEU2*	This work
BY42-*ypl100w*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 ypl100w*Δ::*LEU2*	This work
BY41-*yfr021w*Δ*ygr223c*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 yfr021w*Δ::*URA3* ygr223cΔ::*HIS3*	This work
BY41-*yfr021w*Δ*ypl100w*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0* met15Δ*0 ura3*Δ*0 yfr021w*Δ::*URA3 ypl100w*Δ::*LEU2*	This work
BY41-*ypl100w*Δ*ygr223c*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0* met15Δ*0 ura3*Δ*0 ypl100w*Δ::*LEU2 ygr223c*Δ::*HIS3*	This work
BY41-*yfr021w*Δ*ygr223c*Δ *ypl100w*Δ	*MAT***a** *his3*Δ*1 leu2*Δ*0* met15Δ*0 ura3*Δ*0 yfr021w*Δ::*URA3 ygr223c*Δ::*HIS3 ypl100w*Δ::*LEU2*	This work
FYBL3	*MAT***a**/*MAT*α *ura3*-Δ851/*ura3*-Δ851 *leu2*-Δ1/*leu2*-Δ1 *his3*-Δ200/ *his3*-Δ200 *trp1*-Δ63/+*lys2*-Δ202/+	B. Dujon

media were verified for ORF-deletion by colony PCR. Three different diagnostic PCR reactions were conducted of each deletion: one using primers (Table 2) hybridizing to 5′ upstream and 3′ downstream regions of the deleted ORF to detect a fragment, the size of which was different in the deletion strain compared to the wild-type; a second using the same 5′ upstream primer and a 3′ primer specific for each auxotrophy-complementing marker sequence to obtain a characteristic fragment size and verify the integration of the marker in the correct site within the deleted ORF; and the third diagnostic PCR was similar to the second but concerned the auxotrophy-complementing marker and the 3′ region of each deleted ORF.

Two-hybrid screens

The fusion Gal4pDBD–ORF 'baits' used for the two-hybrid screens were generated by homologous recombination between pGBT9 vector (Clontech) and PCR-synthesized ORF fragments in yeast (Muhlrad *et al.*, 1992). Yeast cells were co-transformed with *Bam*HI–*Eco*RI linearized vector and a DNA ORF fragment bearing 30 bp of vector homologous sequences at its 5′ and 3′ ends (Table 2). Primers were purchased from the Microchemistry Lab (Crete, Greece) and MWG-BIOTECH AG (Ebersberg, Germany). 100 ng gel-purified vector and 500 ng each PCR product (from several PCR reactions) were used to transform the PJ69-4α strain. Transformants grown on the appropriate selective media plates were checked for the correct insert size by colony PCR (Huxley *et al.*, 1990; Wach *et al.*, 1994) using pGBT9 sequence-specific primers and Taq polymerase (Minotech/Crete, Greece). Twenty different correct colonies (~100% correctness) for each of the three baits were pooled together and used in the three separate two-hybrid screens. 'Bait' peptide expression was tested by Western blot analysis (Ausubel *et al.*, 2000) of total protein extracts, using a Gal4pDBD-specific antibody (Santa Cruz Biotechnology Inc., USA).

The two-hybrid screens were performed against a Gal4pAD–yeast genomic library (mechanically sheared genomic DNA fragments to an average size of 800 base pairs inserted into the pACTII vector; A. Ramne and P. Sunnerhagen, unpublished) (**http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_1/b5/index.html**). PJ69-4A library-containing cells were mated (Bendixen *et al.*, 1994; Fromont-Racine *et al.*, 1997) with PJ69-4α bait-containing cells and grown on SC selective medium lacking leucine, tryptophan and histidine and containing 2 μg/ml adenine and 3 mM 3-AT (3-aminotriazole). 6.25×10^7 mated cells were tested for two-hybrid interactions with Yfr021wp bait (after counting ~35% library survival

Table 2. Sequences of the oligonucleotides used as primers for bait constructions, gene deletions and verifications

ORF/primer name	Sequence	ORF and deletion position
YFR021w	5′-aacaaaggtcaaagacagttgactgtatcgTCTGGTAC	+1
P1-5′ (bait)	TATGTCTGATTCATCACCTAC-3′	
P2-3′ (bait)	5′-taagaaattcgcccggaattagcttggctgTCAATCCAT	+1503
	CAAGATGGAAT-3′	
P3-5′ (deletion)	5′-CCAGTTAACTCTGTATCCTTTTCTCTTC	+4
	GGCCTGACAATGagattgtactgagagtgcac-3′	
P4-3′ (deletion)	5′-AAGGCAGCGCGAGACACTTCCGTGAT	+1488
	CAATCCATCAAGATctgtgcggtatttcacaccg-3′	
P5-5′ (diagnostic)	5′GACAACGCTAAACATGCG-3′	−610
P6-3′ (diagnostic)	5′GTGAAGTTAACTCTTCCG-3′	+1837
YGR223c	5′-aacaaaggtcaaagacagttgactgtatcgTCTGGTAC	+1
P7-5′ (bait)	TATGGATGTTCGTCGACCTAT-3′	
P8-3′ (bait)	5′-taagaaattcgcccggaattagcttggctgTTAAAGCT	+1347
	CTCTCCATGATT-3′	
P9-5′ (deletion)	5′-TAGAGGACAACTATAAGCATACATAA	+8
	CTAGCAGATGGATGagattgtactgagagtgcac-3′	
P10-3′ (deletion)	5′-GTTACATCACCACTGTTGTCATTTCTG	+1046
	TGAGCGTCAAAGATTctgtgcggtatttcacaccg-3′	
P11-5′ (diagnostic)	5′-GTAGACGTGGTGTTACTA-3′	−566
P12-3′ (diagnostic)	5′-GATGCAGCTGACTATTCG-3′	+1476
YPL100w	5′-aacaaaggtcaaagacagttgactgtatcgTCTGGTAC	+1
P13-5′ (bait)	TATGAAAGTATTACAATTCAA-3′	
P14-3′ (bait)	5′-taagaaatacgcccggaattagcttggctgTTATGTAAA	+1491
	TTTATTATTTT-3′	
P15-5′ (deletion)	5′-ACTCCTTTGGATTTGAAATAGACAGAT	+4
	AGAAAAGGATATGagattgtactgagagtgcac-3′	
P16-3′N (deletion)	5′-ATAAGCAAAGTTACGGCTCAGGTTCT	+1293
	GGTTTGGTATTGAAGActgtgcggtatttcacaccg-3′	
P17-5′ (diagnostic)	5′-GGGAAATATACGGCATGC-3′	−273
P18-3′ (diagnostic)	5′-GAAGCCTTGGTATAGGAG-3′	+1679
LEU2 (reverse)	5′-ACCTGTAGCATCGATAGC-3′	+155
P19-5 (diagnostic)		
P20-3′ (diagnostic)	5′-CCTCTTTGCCAGACAAGA-3′	+815
HIS3 (reverse)	5′-TCACTTGCGATTGTGTGG-3′	+157
P21-5′ (diagnostic)		
P22-3′ (diagnostic)	5′-TTGCAGAGGCTAGCAGAA-3′	+500
URA3 (reverse)	5′-CAACAGGACTAGGATGAG-3′	+52
P23-5′ (diagnostic)		
P24-3′ (diagnostic)	5′-GGATGATGTGGTCTCTAC-3′	+610
pGBT9	5′-TCATCGGAAGAGAGTAGT-3′	827
P25-5′ GAL4B (diagnostic)		
P26-3′ (diagnostic)	5′-CGTTTTAAAACCTAAGAGTCAC-3′	1015
pACT2	5′-GGAATCACTACAGGGAT-3′	5176
P27-5′ GAL4AD (diagnostic)		
P28-3′ (diagnostic)	5′-TTGCGGGGTTTTTCAGTATCTACG-3′	4951
P29-5′ GAL4ADF (sequencing)	5′-CGATGATGAAGATACCCCACCAAACCC-3′	5118

Vector homologous sequences in chimeric primers are given in low case.

and ~60% mating efficiency); 7.6×10^7 cells with Ygr223cp bait (~73% library survival and ~30% mating efficiency); and 5.09×10^7 cells with Ypl100wp bait (~35% library survival and ~48% mating efficiency). Grown colonies were examined for their pACTII-insert size by colony PCR. Rescued 'prey' plasmids were retransformed into PJ69-4A along with bait plasmids and two-hybrid interactions

were verified in: (a) SC lacking leucine, tryptophan and histidine and supplemented with 3 mM 3-AT (providing growth due to *HIS3* activation); (b) SC lacking leucine, tryptophan, histidine and adenine (white instead of red colonies due to *ADE2* activation); and (c) SC lacking leucine and tryptophan and supplemented with 40 μg X-gal/ml (Guthrie and Fink, 1991) (colonies of light or strong blue colour, due to activation of the *lacZ* gene). Yeast prey DNAs of clones positive by all three criteria were sequenced and analysed for homologies with the BLAST program of SGD (Saccharomyces Genome Database). DNA and protein sequence information was found at: MIPS (Munich Information Centre for Protein Sequences) (**http://speedy.mips.biochem.mpg.de/mips/yeast**); SGD (**http://genome-www.stanford.edu/Saccharomyces**); YPD (Yeast Protein Database) (**http://quest7.proteome.com/YPDhome.html**); Yeast Gene Duplications (**http://acer.gen.tcd.ie/khwolfe/yeast/topmenu.html**); NCBI, by the PSORT II program (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences) (**http://psort.nibb.ac.jp/form2.html**) and the GCG package software.

Results and discussion

Known features of the paralogous ORF family

YFR021w, YGR223c and YPL100w are paralogous ORFs of unknown function. Their amino acid sequence similarities are shown in Figure 1. YFR021w is more similar to YPL100w (28%) than to YGR223c (26%) but YPL100w is equally similar (28%) to both. All three ORFs exhibit similarities with restricted sequence regions in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *A. thaliana*, mouse and human hypothetical proteins (YPD and NCBI databases). For Yfr021wp, PSORT II subcellular localization prediction analysis suggests 65.2% nuclear (reliability 89) and 13% plasma membrane and other features, a potential transmembrane region, a long tyrosine tail and dileucine motifs in the tail; for Ygr223cp, the prediction is 52.2% nuclear (reliability 89), 21.7% mitochondrial and 21.7% cytoplasmic; and for Ypl100wp, 69.6% nuclear (reliability 94.1), 13% mitochondrial and 13% cytoplasmic. Ypl100wp contains an ER retention motif-NNKF (KKXX-like motif) in the C-terminus. Limited experimental data are available for the expression and function of the three ORFs. A systematic high-throughput assay of yeast deletants (Winzeler *et al.*, 1999) showed that the three single-ORF deletions were viable. Reduced growth of *ygr223c*Δ FY *MAT***a** cells and heterozygous diploids was observed in glycerol-rich media at 15°C and of *ygr223c*Δ FY *MAT*α and homozygous diploid cells at both 15°C and 37°C (EUROFAN I: D. Gallwitz). *YFR021w*-specific RNA was first detected in the transcription analysis of chromosome VI (Murakami *et al.*, 1995; Naitou *et al.*, 1997) and *YGR223c* mRNA abundance was increased by 4.2-fold following methylmethane sulphonate (MMS) treatment, as detected in an oligonucleotide microarray experiment (Jelinsky and Samson, 1999).

Interacting proteins in two-hybrid screens

To obtain some insights into the function of the three paralogous ORFs, we first performed two-hybrid screens to identify specific protein interaction partners. None of the baits was found to transactivate any of the three strain reporters (data not shown). Identification of interacting proteins of known function is a starting point for further investigation of the role of unknown function proteins within known pathways. We have considered as 'real' only the interactions that gave strong signals from all three reporters. The insert size of each positive interactant clone, the number of different clones containing the same ORF and the fusion point of each prey with Gal4pAD were determined. Eleven different ORFs in-frame with the Gal4pAD were found as prey for Yfr021wp and one for Ygr223cp bait (Table 3). The Ygr223cp-specific prey did not interact with Yfr021wp or Ypl100wp baits in individual two-hybrid assays. Ypl100wp bait gave no 'real' interactants, either because it does not associate strongly with other proteins or due to library-specific limitations. Its amino acid sequence does not indicate any hydrophobic regions that would prevent the fusion product from entering the nucleus where two-hybrid interactions were assayed. The only interactants obtained by YPL100w were clones of intergenic regions and the false-positive prey Yaf1p (Oaf1p/YAL051w) (data not shown), identified in almost all two-hybrid screens that we performed with different baits, especially in those that gave very few 'true' interactants. This is probably attributed to the ability of Yaf1p transcriptional activator to recognize and bind to

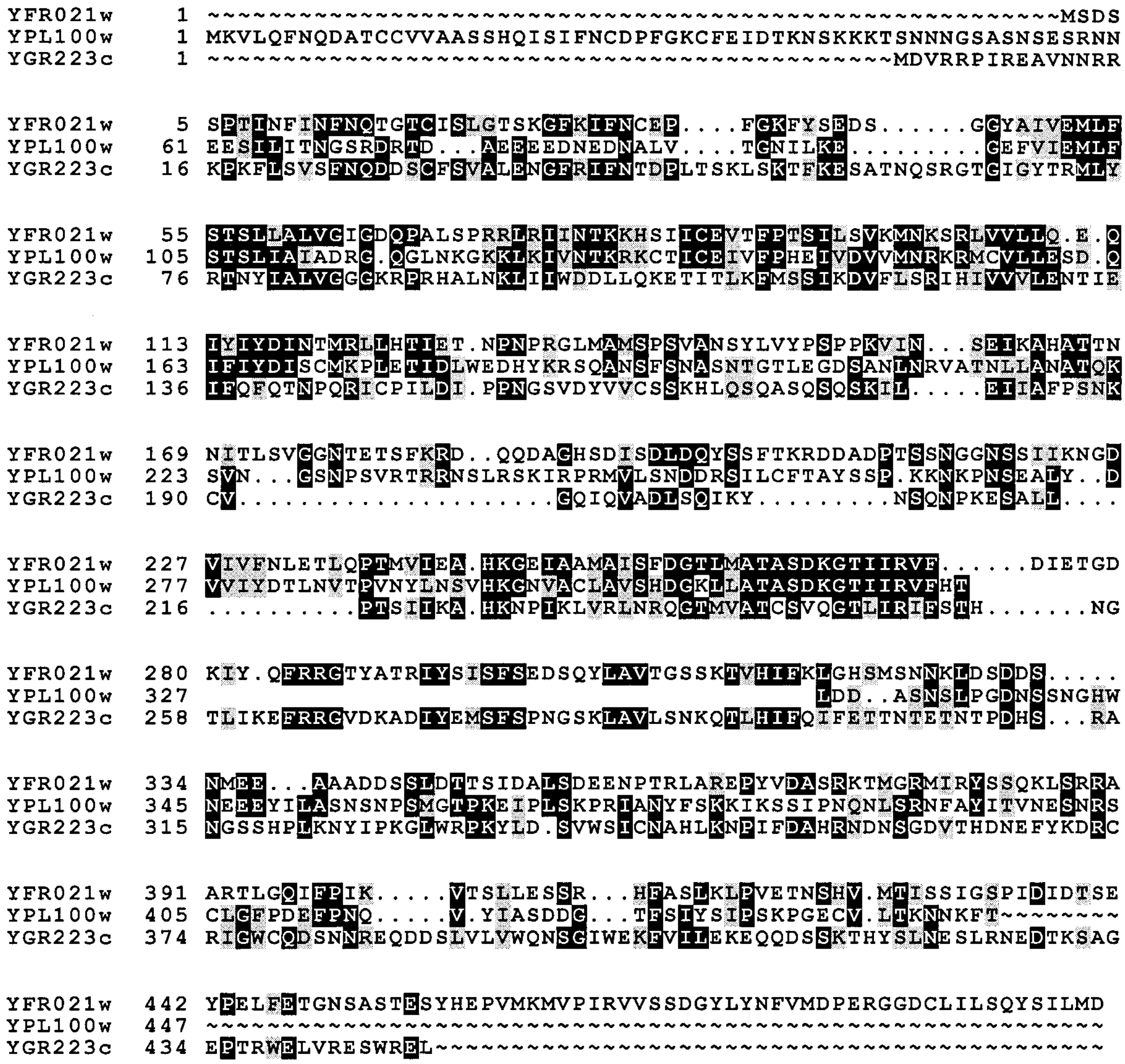

Figure 1. Amino acid residue alignment of the three-ORF family. Alignment was performed by the Pileup program of the GCG package and displayed by the BOXSHADE 3.21 program (Pretty Printing and Shading of Multiple-Alignment files; **http://www.ch.embnet.org/software/BOX_form.html**). Black boxes indicate residue identity and grey boxes indicate residue similarities

almost identical binding sites with those of Gal4p. We also tested all prey found in the YFR021w screen individually with Ypl100wp bait and found three positive interactions, albeit less strong, as indicated by a lighter blue colour in X-gal assays. It should be noted that the tested prey were partial ORF sequences, therefore additional or stronger interactions with entire prey ORF sequences could not be excluded. Finally, none of the Yfr021wp prey interacted with Ygr223cp bait in individual two-hybrid assays.

Construction of ORF-deletion and overexpression strains

Single-, double- and triple-deletion strains for YFR021w, YGR223c and YPL100w ORFs were constructed as described in Materials and methods (Table 1). The PCR-synthesized deletion cassette for each ORF was used to transform haploid (*MAT***a** and *MAT*α) and diploid cells. None of the single-ORF deletions was lethal. Correct replacement of each ORF in the genome was verified by colony PCR

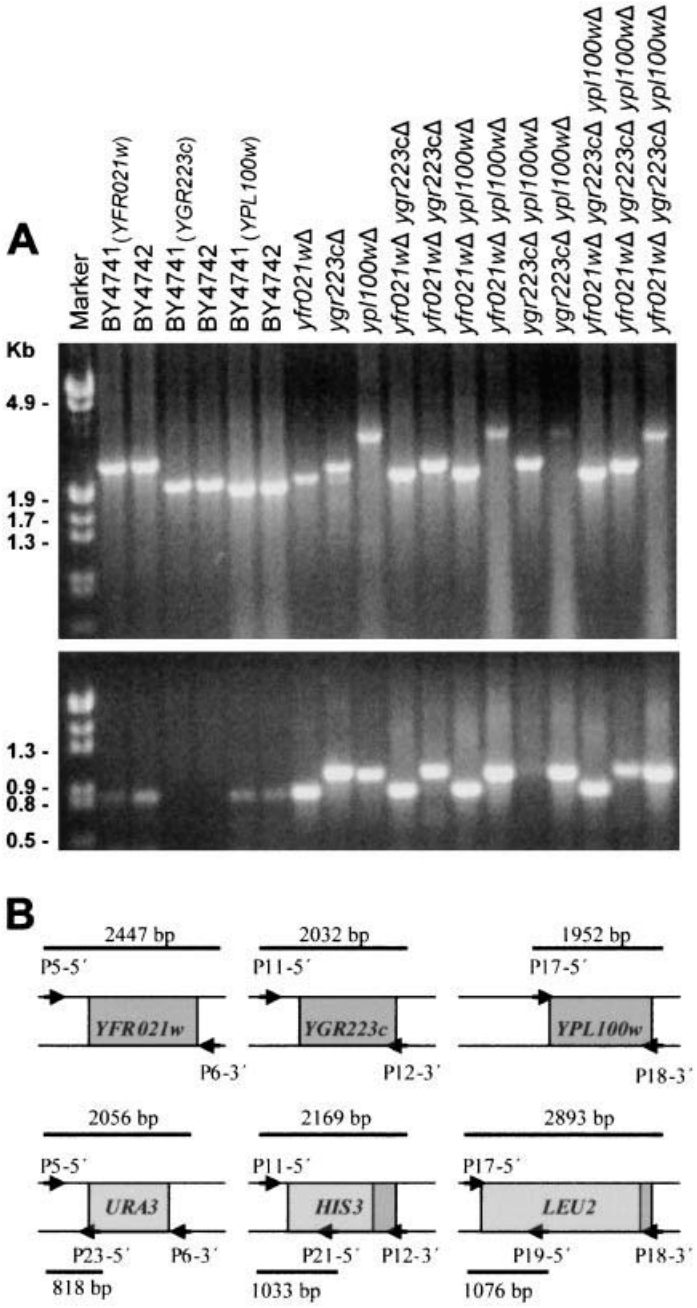

Figure 2. Molecular verification of ORF deletions. (A) Electrophoretic patterns of amplified DNAs from the indicated strains using 5′ and 3′ primers specific for ORF flanking sequences (upper part) or 5′ primers specific for ORF flanking sequence and 3′ primer specific for replacement marker sequences (lower part). The tested ORFs in the wild-type strains are indicated in parentheses. Two successive lanes of each double deletant exhibit the result for the first and second deleted ORF, respectively. The three lanes of the triple deletant show the result for the first, second and third ORF deletion, respectively. The faint bands in the lower part in BY (wild-type) lanes are non-specific products obtained under the PCR conditions used. (B) Schematic representation of the wild-type (top row) and deleted alleles (bottom row) indicating the oligonucleotide primers used and the expected sizes of PCR products

Table 3. ORFs identified in two-hybrid screens

ORF/gene prey	Number of clones in frame identified	Number of different clones in frame retransformed	β-galactosidase	Insert size in nt	Position within ORF for first nucleotide	Coordinates (ORF size in nt)	Chromosome	Size of predicted protein in aa	Features/function
YFR021w bait (500 aa)									
YHR103w/ SBE22	18	3	L[#]	~1500	1146	320416–322974 (2559)	VIII	852	Protein required for bud growth/cell wall maintenance
			L	~1200	1146				
			L	~1100	564				
YBL103c/ RTG3*	9	1	B	~900	255	23530–22070 (1461)	II	486	Basic helix–loop–helix (bHLH)-leucine zipper transcription factor involved in controlling metabolic interactions between mitochondria and peroxisomes
YCL063w	9	3	L	~1200	963	17273–18544 (1272)	III	423	Protein involved in sensitivity to certain drugs; has similarity to plant aminocyclopropane-1-carboxylate synthase
			B	~750	1212				
			B	~700	1215				
YML059c*	5	3	L	~1200	1392	158258–153219 (5040)	XIII	1679	Protein of unknown function
			B	~850	1254				
			L	~500	1824				
YNR058w/ BIO3	3	1	L	~500	360	734286–735728 (1443)	XIV	480	DAPA aminotransferase component of the biotin biosynthesis pathway
YLR094c/ GIS3	2	2	B	~800	624	329239–327731 (1509)	XII	502	Protein of unknown function, multicopy suppressor of galactose and raffinose utilization mutations
			B	~700	474				
YER162c/ RAD4	2	1	L	~500	1629	502890–500626 (2265)	V	754	Component of the nucleotide excision repairsome, homologue to the human XPC xeroderma pigmentosum gene product
YJR140c/ HIR3	2	2	L	~800	66	695385–690439 (4947)	X	1648	Histone transcription regulator
			L		282				

Table 3. Continued

ORF/gene prey	Number of clones in frame identified	Number of different clones in frame retransformed	β-galactosidase	Insert size in nt	Position within ORF for first nucleotide	Coordinates (ORF size in nt)	Chromosome	Size of predicted protein in aa	Features/function
YFR029w/ PTR3	1	1	L	~500	630	210924–212960 (2037)	VI	678	Peptide permease regulator
YFR019w/ FAB1	1	1	W	~700	5076	184489–191325 (6837)	VI	2278	Phosphatidyinisitol-3-phosphate 5-kinase involved in orientation or separation of mitotic chromosomes; may regulate vacuolar function and morphology
YJR025c/ BNA1*	1	1	L	~500	360	471359–470826 (534)	X	177	3-Hydroxyanthranilate 3,4-dioxygenase, involved in biosynthesis of nicotinic acid from tryptophan
YGR223c bait (448 aa)									
YBR138c/ HDR1	1	1	B	~600	387	515295–513721 (1575)	IV	524	Protein involved in meiotic segregation
YPL100w bait (496 aa)/none									

*Also interacting with YPL100w bait in individual two-hybrid assays.
aa, Amino acids.
#Light blue, Blue, White.

(see Materials and methods; Figure 2). Double-ORF deletion strains were generated by mating of the appropriate single-mutant strains, sporulation and subsequent tetrad analysis. None of the double-ORF deletions was lethal. Finally, the triple-ORF deletion strain was generated by mating of *yfr021wΔ ygr223cΔ* with an opposite mating type *ypl100wΔ*, sporulation and tetrad analysis. The double- and triple-ORF deletion strains were also verified molecularly (Figure 2). The triple deletion was also viable. The *yfr021wΔura3* strain, used for phenotypic complementation, was derived from *yfr021wΔ* by replacement of the *URA3* marker with a coding frame disrupted *URA3* DNA fragment (*URA3* digested with *Nco*I, treated with Klenow polymerase and religated). *yfr021wΔura3* cells were selected on FOA plates. ORF-overexpression strains were generated by transformation of wild-type cells with the tetracycline-regulated pCM190–ORF-containing plasmids, as described in Materials and methods. Analysis of the ORF-specific RNA levels confirmed overexpression, compared to the endogenous mRNA levels (data not shown).

Growth phenotypes of deletion strains suggest mitochondrial and/or peroxisomal functions for the YFR021w/YGR223c/YPL100w ORF family

Serial dilutions of cultures of the same mating type for single-, double- and triple-deletion and wild-type strains were examined in parallel for growth under standard conditions on YPD, YPG (to detect mitochondrial defects) and SC plates (to enhance leaky phenotypes), at 15°C, 30°C and 37°C (to detect thermosensitivity) (Hampsey, 1997). Compared to the wild-type cells, the only phenotypic difference observed was for all strains carrying a YFR021w ORF deletion that exhibited reduced growth in YPG medium, more pronounced at 37°C (data not shown). This indicated a role for *YFR021w* in respiratory competence of the cells (mitochondrial function) grown in a non-fermentable carbon source such as glycerol. We did not detect the *ygr223cΔ* phenotypes reported by Gallwitz (mentioned above), probably due to genetic background differences between the two S288C derivatives (BY and FY). Growth of ORF-overexpression strains was similarly tested and no distinct phenotype was observed (data not shown). Therefore, we further used the ORF-overexpression plasmids for phenotypic complementation assays of ORF-deleted strains (see below).

Given the growth phenotype of *yfr021wΔ* in YPG and the detected two-hybrid interactions of both Yfr021wp and Ypl100wp with Rtg3p, we further explored the possibility that the ORF family participates in mitochondria-related functions and in *RTG*-dependent pathways. This could also test the biological significance of the aforementioned two-hybrid protein interactions. The Rtg3p peptide identified in our two-hybrid screen includes ~300 residues and lacks the N-terminal 85 residues (containing an LDFS motif involved in SAGA recruitment; Massari *et al.*, 1999) and ~100 C-terminal residues of unknown function. Rtg3p is a bHLH/Zip family transcriptional activator, which heterodimerizes with another bHLH/Zip protein, Rtg1p, and regulates both basal and elevated expression of the *CIT2* and *DLD3* (*YEL071w*) genes by direct binding to their promoters. *CIT2* encodes the peroxisomal isoform of citrate synthase, enzyme of the glyoxylic cycle (Jia *et al.*, 1997), and *DLD3* encodes a cytoplasmic D-lactate dehydrogenase of unknown role (Chelstowska *et al.*, 1999). In cells with reduced or eliminated mitochondrial respiratory capacity, Rtg1p/Rtg3p are also responsible for the expression of *CIT1*, *ACO1*, *IDH1* and *IDH2*, encoding the first three steps of the TCA cycle, leading to synthesis of α-ketoglutarate, precursor of glutamate (Liu and Butow, 1999). In cells with fully functional mitochondria, such as those in diauxic shift, these TCA cycle genes are regulated by the Hap2p-5p transcriptional complex (Liu and Butow, 1999). Rtg1p/Rtg3p transcription factors are also essential for the expression of genes involved in peroxisome biogenesis and proliferation in cells grown in medium containing oleic acid (Jia *et al.*, 1997). *rtg1Δ* and *rtg3Δ* strains are viable and respiratory-competent, but they are auxotrophic for glutamate or aspartate in minimal glucose medium, show limited growth in oleic acid-based media and cannot use acetate as a sole carbon source (Liao and Butow, 1993, 1999; Rothermel *et al.*, 1997). When the two cycles are defective, cells do not grow on media containing ammonia as the sole nitrogen source and this defect can be rescued by the addition of glutamate in the media (Hampsey, 1997; Liao and Butow, 1993, 1999).

Based on these data, we tested all deletants on various media requiring functional TCA and glyoxylate cycles, at three temperatures, seeking phenotypes concerning direct or indirect defects in these pathways. All *yfr021wΔ* strains showed a slight but observable growth reduction (less in *yfr021wΔ*

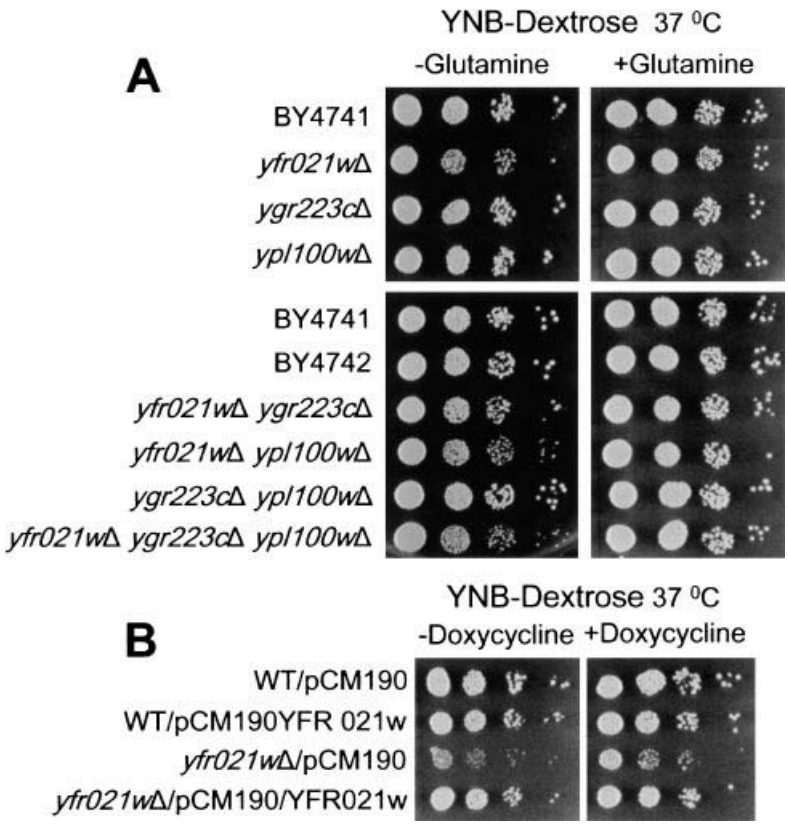

Figure 3. Effect of ORF deletions in cells grown in minimal glucose medium in the presence or absence of glutamine. (A) Serial dilutions (from left to right) of exponential cultures spotted on YNBD plates and incubated for 2 days. Glutamine was added to a final concentration of 0.02%. (B) Phenotypic complementation of YFR021w ORF deletion. Serial dilutions of exponential cultures transformed with pCM190 vector or with YFR021w subcloned into pCM190 spotted on YNBD plates or YNBD supplemented with 0.1 μg/ml doxycycline (to avoid muticopy effects) and incubated for 2 days. (The pCM190 vector alone caused slow growth of the *yfr021w*Δ strain.)

*ygr223w*Δ) on YNBD (containing ammonia as the sole nitrogen source) at 37°C, and growth was restored in YNBD supplemented with glutamine (Figure 3A) or glutamate or aspartate (data not shown). Phenotypic complementation was accomplished by the expression of YFR021w ORF in *yfr021w*Δ*ura3* (Figure 3B). Although not identical to *rtg* phenotypes, *yfr021w*Δ phenotype at 37°C, at which mitochondrial contribution is increased, was in agreement with that observed in YPG medium. Similarly reduced growth phenotype of *yfr021w*Δ strains only was also observed on media containing, as sole carbon source, oleic acid at 37°C (Figure 4). Growth on acetate-based medium at 15°C revealed wild-type phenotype for the *yfr021w*Δ single deletant but slight growth reduction for all *ypl100w*Δ strains, more growth reduction for *ygr223c*Δ *ypl100w*Δ and almost no growth for the triple deletant (Figure 4). Therefore, all three ORFs might have a role related to mitochondrial and/or peroxisomal functions.

At growth conditions even more demanding for mitochondrial competence, such as minimal (YNB) glycerol-based medium, all strains, except *ygr223w*Δ single deletant, showed a definite inability to grow at 30°C (Figure 5A). *ygr223w*Δ showed significantly reduced growth compared to wild-type cells and intermediate phenotype in *ygr223w*Δ *ypl100w*Δ. For the same incubation time, no growth was detected at 37°C and 15°C, even for wild-type cells. Addition of glutamate or aspartate in the above medium restored growth of *ypl100w*Δ, *ygr223c*Δ and *ypl100w*Δ *ygr223c*Δ strains but not of y*fr021w*Δ strains (Figure 5B). The triple deletant appeared with partially restored growth. These phenotypes, although not identical to the known *rtg* phenotypes,

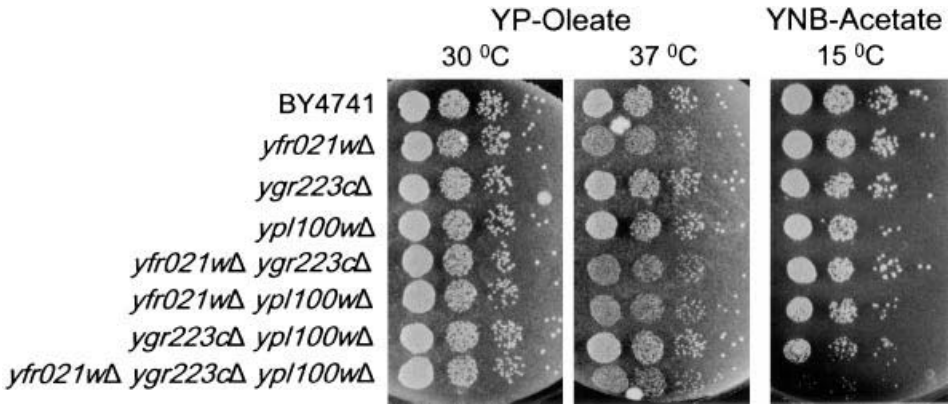

Figure 4. Effect of ORF deletions in cells grown in oleate complete or acetate-based medium. Serial dilutions of exponential cultures spotted on YPOl plates and incubated at 30°C for 2 days and at 37°C for 4 days, or spotted on YNBAc plates (containing 0.5% yeast extract) and incubated at 15°C for 10 days

demonstrated a major defect for the strains carrying deletions of YFR021w and YPL100w ORFs, and less severe defect for the single YGR223c ORF deletant, under conditions that required functional mitochondria.

ORF deletions of the *YFR021w/YGR223c/ YPL100w* family affect *RTG*-dependent gene expression

To investigate the relation of the observed mitochondrial and/or peroxisomal defect phenotypes to the known *RTG*-dependent expression, we analysed the levels of *CIT1*, *CIT2* and *DLD3* mRNAs in all ORF-deleted strains. *CIT2* transcription is upregulated as part of a stress response pathway of nuclear genes induced by mitochondrial dysfunction or elimination (petites), termed retrograde regulation (Liao and Butow, 1993; Liao *et al.*, 1991). It is associated with the cytoplasmic-to-nuclear translocation of Rtg1p/ Rtg3p heterodimer, dephosphorylation of Rtg3p, and depends on the cytoplasmic protein Rtg2p (Sekito *et al.*, 2000). In respiratory-competent cells, the expression of *CIT2* is low but still dependent on the Rtg proteins (Jia *et al.*, 1997; Liu and Butow, 1999) and can be elevated up to 40-fold, depending on the severity of mitochondrial disfunction (Liao *et al.*, 1991; Small *et al.*, 1995) to ensure the synthesis of sufficient levels of glutamate for biosynthetic processes and sufficient metabolites from the glyoxylate to the

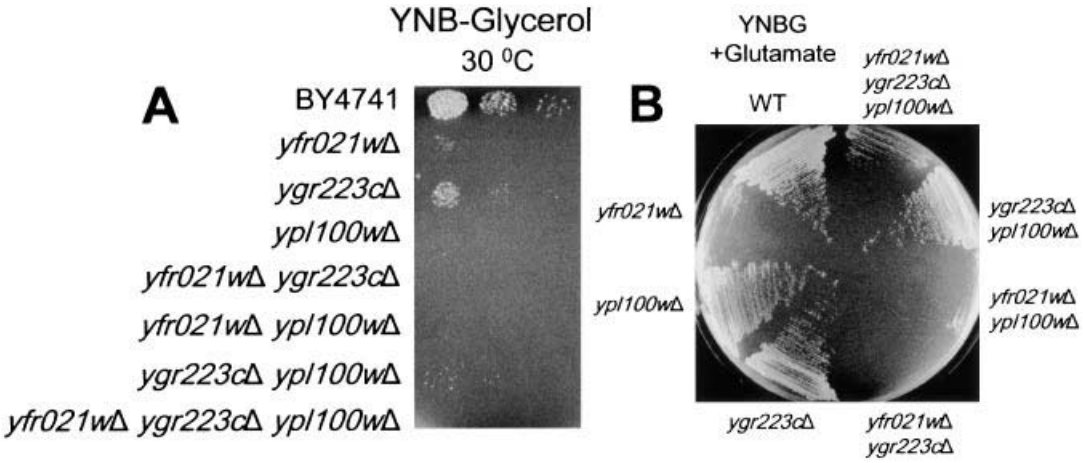

Figure 5. Effect of ORF deletions in cells grown in glycerol minimal medium. (A) Serial dilutions of exponential cultures spotted on YNBG plates and incubated at 30°C for 13 days. (B) Streaks of the same cultures on YNBG plates in the presence of glutamate incubated at 30°C for 13 days

TCA cycle to support anabolic pathways. Glutamate acts as a feedback regulator of the RTG system, being a potent repressor of *RTG*-dependent expression (Liu and Butow, 1999).

Our mRNA analysis showed that under poor growth conditions (YNBG at 30°C), at which wild-type cells rely on mitochondrial functions, *CIT2* mRNA levels were higher compared to those in cells grown in dextrose-based media. Under these conditions, *CIT2* mRNA was further elevated in all *yfr021w*Δ and *ypl100w*Δ strains, except in *yfr021w*Δ *ygr223c*Δ, which showed wild-type levels similar to *ygr223c*Δ cells (Figure 6). This result implied that deletion of YFR021w or YPL100w ORF resulted in mitochondrial dysfunction, in agreement with the reduced growth phenotypes observed in *yfr021w*Δ and *ypl100w*Δ strains under these conditions (Figure 5). Deletion of YGR223c did not result in mitochondrial dysfunction and apparently reversed the *yfr021w*Δ effect on *CIT2* mRNA. In cells grown in the less poor YNBD medium, but at 37°C (which requires more efficient function of mitochondria than at 30°C), *CIT2* mRNA was elevated in all *yfr021w*Δ strains (but not to the extent of the previous condition), in accordance with the observed reduced growth of all *yfr021w*Δ strains at these conditions (Figures 3, 6). In contrast, in YNBD medium at 30°C, where all strains grew as well as the wild-type (see above), *yfr021w*Δ and *ypl100w*Δ single deletants showed lower than wild-type *CIT2* mRNA levels and only in double and triple mutants were these levels higher than wild-type. *ygr223c*Δ did not affect *CIT2* expression but reversed the *ypl100w*Δ effect in the double deletant *ygr223c*Δ *ypl100w*Δ (Figure 6). On the same RNA blots, *DLD3* mRNAs exhibited profiles that matched those of *CIT2*, although *CIT1* mRNAs were at equally low levels across all samples and conditions (data not shown).

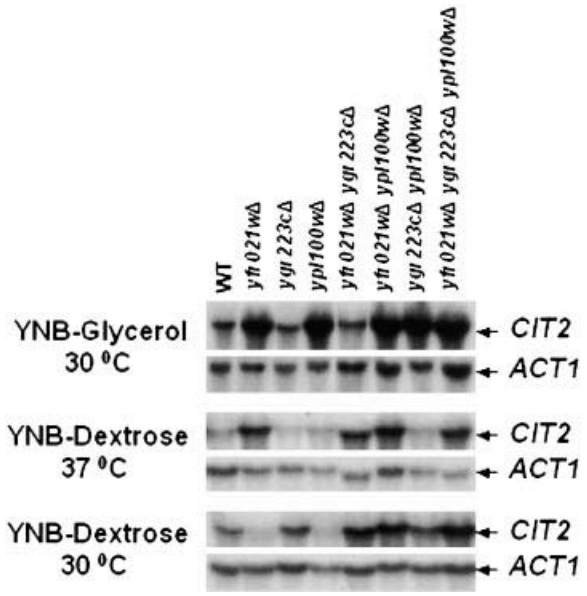

Figure 6. Effects of ORF deletions on the *CIT2* mRNA accumulation. Total RNA was isolated from cultures of the indicated strains and probed with *CIT2* and *ACT1* (actin/internal quantitative control) radiolabelled DNA probes. Cells were grown exponentially at the indicated conditions, except those in YNBG, which were first grown in YNBD and then transferred in YNBG medium for 6 h

These results showed that, under conditions of 'minimal' mitochondrial function, under glucose repression (YNBD/30°C), deletion of YFR021w or YPL100w ORF resulted in reduced *CIT2*/*DLD3* expression, attributing to each ORF a positive role in the constitutive *RTG*-driven expression. However, deletion of both ORFs resulted in mitochondrial defects severe enough to cause retrograde response, monitored by the elevated levels of the *CIT2*/*DLD3* mRNAs. Under conditions where cells relied more (YNBD/37°C) or exclusively (YNBG, glucose derepression) on mitochondrial functions, even single-ORF deletions resulted in retrograde response of the *CIT2*/*DLD3* expression. Therefore, Ypl100wp and Yfr021wp, additionally to their positive role in the Rtg3p-dependent constitutive *CIT2*/*DLD3* expression, participate in other unknown mitochondrial functions. Furthermore, we can conclude that Ypl100wp and Yfr021wp are not required for the *RTG*-dependent transcriptional induction of *CIT2*/*DLD3* under retrograde response. In accordance with this, we found that under the extreme conditions of mitochondrial DNA elimination (cells grown in the presence of ethidium bromide: Guthrie and Fink, 1991) and subsequent induction of retrograde response (cells transferred in raffinose-based medium containing ethidium bromide), *CIT2* mRNA was elevated to equally high levels in wild-type and *yfr021w*Δ or *ypl100w*Δ cells (data not shown).

Connections of the YFR021w/YGR223c/YPL100w ORF family to the *Ssy1p*/*Ptr3p* amino acid sensor

In addition to Rtg3p, Yfr021wp also interacted in two-hybrid context with Ptr3p, a unique structure protein localized on the cytosolic phase of the

plasma membrane (Barnes *et al.*, 1998; Klasson *et al.*, 1999). Ptr3p is functionally associated with a plasma membrane member of the AAP family, Ssy1p (Klasson *et al.*, 1999) and both are probably components of an extracellular amino acid sensor. *ssy1* and *ptr3* mutations pleiotropically affect amino acid uptake, vacuolar amino acid pools (Klasson *et al.*, 1999) and the steady-state levels of specific amino acid permease mRNAs (Iraqui *et al.*, 1999; Klasson *et al.*, 1999). Ptr3p is a candidate for binding cytoplasmic amino acids directly and transporting them to other cellular regions to exert gene regulatory functions (Klasson *et al.*, 1999). Both *ssy1*Δ and *ptr3*Δ are resistant to toxic levels of histidine that kill wild-type cells, due to decreased levels of histidine uptake by the Hip1p (histidine-specific permease) and mainly due to increased compartmentalization of histidine in the vacuole (Klasson *et al.*, 1999). We have tested our ORF-deleted strains for such phenotype by growth in YNBD supplemented with 30 mM histidine and found, surprisingly, that only *ypl100w*Δ strains were viable (Figure 7A). Compared to *ypl100w*Δ, viability was somewhat reduced in *ypl100w*Δ *ygr223c*Δ strain and further reduced in the triple mutant. The *ypl100w*Δ phenotype was complemented by the expression of YPL100w ORF in that strain (Figure 7B). We should note that we found no significant two-hybrid interaction between Ypl100wp and the Ptr3p portion isolated by the Yfr021wp two-hybrid screen. However, since that included only 170 internal residues, lacking the 210 N-terminal and about 300 C-terminal residues, we cannot exclude the possibility of Ypl100wp interaction with a missing Ptr3p region. Additional evidence indicating that *YPL100w* function might be related to amino acid-sensing pathways was obtained by the response of a *HIS3*UAS–*lacZ* reporter (Georgakopoulos *et al.*, 1995) in the singly-deleted strains. Induction of β-galactosidase levels was only detected in *ypl100w*Δ (not in *ygr223c*Δ or *yfr021w*Δ), implying induction of the GCN system by amino acid starvation in that strain (Figure 7C).

Conclusions

Our functional analysis of the YFR021w/YGR223c/YPL100w ORF family consists of the combination

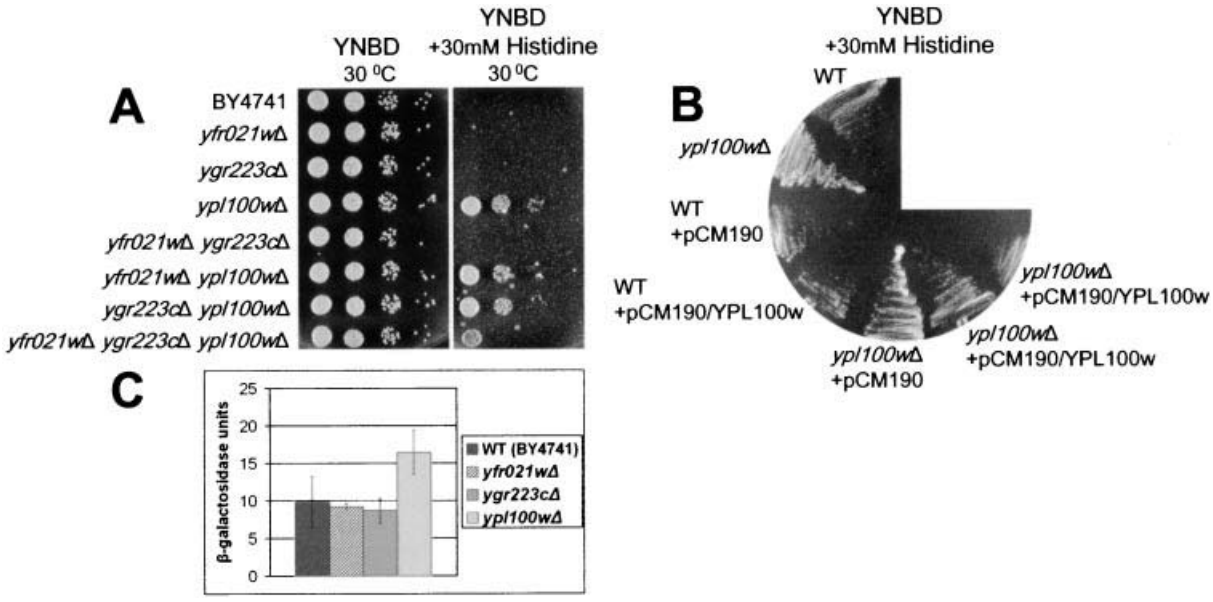

Figure 7. (A) Effect of ORF deletions in cells grown in toxic levels of histidine. Serial dilutions of exponential cultures spotted on YNBD plates containing 30 mM histidine and incubated for 4 days. (B) Phenotypic complementation of YPL100w ORF deletion. Streaks of strains transformed with pCM190 vector or with YPL100w subcloned into pCM190 on YNBD plates containing 30 mM histidine in the presence of 0.1 μg/ml doxycycline and incubated for 4 days. (C) Effect of ORF deletions on *HIS3*UAS–*lacZ* reporter. β-galactosidase units obtained from assays on strains transformed with *HIS3*UAS–*lacZ*-bearing plasmids and grown in YNBD exponentially. Average values from three independent experiments are presented

of results from two-hybrid screens and from phenotypic and molecular marker assays of the ORF-deleted strains. One major conclusion from this analysis is that the three ORFs appear to have pleiotropic functions. Several specific conclusions concerning these functions can be derived.

First, all three ORFs are related to mitochondrial and/or peroxisomal functions. Single- or triple-ORF deletions exhibited reduced or no growth phenotypes under several conditions requiring functional mitochondria and peroxisomes. Cells monitor the state of organelles and respond to changes by modulating the transcription patterns of specific nuclear genes. We found that in cells relying on mitochondrial functions (end products), deletion of YFR021w or YPL100w ORFs resulted in retrograde regulation of the peroxisomal citrate synthase, detected as induced *CIT2* and *DLD3* expression. The effects of YGR223c ORF deletion on *CIT2* (and *DLD3*) expression were only detected as reversal of the effects of the other two ORF deletions.

Second, Yfr021wp and Ypl100wp are positive regulators of *CIT2*/*DLD3* basal transcription, possibly by interacting with the transcriptional activator Rtg3p, identified as a two-hybrid interactant of both Yfr021wp and Ypl100wp. Although this interaction needs to be proved by further biochemical and genetic assays, we detected a significant reduction of the basal *CIT2* (and *DLD3*) mRNA levels in the two ORF single deletants. The role of Yfr021wp or Ypl100wp could be in the nucleus on transcriptional complexes, or in the cytoplasm, related to the mobilization and translocation of the Rtg1p/Rtg3p heterodimer under conditions of basal *RTG*-driven expression.

Third, the results of the phenotypic assays suggested that the three ORFs are not parts of the same pathway. In three different conditions examined (YPG/37°C, YPOl/37°C and YNBD/37°C), only the single deletion of YFR021w resulted in detectable phenotype, and in two other conditions (YNBAc/15°C and 30 mM histidine) only the single deletion of YPL100w exhibited detectable phenotype. In another condition (YNBG/30°C) both YFR021w and YPL100w single deletions were lethal and YGR223c deletion resulted in reduced growth phenotype, while addition of glutamate or aspartate reversed only the phenotypes of YPL100w and YGR223c single deletions.

Fourth, the functions of the three ORFs have different but interacting contributions in the cell. We found no condition under which only the single deletion of YGR223c exhibited a phenotypic effect. However, in two instances the triple mutant exhibited phenotype different than those of the single- or double-gene deletions (YNBAc/15°C, 30 mM histidine). Similarly, *ygr223c*Δ *ypl100w*Δ resulted in intermediate phenotype in YNBAc/15°C and YNBG/30°C. Also, *ygr223c*Δ in combination with *yfr021w*Δ showed phenotypic reversion of some *yfr021w*Δ effects (reduced growth of *yfr021w*Δ in YNBD/37°C and *CIT2* mRNA induction in *yfr021w*Δ grown in YNBG/30°C).

Finally, we found connections of the ORF family with amino acid signalling pathways. We identified a two-hybrid interaction of Yfr021wp with the amino acid sensor component Ptr3p. This interaction needs to be proved, but combining the identified effects in *ypl100w*Δ (30 mM histidine, *HIS3*UAS–*lacZ*) and the fact that the *RTG* system is regulated by amino acids, we propose that the presented ORF family may be related to pathways that convey signals arising from the extra- and intracellular amino acid levels to transcriptional events of genes related with the import or synthesis or catabolism of particular amino acids (Iraqui *et al*., 1999). Further experiments will investigate this hypothesis. In agreement with our proposal, recently reported genetic evidence suggested other connections between *RTG*s and the Ssy1p/Ptr3p amino acid sensor (Liu Z, Sekito T, Butow R. In *Yeast Genetics and Molecular Biology 2000*, Seattle, WA; **http://genome-www.stanford.edu/Saccharomyces/yeast00/abshtml/218.html**).

New leads to the functions of YFR021w, YGR223c and YPL100w ORFs may be obtained from the analysis of several protein interactions identified in our two-hybrid screens, in addition to Rtg3p and Ptr3p. Ygr223cp interacted with Hdr1p, recently found to interact in two-hybrid context (Uetz *et al*., 2000) with the nuclear Met30p F-box protein that regulates sulphur assimilation genes in response to S-adenosylmethionine levels, is required for S-adenosylmethionine inhibition of Met4p activation function and is regulated by the availability of methionine (Smothers *et al*., 2000). Yfr021wp interacted with: (a) Bio3p, regulator of vacuole homeostasis, part of the bio-cluster containing Bio5p, involved in vitamin uptake (Phalip *et al*., 1999); (b) Fab1p, a vacuolar PI(3)P 5-kinase, also regulator of vacuole homeostasis (Yamamoto *et al*., 1995); (c) Bna1p oxidoreductase, part of the kynurenine pathway for the degradation of

tryptophan and the biosynthesis of nicotinic acid (Kucharczyk *et al*., 1998); (d) Rad4p, involved in DNA repair (Guzder *et al*., 1998); and (e) Hir3p, involved in the cell cycle-regulated repression of *HTA1-HTB1* histone genes and HML/sHMR loci (Kaufman *et al*., 1998). The significance of these interactions needs to be investigated.

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