Functional analysis of the *Saccharomyces cerevisiae* DUP240 multigene family reveals membrane-associated proteins that are not essential for cell viability

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The DUP240 gene family of *Saccharomyces cerevisiae* is composed of 10 members. They encode proteins of about 240 amino acids which contain two predicted transmembrane domains. Database searches identified only one homologue in the closely related species *Saccharomyces bayanus*, indicating that the DUP240 genes encode proteins specific to *Saccharomyces sensu stricto*. The short-flanking homology PCR gene-replacement strategy with a variety of selective markers for replacements, and classical genetic methods, were used to generate strains deleted for all 10 DUP240 genes. All of the knock-out strains were viable and had similar growth kinetics to the wild-type. Two-hybrid screens, hSos1p fusions and GFP fusions were carried out; the results indicated that the Dup240 proteins are membrane associated, and that some of them are concentrated around the plasma membrane.

Keywords: gene disruption, membrane protein, gene tandem repeats, two-hybrid system, yeast

INTRODUCTION

Multigene families have been found in all sequenced organisms. In *Arabidopsis thaliana* gene duplication is as high as 65% (Arabidopsis Genome Initiative, 2000). These paralogous genes have similar sequences. There are numerous, more or less ancient, examples that reflect different situations: tRNA genes dispersed throughout the genome; rRNA genes gathered in clusters; dispersion and lower duplication levels for the genes encoding ribosomal proteins. The duplication phenomenon is observed for genes with known and unknown functions. Studying these multigenic families should provide useful information about the functions of the different copies and also about how they are involved in genome dynamics.

Saccharomyces cerevisiae S288C potentially encodes 5651 ORFs (Malpertuy *et al.*, 2000). Of these, 914

(16.2%) belong to two-gene families and 1544 (27.3%) belong to multigene families with between three and over 20 members (Blandin *et al.*, 2000). In some cases, there are physiological reasons for the presence of multigene families. For example the cytoplasmic and mitochondrial methionyl-tRNA synthetases are encoded by two related but different nuclear genes, resulting in different localizations (Schneller *et al.*, 1978). The genes encoding alcohol dehydrogenase (*ADHI* and *ADHII*) are very similar, but *ADHI* catalyses the formation of ethanol from acetaldehyde whereas *ADHII* catalyses the formation of acetaldehyde from ethanol (Johnston & Carlson, 1992). Another well-studied case is the *CUP1* locus, which encodes a copper- or cadmium-chelating metallothionein expressed after induction with metals. The reference strain, S288C, contains only two copies of *CUP1*, but copper- or cadmium-resistant strains can contain up to 15 copies (Karin *et al.*, 1984). The expression of most hexose transporters is tightly regulated by glucose concentration, starvation, osmotic pressure and the physiological state of the cells. In fact, to abolish glucose consumption and transport activity completely, all 18 members of the hexose transporter family, *HXT1–17*, *GAL2* and three members of the

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Abbreviation: GFP, green fluorescent protein.

Fig. 1. Map of the genetic organization, chromosome localization and predicted amino acid sequence identities of the 10 *DUP240* ORFs of *S. cerevisiae*. The ORFs are represented by rectangles, their orientation with respect to the centromeres by arrows. The percentages show the level of amino acid identities between the indicated ORF products.

maltose transporter family (*AGT1*, *YDL247w* and *YJR160c*) have to be deleted (Wieczorke *et al.*, 1999).

In comparison with these established examples and with the exception of the *PAU* and *OSBP* families (Rachidi *et al.*, 2000; Beh *et al.*, 2001), the large gene families identified by sequencing programmes remain mostly unstudied. This is the case for the *DUP240* gene family. This family is particularly interesting because of its unusually high copy number (10), the high level of nucleotide identity between some of its members and the specific chromosomal organization of the members (Fig. 1). The *YAR027w*, *YAR028w*, *YAR029w*, *YAR031w* and *YAR033w* ORFs are arranged as tandem repeats on chromosome I and the *YGL051w* and *YGL053w* ORFs are arranged as tandem repeats on chromosome VII. The nucleotide sequences of *YAR033w* and *YAR031w* are 98% identical to those of *YGL051w* and *YGL053w* (Feuermann *et al.*, 1997). Most of the corresponding proteins are approximately 240 aa long. Moreover, these ORFs appear to be specific for the *Saccharomyces sensu stricto* group (Bon *et al.*, 2000).

We carried out a functional analysis of these genes to get some insight into the role of the proteins which they encode. We replaced all 10 members of the *DUP240* family and screened for associated phenotypes using the criteria defined by the EUROFAN network (Dujon, 1998; Oliver *et al.*, 1998). Green fluorescent protein (GFP) fusion fluorescence was subsequently used to study the subcellular localization of the different ORF products. Activation of the RAS signalling pathway through hSos1p–Dup fusions and the classical *GAL4*-based nuclear two-hybrid system were used to determine whether some of the Dup240 proteins were localized at the plasma membrane and to search for partners that interact with the products of the *YGL051w* and *YGL053w* genes. Our results indicated that the *DUP240*

gene family encodes membrane-associated proteins that are not essential for cell viability.

METHODS

Yeast strains and media. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Cells were grown on YPD [1% yeast extract, 2% peptone, 2% glucose (dextrose) and 2% agar], YPG (1% yeast extract, 2% peptone, 2%, v/v, glycerol and 2% agar) and SD [6.7 g yeast nitrogen base l⁻¹, 2% glucose (dextrose) and 2% agar] supplemented with the amino acids and bases not required for the selection. The same media without agar were used for liquid cultures. Synthetic complete glucose medium (SC) was used for the two-hybrid selections (Sherman *et al.*, 1986). Sporulation of diploids required growth on YPD overnight at 30 °C prior to transfer onto sporulation plates (1% potassium acetate and 2% agar) for 3–5 days at 30 °C.

PCR-mediated gene replacements. The primers used for PCR-mediated gene disruption consisted of 40–60 nt specific to sequences just upstream or downstream from the ORF to be deleted followed at the 3' end of the primers by 18–20 bases homologous to the upstream or downstream flanking region of a selectable marker gene located in a yeast integrating vector (Table 2). PCR conditions were: 94 °C for 2 min, (94 °C for 30 s, 54 °C for 30 s, 72 °C for 2 min) × 30 cycles, 72 °C for 10 min. The specific PCR products were treated with phenol/chloroform prior to ethanol precipitation. Usually 1–5 µg amplified DNA was used to transform *S. cerevisiae*. High-efficiency transformations were performed by the method described by Gietz *et al.* (1995). Correct ORF replacements were checked by PCR analysis. Oligonucleotides were designed to bind outside the target loci (A1 and A4), within the target loci (A2 and A3) or within the marker genes (M2 and M3). In diploid yeast transformants with correctly integrated markers, one of the two copies of the target locus was replaced by the marker module. In PCR experiments with genomic yeast DNA and using either A1, A2 and M2 or M3, A3 and A4, the correct integration of the marker DNA was

Table 1. *S. cerevisiae* strains

Strain	Genotype	Source*
SFY526	MATa <i>trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2-801 ade2-101 URA3::GAL1-lacZ can</i>	Bartel <i>et al.</i> (1993b)
PJ69-4a	MATa <i>trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
PJ69-4x	MATα <i>trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	P. James
CDC25H	MATa <i>ade2-801 cdc25-2 his3-200 leu2-3,112 trp1-901 ura3-52</i>	Petitjean <i>et al.</i> (1990)
(All subsequent strains are isogenic to S288C)		
BY4709	MATα <i>ura3Δ0</i>	ATCC 200872
FY1679	MATa/MATα <i>his3Δ200/HIS3 leu2Δ1/LEU2 trp1Δ63/TRP1 ura3Δ52/ura3Δ52</i>	ATCC 96604
FYBL1-17B	MATa <i>his3Δ200 trp1Δ63 ura3Δ851</i>	B. Dujon
FYBL1-23D	MATα <i>his3Δ200 trp1Δ63 ura3Δ851</i>	B. Dujon
LD073	MATa <i>YGL053w-YGL051wΔ::kan his3Δ200 trp1Δ63 ura3Δ851</i>	This study
LD076	MATα <i>YAR027w-YAR033wΔ::HIS3 his3Δ200 trp1Δ63 ura3Δ851</i>	This study
LD078	MATa <i>YAR027w-YAR033wΔ::HIS3 YGL053w-YGL051wΔ::kan his3Δ200 trp1Δ63 ura3Δ851</i>	This study
BY4734	MATα <i>his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0</i>	ATCC 200896
LD084	MATα <i>YGL053w-YGL051wΔ::kan his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD099	MATa <i>YAR027w-YAR033wΔ::HIS3 YGL053w-YGL051wΔ::kan his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD100	MATα <i>YAR027w-YAR033wΔ::HIS3 YGL053w-YGL051wΔ::kan his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD102	MATa <i>YAR023cΔ::MET15 YAR027w-YAR033wΔ::HIS3 YGL053w-YGL051wΔ::kan his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD103	MATα <i>YAR023cΔ::MET15 YAR027w-YAR033wΔ::HIS3 YGL053w-YGL051wΔ::kan his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD107	MATa <i>YAR023cΔ::MET15 YAR027w-YAR033wΔ::HIS3 YGL053W-YGL051wΔ::kan YHL044wΔ::LEU2 his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD108	MATα <i>YAR023cΔ::MET15 YAR027w-YAR033wΔ::HIS3 YGL053w-YGL051wΔ::kan YHL044wΔ::LEU2 his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD115	MATa <i>YAR023cΔ::MET15 YAR027w-YAR033wΔ::HIS3 YCR007cΔ::TRP1 YGL053w-YGL051wΔ::kan YHL044wΔ::LEU2 his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study
LD114	MATα <i>YAR023cΔ::MET15 YAR027w-YAR033wΔ::HIS3 YCR007cΔ::TRP1 YGL053w-YGL051wΔ::kan YHL044wΔ::LEU2 his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ</i>	This study

* ATCC, American Type Culture Collection, Manassas, VA, USA; P. James, University of Wisconsin, Madison, WI, USA; B. Dujon, Institut Pasteur, Paris, France.

confirmed by the appearance of two PCR products of the predicted length: one characteristic for the wild-type allele (A1-A2 or A3-A4 amplification product) and a second fragment characteristic for the mutated allele (A1-M2 or M3-A4 amplification product). Incorrect transformants yielded only the amplification products of the wild-type allele.

DNA preparations and *Escherichia coli* transformation. Bacterial plasmid DNA preparations, bacterial transformations and DNA manipulations were carried out according to standard protocols (Sambrook *et al.*, 1989). Yeast genomic DNA and plasmid DNA were purified according to the method described by Hoffman & Winston (1987).

Cloning of a PCR product in a linearized plasmid by homologous recombination in yeast. Plasmids were linearized by two different endonucleases and dephosphorylated with calf intestine phosphatase (Boehringer). The sequences to be cloned were amplified by PCR (Saiki *et al.*, 1985) from genomic DNA of yeast strain FY1679 with oligonucleotide

primers having 20 additional nucleotides at the 5' ends. These ends were homologous to the ends of the linearized plasmid and used for cloning by homologous recombination in this plasmid (Muhlethaler *et al.*, 1992). The PCR conditions were: 94 °C for 1.5 min, (94 °C for 30 s, 48 °C for 2 min, 70 °C for 2.5 min) × 10 cycles, (94 °C for 30 s, 60 °C for 2 min, 70 °C for 2.5 min) × 35 cycles, 70 °C for 10 min. The PCR product and the linearized plasmid were treated with phenol/chloroform prior to ethanol precipitation. High-efficiency transformations were performed according to the method described by Gietz *et al.* (1995). For cloning in pGBT9 (Bartel *et al.*, 1993a), pSOS (Stratagene) and pGRU1 (see below), the vector was linearized by *EcoRI* and *Sall*, *NcoI* and *MluI*, and *EcoRI* and *Sall*, respectively. The PCR product and the corresponding linearized plasmid were introduced into the yeast cells simultaneously. The oligonucleotide sequences are given in Table 2. We ensured that there were no errors in the junctions between the plasmid and the inserts for pGBT9-YGL051w and pGBT9-YGL053w by sequencing.

Table 2. Oligonucleotides used as primers to generate PCR products

Name	Oligonucleotides used for PCR-based ORF disruptions	Plasmid†	ORF replacement‡
	Sequence (5'→3')*		
53S1	CATAACAACCTCCAAAACCATATAATAACCTTACACAAGA-CAAGA	pFA6a-kanMX4	<i>YGL053w</i> – <i>YGL051wΔ::kan</i>
51S2	TATCAATTCAACATGCGTACGCTGCAGGTCGAC	pFA6a-HIS3MX6	<i>YAR027w</i> – <i>YAR033wΔ::HIS3</i>
	CTGATTATATATCATACTCTAGTTTATGTTTCGCTTTACGT-ATGGCAG	pFA6a-kanMX4	<i>YGL053w</i> – <i>YGL051wΔ::kan</i>
23S1	TGTCTCTAAGCTAATCGATGAATTCGAGCTCG	pFA6a-HIS3MX6	<i>YAR027w</i> – <i>YAR033wΔ::HIS3</i>
	TGCGTTCCTTTTATACCAATATATTAGATACGTAAACTC-TACTCAAGATTGTAAGTGCAGAGTGCAC	pRS401	<i>YAR023cΔ::MET15</i>
23S2	CAGAGTTATTTGCTTTACGAAATTGTACGCGCCAAGTATA-TAATGCTGTGCGGTATTTTACACCG	pRS401	<i>YAR023cΔ::MET15</i>
07S1	CGCTCAGTATAAGTGCTAAATAAAATTATCAAGATTATATA-TTTCAAGAGAGAGGGCCAAGAGGGA	pRS404	<i>YCR007cΔ::TRP1</i>
07S2	GTCTATACCATATCAAGACAAGAAACAAACTCCGTATCT-GCATGGGCAAGTGCACAAACAATAC	pRS404	<i>YCR007cΔ::TRP1</i>
44S1	TATAAAGACAAATTCAAAAGCAAGTGAGGGCCCGCTAAGG-CTATGAAGGCCGTTTCTGACAGAG	pRS405	<i>YHL044wΔ::LEU2</i>
44S2	GC GTTATAAAAACTAAAGTAGAACCTAGAAATACCTTCC-AATCAATCGCACAGAATCAAATTCG	pRS405	<i>YHL044wΔ::LEU2</i>
Name	Oligonucleotides used for cloning by recombination	Plasmid§	Insert name
	Sequence (5'→3')§		
51P1	ACCAAAGGTCAAAGACAGTTGACTGTATCGATGCAGACCCCTCTAGAAAAG	pGBT9	<i>YGL051w</i>
51P2	TAAGAAATTCGCCCCGGAATTAGCTTGGCTGCTATTCCGCTCTTTTAAGAAGC		
53P1	ACCAAAGGTCAAAGACAGTTGACTGTATCGATGCAGACCCCTTCAGAAAAATAC	pGBT9	<i>YGL053w</i>
53P2	TAAGAAATTCGCCCCGGAATTAGCTTGGCTGCTAAAAAACTCATCGACACC		
29SOS1	ATTAGTTATAGTAGGATCCCCATGAATAAAATATCTATTTGACC	pSOS	<i>YAR029w</i>
29SOS2	CGCGGCGGCCGCGAGCTCACTACAATATCCGCTGTCTTGG		
31SOS1	ATTAGTTATAGTAGGATCCCCATGTCGCTCAATACCATTT	pSOS	<i>YAR031w</i>
31SOS2	CGCGGCGGCCGCGAGCTCACTAAAAAACTCATCGACACC		
33SOS1	ATTAGTTATAGTAGGATCCCCATGCAGACCCCTCCAGAAAAG	pSOS	<i>YAR033w</i>
33SOS2	CGCGGCGGCCGCGAGCTCACTATTCCGCTCTTTTAAGAAGC		
51SOS1	ATTAGTTATAGTAGGATCCCCATGCAGACCCCTCTAGAAAAG	pSOS	<i>YGL051w</i>
51SOS2	CGCGGCGGCCGCGAGCTCACTATTCCGCTCTTTTAAGAAGC		
53SOS1	ATTAGTTATAGTAGGATCCCCATGCAAACCCCTTCAGAAAAAT	pSOS	<i>YGL053w</i>
53SOS2	CGCGGCGGCCGCGAGCTCACTAAAAAACTCATCGACACC		
adhGRUf	GCGTTGGCCGATTCATTCCCGTTGCTTGCATGCAACTTC	pGRU1	<i>Padh1</i>
adhGRUr	CGGAGCTTGCATGCCTGCAGCGGCCGCATCTTTCAGGAGGCTTGCTTCT		
29ADHf	GAAGCAAGCCTCCTGAAAGATGAATAAAATATCTATTTGACC	pGRU1-Padh1	<i>YAR029w</i>
29ADHr	CGGAGCTTGCATGCCTGCAGCAATATCCGCTGTCTTGGA		
31ADHf	GAAGCAAGCCTCCTGAAAGATGTCGCTCAATACCATTTT	pGRU1-Padh1	<i>YAR031w</i>
31ADHr	CGGAGCTTGCATGCCTGCAGAAAAAACTCATCGACACCAGG		
33ADHf	GAAGCAAGCCTCCTGAAAGATGCAGACCCCTCCAGAAAAG	pGRU1-Padh1	<i>YAR033w</i>
33ADHr	CGGAGCTTGCATGCCTGCAGTTCCGCTCTTTTAAGAAGCGC		
51ADHf	GAAGCAAGCCTCCTGAAAGATGCAGACCCCTCTAGAAAAG	pGRU1-Padh1	<i>YGL051w</i>
51ADHr	CGGAGCTTGCATGCCTGCAGTTCCGCTCTTTTAAGAAGCGC		
53ADHf	GAAGCAAGCCTCCTGAAAGATGCAAACCCCTTCAGAAAAAT	pGRU1-Padh1	<i>YGL053w</i>
53ADHr	CGGAGCTTGCATGCCTGCAGAAAAAACTCATCGACACCAGG		
44ADHf	GAAGCAAGCCTCCTGAAAGATGAGTTTCAAGATTATTAATATC	pGRU1-Padh1	<i>YHL044w</i>
44ADHr	CGGAGCTTGCATGCCTGCAGAACTGTGACCCCATCTCTG		

* The nucleotides at the 5' end are homologous to the genomic DNA region just upstream or downstream from the gene to be replaced. The initiator or stop codon is shown in bold. The underlined nucleotides are homologous to the plasmid sequence upstream or downstream from the selectable marker gene.

Determination of Dup240–GFP localization *in vivo*. The PCR-amplified *DUP240* ORFs were cloned in-frame at the C-terminal end of the green fluorescent protein-S65T (GFP-S65T) gene of pGRU1 (Michel Aigle, IBGC, Bordeaux, France) and of pGRU1-Padh1. Transformed yeast cells were selected in SD medium without uracil because *URA3* was used as the marker gene for selection of pGRU1. Dup240–GFP and control GFP were excited with a 488 nm laser and viewed under a Leica confocal microscope equipped with a $\times 63$ objective.

Activation of the RAS signalling pathway through hSos1p–Dup240 fusion. We modified the cytoplasmic two-hybrid system (Aronheim *et al.*, 1997) marketed by Stratagene (CytoTrap) so that it would test for the presence of a hybrid protein at the plasma membrane. This system uses the *S. cerevisiae* mutant CDC25H, containing a thermosensitive mutation of the *CDC25* gene product (Petitjean *et al.*, 1990). This gene is homologous to the human *hSOS1* gene (Chardin *et al.*, 1993) and encodes a GDP/GTP exchange factor. By stimulating the exchange of GDP associated with the Ras1 and Ras2 proteins for GTP, Cdc25p stimulates the signalling pathway involving these proteins. hSos1p is cytoplasmic in the system. The *cdc25H* mutation prevents cell growth at 37 °C while growth is normal at 25 °C. Cell growth can be restored by targeting hSos1p to the plasma membrane (Aronheim *et al.*, 1994). To test whether Dup240 proteins can target hSos1p to the plasma membrane we constructed hSos1p–Dup240 fusion proteins by introducing PCR-amplified *DUP240* ORFs into the pSOS vector (CytoTrap) in-frame at the 5' end of the *hSOS1* gene. We then tested the ability of the resulting constructions to restore the growth of the CDC25H yeast strain (CytoTrap) at 37 °C. Drops of liquid control and transformed yeast cultures were deposited onto YPD plates and incubated for 2–3 days at 37 °C to evaluate growth.

Two-hybrid screen strategy. Two-hybrid screens were carried out using a system based on that described by Fields & Song (1989). We used the Gal4pAD–yeast genomic library (mechanically sheared genomic DNA fragments with a mean size of 800 bp inserted into the pACTII vector which bears the marker gene *LEU2*; A. Ramne & P. Sunnerhagen, http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_1/b5/index.html). This library was introduced (Georgakopoulos *et al.*, 2001) into the haploid yeast PJ69-4a (Table 1). This strain contains three markers (*HIS3*, *ADE2* and *lacZ*) controlled by three different *GAL* promoters (*GAL1*, *GAL2* and *GAL7*, respectively), which can each be activated in the two-hybrid system (James *et al.*, 1996). This library was composed of 2.1×10^7 independent transformed yeast cells. It was amplified in liquid culture and aliquot vials containing 6.5×10^8 yeast cells were stored at –80 °C. A mating strategy inspired by Bendixen *et al.* (1994) and modified by Fromont-Racine *et al.* (1997) was used to obtain a wide range of diploids containing both bait and target plasmids. High mating efficiency enabled us to test over 5×10^7 interactions per experiment. The haploid yeast PJ69-4a (Table 1) was transformed with the bait cloned by homologous recombination

and fused with the Gal4pBD (Gal4p-DNA binding domain) of the pGBT9 vector bearing the marker gene *TRP1*. For each screen, one vial containing 6.5×10^8 transformed PJ69-4a was mixed with 10^9 PJ69-4a cells. Cells were concentrated onto filters and incubated on rich medium for 4.5 h at 30 °C prior to collection. The cells were diluted and spread onto SC–Leu, SC–Trp and SC–Leu–Trp plates to count the number of parental cells and the number of diploids. The rest of the cell suspension was spread onto 24 SC–Leu–Trp–His plates (24 cm $\times$ 24 cm) containing 2 mM 3-aminotriazole. The plates were incubated at 30 °C for 4 days. His-positive clones were subjected to a second selection on SC–Leu–Trp–adenine plates. Plasmids were rescued in *E. coli* HB101. Insert junctions with Gal4pAD were sequenced and precisely identified in the yeast genome using the MIPS (Munich Information Centre for protein sequences; <http://mips.gsf.de>) Yeast Genome Database (MYGD) or the *Saccharomyces* Genome Database from Stanford University (<http://genome-www.stanford.edu/Saccharomyces>).

β -Galactosidase filter assays to test the two-hybrid interactions in another genetic background. Yeast strain SFY526 was transformed by the LiAc method (Gietz *et al.*, 1995) simultaneously with two plasmids which gave a two-hybrid interaction in PJ69-4 and spread onto SD–Leu–Trp agar in 150 mm diameter Petri plates. After 2 days at 30 °C these plates were replicated onto 125 mm diameter filters, which were incubated on YPD medium plates for 1 day at 30 °C. The filters were frozen quickly in liquid nitrogen and put onto plates containing, in a total of 3.5 ml: 58.5 μ l 2% X-Gal in dimethylformamide, 9.5 μ l β -mercaptoethanol, 16.1 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ l⁻¹, 5.5 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ l⁻¹, 0.75 g KCl l⁻¹ and 0.246 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ l⁻¹ at pH 7.0. The time required for yeasts to stain blue was measured at room temperature (22 °C).

RESULTS

In silico analysis

The 10 *DUP240* ORFs constitute a large multigene family in the *S. cerevisiae* S288C strain. The relationships between the 10 encoded proteins are depicted in Fig. 2. The alignment of the protein sequences (Fig. 3) revealed five well-conserved domains: C1 (amino acids 1–44), C2 (amino acids 109–150), C3 (amino acids 206–224), H1 (amino acids 51–67) and H2 (amino acids 78–94). H1 and H2 are hydrophobic domains and because of their length they are predicted to be transmembrane domains (Klein *et al.*, 1985). The ORF products Yar033p and Ygl051p have a very high level of identity (96.6%), with only eight amino acid differences in a total of 234. Furthermore, six of these differences were within the 17 residues of the second predicted transmembrane domain (Fig. 3), but they did not affect the hydrophobicity of the domain. A genetic conversion

† The pFA6a series are reporter/marker plasmids (pFA, plasmids for Functional Analysis; Wach *et al.*, 1994). Information on the sequences and availability of the pRS400 series of vectors is given in Brachmann *et al.* (1998).

‡ The ORF replacement cassette amplified with both 53S1 and 51S2 oligonucleotides allowed the replacement of either the region of chromosome VII between the *YGL053w* ORF and the *YGL051w* ORF or the region of chromosome I between the *YAR027w* ORF and the *YAR033w* ORF.

§ The sequence corresponding to the insert is underlined; the rest of the sequence is homologous to the plasmid and was used for homologous recombination in yeast.

Fig. 2. Relationships between the amino acid sequences of the *DUP240* ORF products from *S. cerevisiae* and the *DUP240* orthologue from *S. bayanus*. The amino acid sequences were aligned by use of the CLUSTAL X (1.8) program using the PAM protein matrix 40. The tree was generated by the neighbour-joining method using the CLUSTAL W program. The YAR031p sequence was deleted of 62 N-terminal residues.

event encompassing the corresponding nucleotide area, as observed for the *FCY2* cluster (Wagner *et al.*, 2001), could explain this local accumulation of point mutations, although we were unable to identify which sequence might have acted as the donor. Most of the ORF *DUP240* products exhibit greater than 25% amino acid identity, suggesting that they share common architectural features (Sander & Schneider, 1991). Until recently this protein family had only been detected in *S. cerevisiae*. However, an orthologue has now been identified in *Saccharomyces bayanus* var. *uvarum* (Bon *et al.*, 2000) (Fig. 2). As the genome of *S. bayanus* was analysed by a random sequencing strategy with a genome coverage of 0.3 $\times$, it is not possible to speculate whether this ORF also belongs to a multigenic family in this species.

The complete deletion of the 10 *DUP240* ORFs does not result in an altered phenotype

To determine the role of the *DUP240* multigenic family, all ORFs of this family were replaced by different marker genes and the phenotype of the corresponding strains was systematically analysed. The strategy employed to create a strain with a complete set of deletion alleles of *DUP240* ORFs is summarized in Fig. 4. We used the PCR-mediated gene replacement method to replace the two tandemly repeated *DUP240* ORFs of chromosome VII (*YGL053w* and *YGL051w*) with the *kanMX4* gene. The five tandemly repeated *DUP240*

ORFs of chromosome I (*YAR027w* to *YAR033w*) were replaced with the *HIS3* allele, and *YAR023c*, *YHL044w* and *YCR007c* were replaced by *MET15*, *LEU2* and *TRP1* markers, respectively. These different replacements were made in a diploid context. The LD114 and LD115 strains obtained by sporulation corresponded to haploid strains of opposite mating type and contained none of the *DUP240* ORFs (Table 1, Fig. 4). Both strains were viable, showing that the 10 *DUP240* ORFs are not essential for cell viability. These two strains grew similarly to the wild-type control strains with the same genetic background in all of the conditions tested (Fig. 5). Mating was carried out between *MAT α* LD114 and *MATa* LD115 strains to obtain homozygous deletants. The sporulation efficiency of the resulting diploids was similar to that of the wild-type and spores were able to produce colonies after germination. Thus, none of the *DUP240* gene deletions resulted in an altered phenotype in any of the conditions tested (fermentable or respiratory carbon sources, growth temperature, mating, sporulation). In addition, because *YGL052w* is localized between *YGL051w* and *YGL053w*, the three ORFs were replaced simultaneously, allowing the conclusion that in the conditions tested the replacement of *YGL052w* does not alter the phenotype. No other hemiascomycetous yeasts contain a sequence corresponding to *YGL052w* (Souciet *et al.*, 2000), and *YGL052w* overlaps with *YGL051w*; thus this ORF (Malpertuy *et al.*, 2000) should no longer be considered as a coding sequence. The same is true for ORF *YAR030c*.

As the strain deleted for the 10 *DUP240* ORFs did not have an altered phenotype in the tested conditions, we examined the subcellular localization of the 10 proteins to get further insight into their functions.

Subcellular localization of different *Dup240*-GFP fusion proteins

The ORFs *YGL051w* and *YGL053w* under the control of their own promoters were each individually cloned in-frame with the gene encoding GFP-S65T. The fluorescent signal yielded by the subsequent constructions on the multicopy plasmids (pGRU1-P-*YGL051w* and pGRU1-P-*YGL053w*, respectively) were hardly detectable. This result could be related to a low expression level as previously reported by Northern blot (Barton *et al.*, 1997) and DNA array data (Jelinsky & Samson, 1999). To enhance transcription, the yeast *ADH1* promoter was introduced into pGRU1, such that the GFP-S65T gene was under the control of this strong promoter; the derivative plasmid was named pGRU1-PadH1. The ORFs *YGL051w*, *YGL053w*, *YAR029w*, *YAR033w*, *YAR031w* and *YHL044w* were cloned in-frame at the 5' end of GFP-S65T ORF into this pGRU1-PadH1, and each one was individually introduced into the reference strain FY1679.

The control experiment with the vector without the insert revealed the expected homogeneous distribution of GFP within the cytoplasm (Fig. 6). *Ygl051p*-, *Ygl053p*-, *Yar033p*-, *Yar031p*- and *Yhl044p*-GFP fu-

Fig. 3. Similarity between the highly conserved proteins encoded by the *DUP240* family. The amino acid sequences were aligned by use of the CLUSTAL X (1.8) program and visually optimized. White on black, amino acid identity; shading, amino acid similarity; boxes, sites of the two predicted transmembrane domains; bold, 15 residues of YAR033p and YGL051p where 6 (outlined) out of 8 amino acid differences between these two sequences are concentrated. YAR031w encodes an additional 62 aa at the N-terminal extremity compared to the other *DUP240* family genes, YAR023c encodes about 50 aa less at the N-terminal end, and YAR029w encodes about 130 aa less at the N-terminal end and about 20 aa less at the C-terminal end, than the other members of the family. The *DUP240* orthologue from *S. bayanus* is shorter because only a part of the sequence was available from the database (<http://cbi.labri.u-bordeaux.fr/Genolevures/Genolevures.php3>).

sion proteins were localized at the plasma membrane. The Yar031p- and Yhl044p-GFP fusion proteins appeared to concentrate as specific spots corresponding to

a higher focal concentration at the plasma membrane. In addition Ygl051p-, Ygl053p- and Yar033p-GFP fusion proteins surrounded the nucleus, a situation typical of

Fig. 4. Strategy used to construct the strains deleted for the 10 *DUP240* ORFs. Lines with arrows indicate that the identified allele was introduced by PCR-mediated gene disruption. Lines connecting two strains define a mating between the two strains and lines without arrows indicate that a spore resulted from the mating. In some instances the genotypes of the spores are indicated. Strains LD114 and LD115 are deleted for all 10 *DUP240* ORFs.

an endoplasmic reticulum localization. Moreover, Ygl053p- and Yar033p-GFP fusion proteins appeared to accumulate into additional membranes that could correspond to the Golgi apparatus. Conversely, the Yar029p-GFP fusion protein showed a similar distribution to GFP alone, but with additional spots corresponding to a higher focal concentration not linked to the plasma membrane. This could be because Yar029p is the shortest member of the Dup240 family and the only one that lacks the predicted transmembrane domain. The localization of the Dup240 protein at the plasma membrane was further addressed by studying the hSos1p-Dup240 fusion RAS signalling pathway activation system.

A new strategy to test the localization of Ygl051p, Ygl053p and Yar033p at the plasma membrane level: hSos1p-Dup240 fusion

We modified the cytoplasmic two-hybrid system developed by Aronheim *et al.* (1994) so that it could be used to detect proteins localized at the plasma mem-

Fig. 5. The 10 *DUP240* ORFs are not essential for cell viability. The LD114 mutant strain (lower rows of each tenfold dilution series of an overnight cell suspension) grew similarly to the BY4709 control strain (upper rows) on complete glucose medium (top panel), complete glycerol medium (middle panel) and minimal medium (bottom panel) after 2–5 days at 30 °C. The same results were obtained at 15 °C and 37 °C (data not shown).

brane. This system is based on the activation of the Ras signalling pathway. The growth of the *cdc25H* mutant (strain CDC25H) at the restrictive temperature requires the targeting of the tested hSos1p-Dup240 fusion protein to the plasma membrane. The *YAR029w*, *YAR031w*, *YAR033w*, *YGL051w* and *YGL053w* ORFs were cloned in-frame with the *hSOS1* gene, resulting in pSOS-YAR029w, pSOS-YAR031w, pSOS-YAR033w, pSOS-YGL051w and pSOS-YGL053w, respectively. These constructs were introduced into the yeast strain CDC25H. The constructions containing the *YAR033w*, *YGL051w* and *YGL053w* ORFs were able to restore the growth of the yeast cells. The correlation between the loss of the plasmid and the inability to grow at the restrictive temperature was verified. Growth was thus truly due to the presence of the coding sequences of one or other of these three ORFs, indicating that the corresponding fusion proteins were targeted to the plasma membrane.

This result can be explained by at least two mechanisms. Firstly, these bait proteins could interact physically with endogenous membrane protein targets, allowing the co-localization of the hSos1p protein at the plasma membrane. hSos1p could then fulfil its deoxyriboguanosine exchange function. Alternatively, the bait proteins Yar033p, Ygl051p and Ygl053p may themselves be membrane proteins. This would localize the fused hSos1p protein at the plasma membrane, allowing it to fulfil its role as an enzyme.

The fusion proteins hSos1p-Yar029p and hSos1p-Yar031p were unable to restore the growth of the yeast CDC25H strain. These results were expected for Yar029p (see preceding section on GFP fusions) but were

Fig. 6. Fluorescence micrographs of cell expressing C-terminal Dup240–GFP fusion proteins. The indicated *DUP240* ORFs were cloned in-frame in front of the N-terminal of the GFP gene in pGRU1-PADH1 and expressed in the yeast strain FY1679. Cells were grown on minimal medium supplemented with the required amino acids. The localization of Dup240–GFP was determined in live cells by use of a Leica confocal microscope. The empty vector, expressing GFP alone, was used as a control.

surprising for Yar031p because the Yar031p–GFP fusion product appeared to be located at the plasma membrane.

Partners that interact with the *YGL051w* and *YGL053w* gene products as identified by two-hybrid screens

Protein–protein interactions are essential for many biological processes. Therefore, the identification of protein partners of proteins of unknown function may help to determine the role played by these proteins in the cell. We used the two-hybrid system to screen for the protein partners of Ygl051p and Ygl053p. The entire sequences of *YGL051w* and *YGL053w* were cloned in-frame with the nucleotide sequence of the Gal4p-binding domain in pGBT9 and introduced into the yeast strain PJ69-4 α . The resulting vectors were named pGBT9-YGL051w and pGBT9-YGL053w, respectively.

For *YGL053w*, the two-hybrid screen was performed on

6.5×10^7 diploid yeast cells obtained by mating. Selection for histidine yielded 384 clones and 103 clones were obtained after selection for adenine. Seven of these clones corresponded to antisense constructs or intergenic regions, and 46 clones to the *OAF1* (= *YAF1*) gene. As *OAF1* encodes a transcription factor involved in the regulation of peroxisome proliferation and inserts of this gene were found in all the two-hybrid screenings performed in the laboratory with this library, these clones were considered to be false-positives. The two-hybrid interactions with the other 50 putative positives, corresponding to 15 different inserts, were verified in another genetic context: yeast strain SFY526 (Bartel *et al.*, 1993b) bearing the *lacZ* gene under the control of the *GAL1* promoter. For this purpose pGBT9-empty or pGBT9-YGL053w were introduced into SFY526 simultaneously with each of the 15 plasmids encoding a putative positive partner. After β -galactosidase filter assays for all these constructs, 45 clones corresponded to

Fig. 7. Two-hybrid interactions with *YGL053w*. Alignments of five different inserts of *YER032w* and six different inserts of *YNL078w*. The region between the dotted lines is the minimal region of interaction. The sequences corresponding to the ORFs are represented in black; the sequences downstream of the stop codons are represented in white.

11 different inserts but only two different genes were retained as positives. Five of the 11 inserts corresponded to a part of the *FIR1* gene (ORF of 2775 nt) which encodes a protein that is thought to be involved in 3'-mRNA processing. The five inserts contained a common sequence of 320 nt corresponding to a region ending at 200 nt upstream from the 3' end of the *FIR1* ORF (Rusnak *et al.*, 1996). The six other inserts corresponded to *YNL078w*, a 1221 nt ORF of unknown function, and all of them contained the last 236 nt of the ORF (Fig. 7).

For *YGL051w*, the two-hybrid screen was performed on 5.7×10^7 diploid yeast cells obtained by mating. Selection for histidine yielded 288 clones and final screening for adenine yielded 32 positive clones. One of these clones corresponding to an antisense construct and three clones of the *OAF1* gene were eliminated. The two-hybrid interactions with the other 28 putative positives, corresponding to 14 different inserts, were verified in SFY526. As for *Ygl053p*, the empty plasmid or the plasmid encoding *Ygl051p* was introduced into SFY526 simultaneously with each of the 14 plasmids encoding a putative positive. After β -galactosidase filter assays, 26 clones corresponding to 12 different inserts and to 12 different ORFs were retained as positives. These positives only activated the transcription of the *lacZ* gene in the presence of *YGL051w*. The products of these 12 ORFs were considered to be two-hybrid partners of the *YGL051w* product. One of these ORFs, *PAN1*, which coordinates regulatory interactions between proteins required for both endocytosis and the organization of the actin-cytoskeleton (Wendland & Emr, 1998), does not encode any predicted transmembrane domain al-

though its product is localized at the plasma membrane. The 11 other ORFs encode proteins with 6 to 14 known or potential transmembrane domains and are classified in the (confirmed or potential) membrane protein family (Goffeau *et al.*, 1997). Three of these proteins are known to be localized in the plasma membrane: *Gls1p*, a catalytic subunit of 1,3- β -D-glucan synthase involved in resistance to papulacandin B and aculeacin A (Eng *et al.*, 1994); *Rgt2p*, a glucose transporter responsible for the induction of gene expression in the presence of high glucose levels, a member of the sugar permease family (Ozcan *et al.*, 1996); and *Agp3p*, a member of the amino acid permease family (Nelissen *et al.*, 1995). The eight other ORF gene products were assigned, by protein identity search, to protein families even though their specific functional roles remain unknown. Three of these, *Yll028p*, *Ybr180p* and *Yor378p*, are members of the multidrug resistance protein family, a subfamily of the major facilitator superfamily (MFS) and have 12, 10 and 9 putative transmembrane domains, respectively. Three others (*Yil166p*, *Yol119p* and *Ykl146p*) are members of MFS subfamilies and have 6, 8 and 9 putative transmembrane domains, respectively: *Yil166p* is similar to allantoin permease (*Dal5p*) and to carboxylic acid transporters and is a member of the allantoin permease family; *Yol119p* shows weak similarity to mammalian monocarboxylate transporters and is a member of the monocarboxylate permease family; *Ykl146p* shows high similarity to the hypothetical C3H1.09c protein of *Schizosaccharomyces pombe*. Finally *Yal022p* shows low similarity to a Na^+/H^+ antiporter and has nine predicted transmembrane domains, and *Ybr147p* has six predicted transmembrane domains. The systematic interaction of *YGL051p* with all of these predicted membrane proteins suggests that it is itself localized close to or at the membrane, corroborating the *hSos1p* fusions and GFP results.

DISCUSSION

Systematic sequencing of genomes has provided a unique opportunity to quantify the level of genetic redundancy in their nucleotide or encoded amino acid sequences. In *S. cerevisiae* most protein families contain two or three members. Large families are rare and can be used to investigate whether primary sequence redundancy is related to functional redundancy. The Dup240 protein family is composed of 10 members. As the first step of the functional analysis, we systematically knocked out all 10 members of the family. As *YGL053w* and *YGL051w* are organized in tandem repeat and *YAR027w*, *YAR028w*, *YAR029w*, *YAR031w* and *YAR033w* represent five direct repeats it was possible to delete all seven of these ORFs with two replacements. The single replacement of multiple ORFs has been termed 'mass-murder' and is an efficient and reliable method for gene replacement (Fairhead *et al.*, 1998). Three single conventional disruptions were required to knock out the last three ORFs. Neither single nor multiple deletions had an altered growth phenotype in the conditions tested (growth on various carbon sources

at different temperatures, mating and sporulation assays). A similar situation has been reported for the *AAD1–7* family, which encodes seven homologues to the arylalcohol dehydrogenase of the lignin-degrading fungus *Phanerochaete chrysosporium* (Delneri *et al.*, 1999). However, the concurrent knock-out of 20 transporter genes was shown to be required to block glucose consumption and transport activity in *S. cerevisiae* (Wieczorke *et al.*, 1999), and only the elimination of the entire *OSBP* gene family (*OSH1–7*) produces a lethal phenotype (Beh *et al.*, 2001).

To learn more about the function of this multigene family we studied the subcellular localizations of the gene products and searched for interacting partners. Fluorescent staining of proteins Ygl051p, Ygl053p, Yar031p and Yar033p fused to GFP revealed that they were localized on the yeast plasma membrane and that Ygl051p, Ygl053p and Yar033p were also located in the endoplasmic reticulum. Takahashi *et al.* (2000) reported that YAR027p fused to GFP was localized at the nuclear envelope and plasma membrane regions. The membrane localization was further supported by the *in silico* analysis, which suggested that all of the proteins coded by the *DUP240* ORFs (except Yar029p, which has no predicted transmembrane domain) possess two 17 aa hydrophobic predicted transmembrane domains. The activation of the RAS signalling pathway by hSos1p–Dup240 fusions confirmed the plasma membrane localization, showing that Ygl051p, Ygl053p and Yar033p were able to target activation in this system, either directly because they are integral membrane proteins, or indirectly by interacting with associated membrane proteins. Yar029p is much shorter than the other Dup240 proteins and Yar029p–GFP was located throughout the cytoplasm, as was GFP alone. This finding is consistent with the inability of Yar029p to target hSos1p to the RAS signalling pathway. The Yar031p protein contains 62 extra amino acids at its N-terminus compared to Ygl053p. The C-terminal parts of these two ORFs are nevertheless highly conserved: 100% identity for the first 93 C-terminal amino acids, and only five differences among the 132 C-terminal amino acids. The identity of the N-terminal domain of Ygl053p and the central region of Yar031p is just 41.7% (48 out of 115 amino acids). When fused to hSos1p, Yar031p was unable to activate the RAS signalling pathway despite its apparent localization as indicated by GFP fusion at the plasma membrane and the presence of two predicted transmembrane domains. This discrepancy might result from the fact that GFP-fused Yar031p always seems to form aggregates at the membrane, and may consequently be unable to target hSos1p correctly for RAS activation.

The Ygl051p and Ygl053p partners identified by screening of the two-hybrid yeast library were in different classes. The product of the ORF *YGL051w* fused to the binding domain of Gal4p interacted with many integral membrane proteins and many proteins of unknown function predicted to have six or more transmembrane domains. This finding further suggests that Ygl051p

is located at the plasma membrane. The two-hybrid partners of Ygl053p, Fir1p and Ynl078p do not appear to be plasma membrane proteins, as they lack predicted transmembrane domains; however, this finding does not exclude a plasma membrane localization for Ygl053p. The common sequence from the different inserts encoding parts of Fir1p or Ynl078p should encode the domains from Fir1p and Ynl078p required for interaction with the Ygl053p protein. Recently, high-throughput two-hybrid screens revealed that Yar031p and Apg12p interact, and that Ygl051p and Yar033p interact (Uetz *et al.*, 2000). We were unable to replicate this finding (data not shown). Apg12p is involved in autophagy and the targeting of proteins to the vacuole. Most free Apg12p seems to be associated with the endoplasmic reticulum (Mizushima *et al.*, 1998). Two-hybrid interactions have also been reported between Yar027p and Cks1p, an essential, physically associated, component that interacts with the protein kinase Cdc28p (Hadwiger *et al.*, 1989), between Yar027p and Yar030p, between Ygl053p and Ylr065p, and between Yhl044p and Ykr035p (Ito *et al.*, 2000). The Yar030p, Ylr065p and Ykr035p proteins have unknown functions but possess two and five putative transmembrane domains, respectively, so these proteins are themselves predicted to be membrane associated. The proteins coded by *YGL051w*, *YGL053w*, *YAR031w* and *YAR033w* are predicted to have structural and/or functional roles at membranes.

Our data suggest that we are dealing with a gene family specific to the genus *Saccharomyces sensu stricto*. This gene family belongs to the set of ascomycete-specific genes, a class of genes that tend to be more sensitive to evolutionary divergence than the average (Malpertuy *et al.*, 2000). Even without specific information on the precise function of the proteins encoded by this gene family, we have shown that when amino acid divergence is detectable, the changes often affect a limited number of amino acids localized within a specific domain of the protein. The proteins encoded by this gene family, with the exception of Yar029p, appear to be located at membranes. We have evidence that they have specialized functions because when they were fused to GFP they did not show identical fluorescence patterns and when fused to hSos1p they did not show identical RAS activation properties. In addition, Ygl051p and Ygl053p both appear to interact with a specific set of non-redundant proteins. Thus, our data suggest that the 10 Dup240 members do not have identical functions.

ACKNOWLEDGEMENTS

We are grateful to P. Sunnerhagen and A. Ramne for providing a yeast genomic two-hybrid library, to D. Alexandraki for providing yeast transformed with this library, to M. Aigle for providing pGRU1-S65TGFP and help with the interpretation of fluorescence data, and to P. James and S. Fields for providing strains PJ69-4a and PJ69-4z. We are also grateful to J. Rommelaere for his kind support throughout this work. This research was supported in part by a contract within the framework of the EUROFAN project of the European Commission (BIO4-CT95-0080) to J.C.J. and by a European

Commission Marie Curie Predoctoral Research Training fellowship BIO4-CT98-5047 to R.P.

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Received 5 December 2001; revised 4 March 2002; accepted 20 March 2002.