RESEARCH ARTICLE

The *Saccharomyces cerevisiae* fermentation stress response protein Igd1p/Yfr017p regulates glycogen levels by inhibiting the glycogen debranching enzyme

Christopher J. Walkey[1], Zongli Luo[1], Christoph H. Borchers[2], Vivien Measday[1] & Hennie J.J. van Vuuren[1]

[1]Wine Research Centre, University of British Columbia, Vancouver, BC, Canada; and [2]University of Victoria – Genome British Columbia Proteomics Centre, Victoria, BC, Canada

Correspondence: Hennie J.J. van Vuuren, Wine Research Centre, University of British Columbia, 2205 East Mall, Vancouver, BC, Canada V6T 1Z4. Tel.: +604 822 0005; fax: +604 822 5143; e-mail: hjjvv@mail.ubc.ca

Received 13 January 2011; revised 11 April 2011; accepted 9 May 2011.
Final version published online 16 June 2011.

DOI:10.1111/j.1567-1364.2011.00740.x

Editor: Isak Pretorius

Keywords
wine yeast; fermentation; stress response; glycogen catabolism.

Abstract

Wine fermentation imposes a number of stresses on *Saccharomyces cerevisiae*, and wine yeasts respond to this harsh environment by altering their transcriptional profile (Marks *et al.*, 2008). We have labeled this change in gene expression patterns the fermentation stress response (FSR). An important component of the FSR is the increased expression of 62 genes for which no function has been identified for their protein products. We hypothesize that a function for these proteins may only be revealed late in grape must fermentation, when the yeast cells are facing conditions much more extreme than those normally encountered in laboratory media. We used affinity copurification to identify interaction partners for the FSR protein Yfr017p, and found that it interacts specifically with the glycogen debranching enzyme (Gdb1p). The expression of both of these proteins is strongly induced during wine fermentation. Therefore, we investigated the role of Yfr017p in glycogen metabolism by constructing wine yeast strains that lack this protein. These YFR017C null cells displayed a significant reduction in their ability to accumulate glycogen during aerobic growth and fermentation. Moreover, Yfr017p inhibits Gdb1p activity *in vitro*. These results suggest that Yfr017p functions as an inhibitor of Gdb1p, enhancing the ability of yeast cells to store glucose as glycogen. Therefore, we propose *IGD1* (for inhibitor of glycogen debranching) as a gene name for the YFR017C ORF.

Introduction

Industrial strains of *Saccharomyces cerevisiae* are widely used to produce ethanol from fermentative processes such as wine-making, beer brewing and biofuel production. Fermentation, including wine production, imposes multiple stresses on *S. cerevisiae*. Grape juice used to make wine can contain more than 25% sugar (as equimolar glucose and fructose), resulting in a formidable osmotic pressure at the onset of fermentation. As the yeast cells ferment sugars via glycolysis, ethanol is produced, increasing to a concentration of 15% v/v or higher. Ethanol alters the intermolecular forces necessary to maintain membrane integrity, and binds free water, thus producing its own osmotic stress. Furthermore, dissolved oxygen is rapidly consumed, resulting in a hypoxic (and eventually anaerobic) environment. We discovered that yeast cells respond to these conditions by a coordinated alteration of their transcriptome, called the fermentation stress response (FSR). Using microarray technology, we identified 2250 genes whose expression was altered during the course of fermentation, including 223 genes, whose expression was upregulated in a statistically significant and sustained profile (Marks *et al.*, 2008). Interestingly, less than half of these genes overlap with those implicated in other environmental stresses, suggesting that the FSR may be a novel response that evolved independently (Gasch *et al.*, 2000; Causton *et al.*, 2001; Marks *et al.*, 2008). As expected, many of these 223 genes are well annotated, and are known to play roles in cellular processes such as glucose uptake, nitrogen regulation, vacuolar stress and detoxification. However, out of these 223 genes, 62 have no described function. Therefore, we are interested in identifying cellular functions for these nonannotated FSR genes, with the goal of gaining an insight into how yeast cells respond to the stress of wine-making.

In order to identify functions for our nonannotated FSR genes, we created industrial wine strains carrying null mutations of each of the FSR genes of interest and followed the changes in fermentation parameters that result. In addition, we have engineered the insertion of a green fluorescent protein (GFP) cassette at the 3′ end of each ORF, resulting in the generation of FSR proteins GFP tagged at the C-terminus. This GFP tagging serves two functions: (1) localization of the GFP-tagged protein in the yeast cell by fluorescence microscopy and (2) coimmunoprecipitation of proteins that interact with our GFP-tagged protein (Trinkle-Mulcahy *et al.*, 2008). Using a novel α-GFP antibody fragment linked to agarose beads (Rothbauer *et al.*, 2008), we have been able to specifically copurify proteins that bind to our FSR proteins and identify them by MS. We hypothesize that identifying interaction partners for our FSR proteins will provide a first clue towards placing them in a metabolic pathway and suggest further experiments that will help discover their function.

This paper outlines the use of this coimmunoprecipitation technique to identify an interaction between one of our FSR proteins, Yfr017p, and the glycogen debranching enzyme Gdb1p. In the *Saccharomyces* Genome Database, the molecular function and biological process for YFR017C are listed as unknown. The experiments presented here suggest that this gene encodes a protein that inhibits Gdb1p activity and serves to preserve glycogen in the yeast cell. Hence, we have named the YFR017C ORF as *IGD1*.

Materials and methods

Strain construction

All strains used in this paper are derived from the commercial diploid M2 wine yeast strain (a gift from R. Gardner) (Richards *et al.*, 2009; Deed *et al.*, 2011). This strain was chosen for its relevance to industrial wine production as well as its amenability to genetic modification and homozygosity. DNA sequences carrying the appropriate modification, along with an antibiotic resistance cassette, were generated by a PCR (see Table 1 for the oligonucleotide sequences and plasmid templates) (Guldener *et al.*, 1996; De Antoni & Gallwitz, 2000). These DNA fragments were introduced into the yeast cells by lithium acetate transformation under standard conditions and recombinants selected by the appropriate antibiotic selection. The fidelity of integration was confirmed by colony PCR. Because the M2 strain is diploid, it was necessary to generate strains that were homozygous for the appropriate genetic modification. Therefore, the cells were induced to sporulate and the resulting tetrads were dissected to isolate individual haploid cells. The resulting colonies, which spontaneously reverted

Table 1. Oligonucleotides used for strain construction

Name	Sequence	Purpose	Template
7(GFP+fwd)	gtacgaggactttaagaaggacatctataaccagcttcacatgtttggagagaagggtgaagctcaaaaacttaat	GFP-tagging YFR017C	pGFP+(NAT)
8(GFP+rev)	ttgacgttacgtacatatgtatgagtgggtaggtgtggacgacactagggaggctgctgacggtatcgataagctt	GFP-tagging YFR017C	pGFP+(NAT)
yfr017c-fc	agtccccatgattagacgg	Confirm YFR017C GFP tag	Genomic DNA
Hf(GFP+fwd)	caccattacggactacggtaaccaggccatacagtacgctaatgagttccaacagggtgaagctcaaaaacttaat	GFP-tagging *HOG1*	pGFP+(NAT)
Hr(GFP+rev)	aaacatcaaaaagaagtaagaatgagtggttagggacattaaaaaaacacgtttagctgacggtatcgataagctt	GFP-tagging *HOG1*	pGFP+(NAT)
H+f	tatctgccacgtttgatg	Confirm *HOG1* GFP tag	Genomic DNA
GFP+chkrev	accatgtggtctctcttttcg	Confirm GFP tag	Genomic DNA
GDB1mycf	agttctgcttgtttgttagatctattttatgatttatgggatgcctacgaagatgattcccagctgaagcttcgtacg	myc-tagging *GDB1*	pU6H2MYC
GDB1mycr	ataaaagtattagcgggtggcaaaaagggaccgttcaattaatttatgctttagctatttatacgactcactataggg	myc-tagging *GDB1*	pU6H2MYC
GDB1upf	tgtgggcaggattgacagagc	Confirm *GDB1* myc tag	Genomic DNA
pUG6intr	accatgcatcatcaggagtacg	Confirm *GDB1* myc tag	Genomic DNA
YFR017CKOf	ataacacatagtaaatcacacatctacgcaacacacacacacacgcatacactcaccctcgccagctgaagcttcgtacg	Null mutation of YFR017C	pUG6
YFR017CKOr	agagattgacgttacgtacatatgtatgagtgggtaggtgtggacgacactagggaggcttaatacgactcactataggg	Null mutation of YFR017C	pUG6
4KOupf	gcgcgagagatctactatcagc	Confirm YFR017C null	Genomic DNA
pUG6intr	accatgcatcatcaggagtacg	Confirm YFR017C null	Genomic DNA
GDB1KOf	acttctcttaacttccagtcaacccgacacccacaatcgtccttagtatataactgctttggtgaagctcaaaaacttaat	Null mutation of *GDB1*	pGFP+(NAT)
GDB1KOr	ataaaagtattagcgggtggcaaaaagggaccgttcaattaatttatgctttagctatttgctgacggtatcgataagctt	Null mutation of *GDB1*	pGFP+(NAT)
GDB1upf	gtactccctttcagttatatgcc	Confirm *GDB1* null	Genomic DNA
GFP+_Nr	acaagaattgggacaactcc	Confirm *GDB1* null	Genomic DNA
YFR017C-1000f	ccgcgtacgcgtcgatagtgctcgctgctcattatgatacc	YFR017C expression	Genomic DNA
YFR017C3′UTRr	attcggtaccgtcgacgaagctaaagagattgacgttacg	YFR017C expression	Genomic DNA
YFRprotf	aattcgagctccgtcacagatccccacttgaacacg	Yfr017p expression	Genomic DNA
YFRprotr	cgcaagcttgtcgacttacttctctccaaacatgtgaagc	Yfr017p expression	Genomic DNA
GDB1protf	aattcgagctccgtcaatagatcattactgctacgtttgtcg	Gdb1p expression	Genomic DNA
GDB1protr	cgcaagcttgtcgactcaggaatcatcttcgtaggcatcc	Gdb1p expression	Genomic DNA

to the diploid karyotype, were again selected by the appropriate antibiotic resistance. For the complementation testing, plasmids were constructed from the pYC250 backbone (a gift from the Carlsberg Laboratory, Denmark) (Olesen *et al.*, 2000; Hansen *et al.*, 2003) and transformed into the relevant M2 yeast strains.

Fermentation

All fermentations were carried out in 200 mL of filter-sterilized Chardonnay grape must (Calona Vineyards, Canada) at 19 °C in glass bottles with vapor locks to prevent oxidation. Samples were removed periodically to measure the glucose, fructose and ethanol contents by HPLC (Adams & van Vuuren, 2010) in order to confirm the progress of fermentation. In some experiments, the strains were grown under standard laboratory conditions, in YPD (1% yeast extract, 2% peptone, 2% dextrose) media at 30 °C with aeration.

Coimmunoprecipitation

Cells were collected by centrifugation and washed twice with cold water. The cells were lysed in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, protease and phosphatase inhibitors) using glass beads in a FastPrep 24 (MP Biomedicals) set for two 40-s pulses at 6.0 ms^{-1}. The beads and cell debris were removed by centrifugation. α-GFP (Chromotek) or α-myc (Roche) agarose beads were added to the lysate and allowed to bind tagged proteins for 1 h at 4 °C. The beads were then pelleted by centrifugation and the depleted lysate was removed. The bead pellet was washed six times with lysis buffer to minimize nonspecific interactions. Next, the bound proteins were eluted by washing the beads briefly in 100 mM glycine at pH 2.5. The denuded beads were pelleted by centrifugation and the elution was neutralized with 1/10 volume of 1 M Tris at pH 8.0.

The purified proteins were separated by SDS-PAGE using precast 4–20% gradient minigels (Bio-Rad). For the identification of Yfr017p-GFP-interacting proteins, the gels were silver stained according to the kit manufacturer's instructions (Invitrogen). Relevant bands were excised, destained and prepared for mass spectrometric analysis. For immunoblot analysis, the purified and separated proteins were transferred to nitrocellulose and immunoblotted with α-GFP or α-myc monoclonal primary antibodies (Roche). Horseradish peroxidase (HRP)-conjugated secondary antibody and the SuperSignal West Femto HRP substrate kit (Pierce) were used to detect the presence of GFP- and myc-tagged proteins.

In direct immunoblotting experiments, 1 $OD_{600\,nm}$ unit of cells were harvested and washed as above. The cell pellet was then resuspended in 10% trichloroacetic acid for 10 min on ice and centrifuged. The acid was removed and the pellet was gently washed with 1 M Tris, pH 8.0, to neutralize the remaining acid. After a second centrifugation and removal of the supernatant, the cells were resuspended in an SDS-PAGE loading buffer and lysed with glass beads as above. The resulting crude lysates were electrophoresed on SDS-PAGE gels and then immunoblotted as above. Before immunoblotting, the nitrocellulose membrane was stained with the protein detection reagent MemCode (Pierce) according to the manufacturer's instructions in order to confirm equivalent lysate loading between the lanes.

MS

To each gel slice, 100 μL of 25 mM ammonium bicarbonate and 10 μL of 200 mM DTT were added and samples were reduced for 45 min at 37 °C. Next, 40 μL of 200 mM iodoacetamide was added to each sample and further incubated for 45 min at 37 °C to alkylate proteins (Parker *et al.*, 2005). Finally, each sample was digested with 5 μg trypsin overnight at 37 °C. Formic acid (500 μL of 0.1% solution) was added to each sample, and the final sample cleanup was performed on HLB columns, with elution by 50% acetonitrile, 0.1% formic acid. Solvent was removed by evaporation.

LC–MS/MS analysis was performed using an integrated Famos autosampler, a SwitchosII switching pump and the UltiMate micro pump (LC Packings, Amsterdam) system with a Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (QStar Pulsar i) equipped with a nanoelectrospray ionization source (Proxeon, Odense, Denmark) and fitted with a 10 μm fused-silica emitter tip (New Objective, Woburn, MA). Chromatographic separation was achieved on a 75 μm × 15 cm C18 PepMap Nano LC column (3 μm, 100 Å, LC Packings), and a 300 μm × 5 mm C18 PepMap guard column (5 μm, 100 Å, LC Packings) was in place before switching inline with the analytical column and the MS. The mobile phase (solvent A) consisted of water/acetonitrile (98 : 2 v/v) with 0.05% formic acid for sample injection and equilibration on the guard column at a flow rate of 100 μL min^{-1}. A linear gradient was created upon switching the trapping column inline by mixing with solvent B, which consisted of acetonitrile/water (98 : 2 v/v) with 0.05% formic acid, and the flow rate was reduced to 200 nL min^{-1} for high-resolution chromatography and introduction into the mass spectrometer.

Samples were brought up to 20 μL with 5% ACN and 3% FA and transferred to autosampler vials (LC Packings). The sample (10 μL) was injected into 95% solvent A and allowed to equilibrate on the trapping column for 10 min to wash away any contaminants. Upon switching inline with the MS, a linear gradient from 95% to 40% solvent A developed for 40 min, and in the following 5 min, the composition of the mobile phase was increased to 20% A before decreasing to 95% A for a 15-min equilibration before the next sample injection. MS data were acquired automatically using the

ANALYST QS 1.0 software Service Pack 8 (ABI MDS Sciex, Concord, Canada). An information-dependent acquisition method consisting of a 1-s TOFMS survey scan of mass range 400–1200 amu and two 2.5-s product spectra of mass range 100–1500 amu was used. The two most intense peaks over 20 counts, with charge states 2–5, were selected for fragmentation and a 6 amu window was used to prevent the peaks from the same isotopic cluster from being fragmented again. Once an ion was selected for MS/MS fragmentation, it was placed on an exclude list for 180 s. Curtain gas was set at 23, nitrogen was used as the collision gas and the ionization tip voltage used was 2700 V. The resulting data files were processed using MASCOT (http://www.matrixscience.com).

Glycogen assay

Intracellular glycogen was measured as described previously (Parrou & Francois, 1997). Briefly, 2 $OD_{600\,nm}$ units of cells were lysed in 125 μL of 250 mM sodium carbonate at 95 °C for 4 h, and then neutralized with 75 mL of 1 M acetic acid and 300 μL of 200 mM sodium acetate, pH 5.2. The glycogen released was digested by adding 0.5 U of amyloglucosidase (Roche) and incubating overnight at 57 °C. Twenty microliters of the resulting glucose was assayed using the glucose oxidase method, according to the manufacturer's instructions (Sigma), and adapted for 96-well plates. The total intracellular protein concentration was assayed using a BCA kit (Pierce) from 10-μL aliquots before glucosidase digestion for normalization.

Production of purified Yfr017p and Gdb1p

YFR017C and *GDB1* ORF sequences were amplified from M2 strain genomic DNA and cloned into the bacterial expression plasmid pET28b(+), resulting in proteins His tagged at the N-terminus. The plasmids were transformed into the BL21 *Escherichia coli* strain. To produce protein, plasmid-containing cultures were grown to the log phase and induced with 1 mM IPTG for 5 h at 30 °C. The bacterial cells were pelleted by centrifugation and lysed by sonication (6 × 10-s pulses, 20% power) in a cold lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole). Ni-NTA agarose beads (Qiagen) were added and allowed to bind His-tagged proteins for 1 h at 4 °C. The lysates plus nickel beads were poured into columns and the denuded lysate was drained. The resulting bead beds were washed twice with a cold wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole). The bound proteins were eluted with four aliquots of elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole). Samples of each aliquot were analyzed by SDS-PAGE and Coomassie staining (with a comparison to an albumin standard) to determine the amount and purity of protein produced.

Glycogen debranching assay

The assays were performed as described in a 200 μL volume of sodium citrate buffer, pH 6.5 (Lee *et al.*, 1970; Teste *et al.*, 2000). The substrate was limit dextrin (phosphorylase-digested glycogen), prepared from 0.3 g of oyster glycogen (Sigma) by glycogen phosphorylase b (Sigma) digestion, followed by dialysis against 10 mM Na_3PO_4, pH 6.8 (Chen *et al.*, 1987). Each reaction contained 60 μg of limit dextrin, 100 ng of Gdb1p and varying amounts of Yrf017p in the appropriate molar ratios. Eluates from pET28b(+) vector-only cells served as negative controls and to normalize assay volumes. The reactions were incubated at 30 °C for 2 h; 400 μL of glucose oxidase reagent (Sigma) was added to measure the glucose produced. After a further 30-min incubation, 400 μL of 12 N sulfuric acid was added to stop the reaction. The amount of glucose produced was determined by measuring the optical absorbance at 540 nm and comparing the values with a glucose standard curve.

Results

Interaction between Yfr017p and Gdb1p

We constructed an M2 wine yeast strain that expressed a C-terminal GFP-tagged version of Yfr017p under the control of its endogenous promoter. This strain was used to ferment Chardonnay grape must under standard conditions. After 6 days, when YFR017C mRNA is known to be elevated and ethanol levels are approaching their maximum, the fermented yeast cells were isolated and gently lysed at 4 °C, in an effort to preserve tertiary protein structure and interactions. Yfr017p-GFP was batch purified using anti-GFP agarose beads and eluted proteins were detected by silver staining. As a control, the same procedure was performed on an M2 wine yeast strain expressing a C-terminal GFP-tagged version of the Hog1p. As shown in Fig. 1a, the immunoprecipitation from the M2 Yfr017p-GFP strain produced two major bands: a smaller band at ~55 kDa (labeled Band A), which corresponds to the predicted size of Yfr017p-GFP, and a larger band at ~170 kDa (labeled Band B). Both of these bands were specific for the Yfr017p-GFP immunoprecipitation, because they did not appear in the Hog1-GFP immunoprecipitation. The bands were excised from the gel, destained and analyzed for peptide identification by MS following tryptic digestion. All seven *S. cerevisiae* peptides identified from the 55 kDa Band A matched the predicted sequence of Yfr017p (Table 2). Also, five peptides corresponding to GFP were identified. From the 170 kDa Band B, 41 of 42 identified *S. cerevisiae* peptides matched the sequence of glycogen debranching enzyme (Gdb1p), suggesting that this enzyme interacts with the FSR protein Yfr017p during fermentation. A second coimmunoprecipitation, where the purified proteins were identified by mass

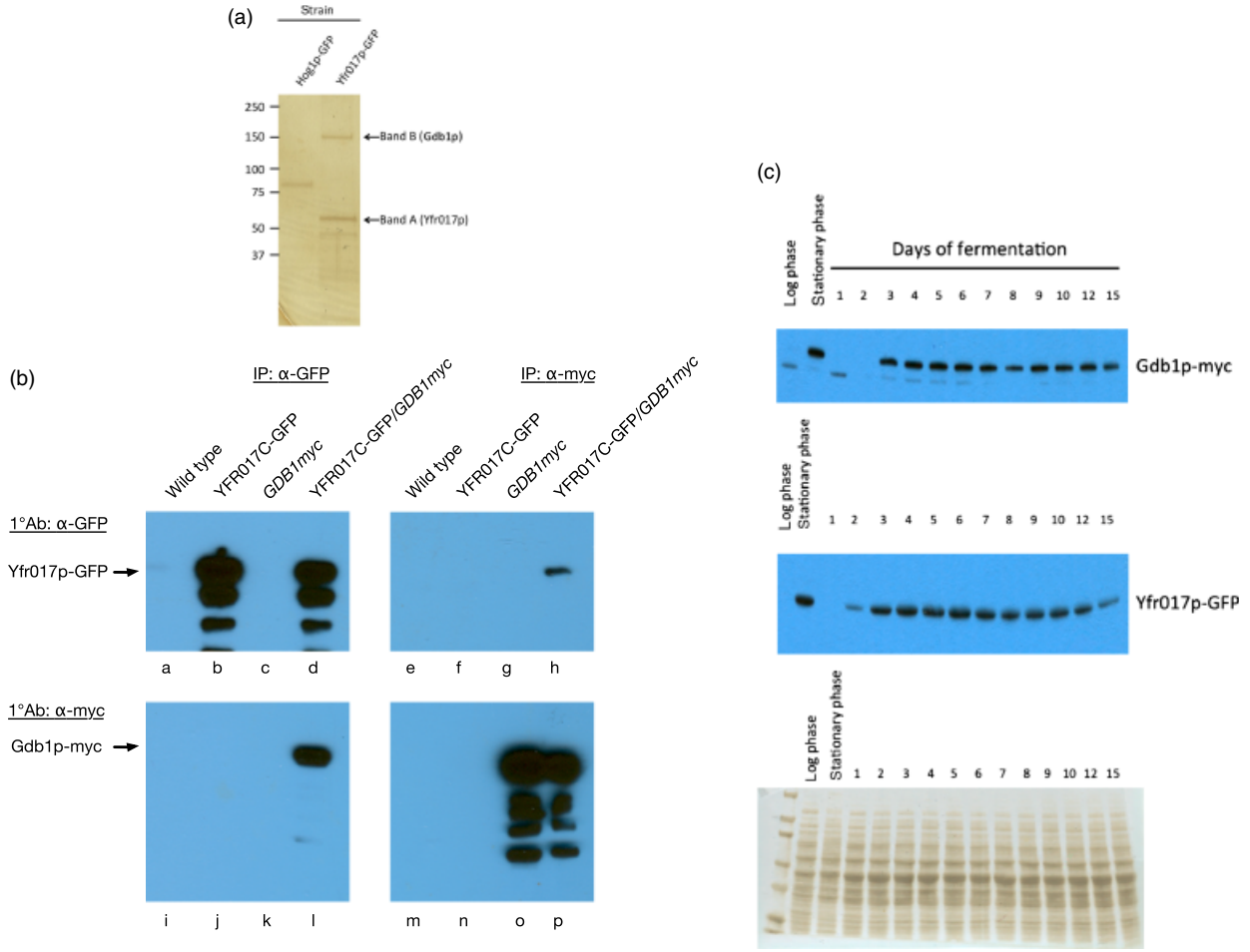

Fig. 1. (a) GFP coimmunoprecipitation from wine yeast during fermentation. Two M2 wine yeast strains, one containing a GFP-tagged version of Hog1p and the second containing a GFP-tagged version of Yfr017p, were used to ferment Chardonnay grape must for 6 days. Cells were lysed and GFP-tagged proteins were immunoprecipitated. Coimmunoprecipitated proteins were separated by SDS-PAGE and detected by silver staining. Two major bands (Band A and Band B) were visible from the Yfr017p-GFP immunoprecipitation. These bands were identified as Yfr017p-GFP (Band A) and Gdb1p (Band B) by mass spectroscopy. (b) Confirmation of Yfr017p and Gdb1p interaction by reciprocal coimmunoprecipitations. Strains expressing Yfr017p-GFP, Gdb1p-myc or both were used to ferment Chardonnay grape must and the proteins were immunoprecipitated as above. Coimmunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting with α-myc and α-GFP antibodies. Strains are organized from left to right: wild type, Yfr017p-GFP, Gdb1p-myc and both Yfr017p-GFP and Gdb1p-myc. In the top left corner, proteins were immunoprecipitated with α-GFP beads and detected with the α-GFP antibody in the immunoblot. Yfr017p-GFP is detected in the appropriate strains (lanes b and d). In the top right corner, proteins were immunoprecipitated with α-myc beads and detected with the α-GFP antibody. The band in lane h confirms that myc-tagged Gdb1p coimmunoprecipitates GFP-tagged Yfr017p. In the bottom left corner, proteins were immunoprecipitated with α-GFP beads and detected with the α-myc antibody. The band in lane l confirms that GFP-tagged Yfr017p coimmunoprecipitates myc-tagged Gdb1p. In the bottom right corner, proteins were immunoprecipitated with α-myc beads and detected with the α-myc antibody. Gdb1p-myc was detected in the appropriate strains (lanes o and p). (c) Time course of Yfr017p and Gdb1p expression during fermentation. An M2 strain of *Saccharomyces cerevisiae* expressing both Yfr017p-GFP and Gdb1p-myc was used for fermentation of grape must and aliquots were removed at different time points. The cells were lysed and the lysates were subjected to SDS-PAGE, followed by immunoblotting with α-GFP and α-myc antibodies. Cells from log- and stationary-phase cultures grown in YPD were also immunoblotted. Blot staining with MemCode (Pierce) demonstrated equivalent protein lysate loading between lanes.

spectroscopy without prior gel separation, only identified Yfr017p-GFP and Gdb1p.

In order to confirm this interaction, we performed additional immunoprecipitation experiments. We constructed M2 strains with Gdb1p myc tagged at its C-terminus, with and without GFP-tagged Yfr017p. Again, these yeast strains were inoculated into Chardonnay grape juice and fermented under standard conditions. After 6 days, the cells were gently lysed as described above and the lysates were immunoprecipitated with either anti-GFP or anti-myc agarose beads. Following elution, the proteins were separated by SDS-PAGE and immunoblotted with either

Table 2. *Saccharomyces cerevisiae* peptides identified by mass spectroscopic analysis

M_r	Peptide	Protein
Band A		
1244.6374	R.VPHGSPPQLGTR.R	Yfr017p
1286.5441	R.STNYMDALNSR.E+oxidation (M)	Yfr017p
1324.6146	R.VPHGSPPQLGTR.R+phospho (ST)	Yfr017p
1509.6743	K.DIYNQLHMFGEK.-+oxidation (M)	Yfr017p
1524.6293	K.NSYLDNNSNGNSAR.V	Yfr017p
1623.7480	R.ERESSIGEHAPGAER.R	Yfr017p
1703.6901	R.ERESSIGEHAPGAER.R+phospho (ST)	Yfr017p
Band B		
851.5148	K.VHVLGSLK.L	Gdb1p
857.5177	R.LGISSLIR.E	Gdb1p
877.4415	K.NIEDLFK.I	Gdb1p
884.4293	R.FSYALER.V	Gdb1p
909.4275	K.DISSDFVK.L	Gdb1p
962.4991	K.DQPLYTVK.L	Gdb1p
985.5334	K.ILQEQVTR.R	Gdb1p
985.5334	K.ILQKQVTR.A+deamidated (NQ)	Yhr015p
1010.5403	K.AGSVGIPGTPR.D	Gdb1p
1016.5318	K.NLVEHIHR.D	Gdb1p
1068.6164	K.LLVSAGSPVAR.D	Gdb1p
1075.5188	R.CVSEIMIPK.I	Gdb1p
1079.5151	K.SWGYPTELK.N	Gdb1p
1091.5079	R.CVSEIMIPK.I+oxidation (M)	Gdb1p
1099.6054	K.AITIADEVLR.G	Gdb1p
1100.4761	R.VGDEIPNDDK.F	Gdb1p
1203.5957	R.EVIVWGDCVK.L	Gdb1p
1209.507	K.FSDVETQDGGR.I	Gdb1p
1212.6551	R.DGAAIEINGLLK.S	Gdb1p
1243.7749	K.GVLTLPPIPLPK.D	Gdb1p
1274.6859	K.HGLIPNLLDAGR.N	Gdb1p
1377.6073	K.SPEDSPYLWER.M	Gdb1p
1431.6572	R.NGHWALDYTISR.L	Gdb1p
1488.7118	R.VGDEIPNDDKFIK.G	Gdb1p
1516.6293	K.ELEGFDISYDDSK.K	Gdb1p
1547.6997	K.DNVTEPNFGTLGER.N	Gdb1p

anti-GFP or anti-myc primary antibodies. As shown in Fig. 1b, both GFP-tagged Yfr017p and myc-tagged Gdb1p were detectable in the anti-GFP immunoprecipitate from the strain expressing both tagged proteins (lanes d and l). The same two proteins were detectable from the immunoprecipitate of the same strain when anti-myc beads were used (lanes h and p). However, in the anti-GFP immunoprecipitation from the strain without GFP-tagged Yfr017p, no myc-tagged Gdb1p was detected (lane k), even though it is present in the lysate (lane o). Moreover, in the anti-myc immunoprecipitation from the strain without myc-tagged Gdb1p, no GFP-tagged Yfr017p was detected (lane f), although it was present in the lysate (lane b). These results confirm a specific interaction between Yfr017p and Gdb1p. It is worth noting that the α-GFP antibody detects several proteins smaller than Yfr017p-GFP. These most likely represent the degradation products of full-length Yfr017p-GFP and account for the low-intensity bands seen in Fig. 1a that migrate below Yfr017p-GFP.

Given the fact that the expression of the YFR017C gene is induced during fermentation, we investigated whether its encoded protein is similarly regulated. We found that the expression of the Yfr017p-GFP protein was fully induced by day 3 of fermentation (Fig. 1c). The expression of Gdp1-myc was also induced at the same time during fermentation. At this stage of fermentation, ethanol levels are starting to increase and sugar is rapidly consumed (Supporting Information, Fig. S1), and the yeast cells are entering the stationary phase. Under standard lab growth conditions, the expression of both Yfr017p and Gdb1p was induced during the stationary phase of growth (Fig. 1c). Under all conditions (logarithmic growth, stationary phase and fermentation), visualizing the GFP-tagged protein by fluorescence microscopy revealed a cytoplasmic localization (data not shown). The cytoplasmic localization of Yfr017p-GFP was also observed previously in logarithmically growing cells (Huh *et al.*, 2003).

yfr017c null mutants have altered glycogen metabolism

Gdb1p is a glycogen catabolic enzyme; thus, the interaction between Yfr017p and Gdb1p suggests that the YFR017C FSR gene plays a role in glycogen metabolism. Therefore, we constructed null mutants of the ORF in the M2 wine strain and tracked glycogen levels in the yeast cells. Our initial experiments were performed under standard laboratory conditions, growing the cells to the stationary phase in YPD media at 30 °C. Intracellular glycogen was assayed at different time points along the growth curve. Previous studies have shown that yeast cells in the log phase contain little glycogen, but as the cultures approach the stationary phase, the cells start to accumulate glycogen. Later, glycogen levels begin to decline, as the cells convert glycogen back to glucose to use as an energy source (Lillie & Pringle, 1980). As shown in Fig. 2a, the wild-type M2 yeast strain displayed this pattern of glycogen accumulation. However, our *yfr017c* null mutant strain revealed significantly lower levels of intracellular glycogen.

We repeated this experiment under wine fermentation conditions. Wild-type M2 yeast and *yfr017c* null mutant yeast were used to ferment Chardonnay grape must. Cells were extracted at different time points along the fermentation, and assayed for intracellular glycogen. The overall glycogen levels were higher than under YPD growth, most likely due to the higher glucose content of the grape juice. As above, we observed that the *yfr017c* null yeast cells were significantly impaired in their ability to store glycogen compared with wild-type cells (Fig. 2b). Despite this change

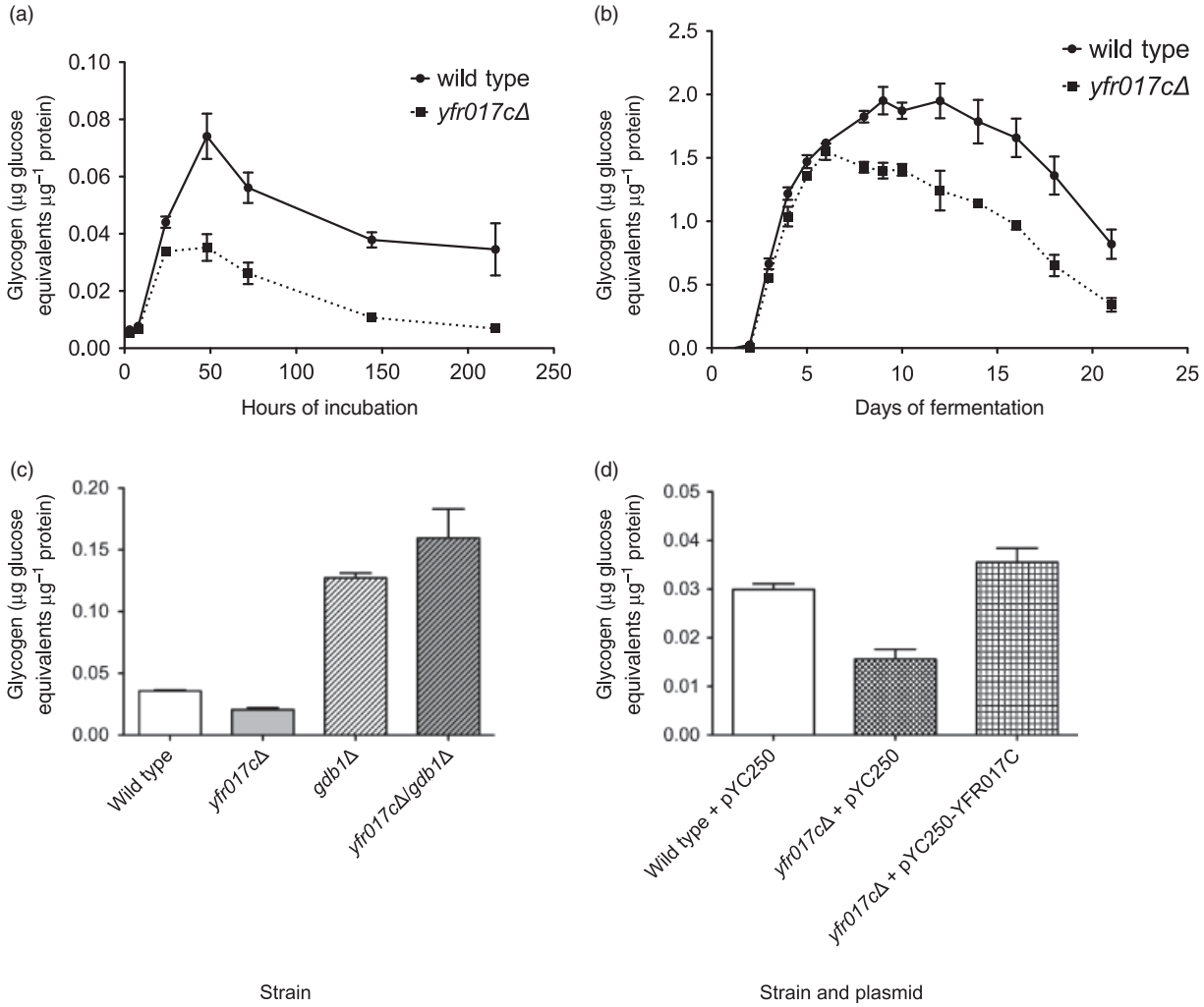

Fig. 2. Cells lacking Yfr017p accumulate less glycogen. (a) Aerobic YPD growth. Triplicate cultures were grown in YPD at 30 °C from two wine yeast strains: Wild-type M2 and M2 carrying a null mutation for YFR017C. At different time points during culture growth, cell samples were extracted and intracellular glycogen was assayed. Cells lacking Yfr017p (squares, dashed lines) accumulated significantly less glycogen than their wild-type counterparts (circles, solid lines). (b) Fermentation. These same two yeast strains were used in triplicate Chardonnay grape juice fermentations. Again, intracellular glycogen was assayed following the extraction of cell samples at different time points. As before, cells lacking Yfr017p (squares, dashed lines) were defective in maintaining glycogen levels compared with wild-type cells (circles, solid lines). (c) Yfr017p alters glycogen metabolism solely through Gdb1p. Four strains were tested in triplicate for their ability to accumulate glycogen: the wild-type M2 strain, M2 carrying the *yfr017c* null mutation, M2 carrying the *gdb1* null mutation and M2 carrying both null mutations. These strains were grown in YPD at 30 °C for 72 h and then assayed for intracellular glycogen. With a functional *GDB1* gene, the lack of Yfr017p produced a decline in glycogen accumulation (gray bar vs. empty bar), as shown above. However, in the absence of Gdb1p, a similar decline was not observed (gray cross-checked bar vs. white cross-checked bar), suggesting that Yfr017p requires Gdb1p for glycogen accumulation. (d) Extrachromosomal complementation of *yfr017c* null mutation restores glycogen accumulation. Three strains were tested in triplicate for their ability to accumulate glycogen: The M2 wild-type strain carrying the empty pYC250 plasmid, M2 carrying the *yfr017c* null mutation plus the empty pYC250 plasmid and M2 carrying *yfr017c* null mutation plus the pYC250 plasmid with a YFR017C cassette (promoter and ORF). The cells were grown for 72 h in YPD at 30 °C and then assayed for intracellular glycogen. Cells carrying the *yfr017c* null mutation plus the empty plasmid (cross-checked bar) accumulated less glycogen than their wild-type counterparts (white bar). However, the expression of Yfr017p from a plasmid restored glycogen levels (vertically checked bar). In all cases, error bars represent the SE.

in glycogen levels, no differences in the fermentation parameters (glucose and fructose consumption, and ethanol production) were detected (Fig. S1).

Two additional experiments were performed to validate these results. The first was to construct an M2 *gdb1* null mutant strain, both with and without the presence of YFR017C. As expected, cells lacking *GDB1* maintained an elevated glycogen level, because this enzyme plays a crucial role in glycogen catabolism (Fig. 2c). However, knocking out YFR017C in addition to *GDB1* made no significant

difference to the glycogen levels. This result suggests that Yfr017p plays its role in glycogen metabolism solely through Gdb1p. Secondly, we complemented the *yfr017c* null mutant strain with a 2 μm plasmid that expresses Yfr017p under the control of the *PGK1* promoter. This complementation restored intracellular glycogen levels to those of wild-type M2 yeast (Fig. 2d). This result confirms that the changes in intracellular glycogen levels are due solely to the absence of Yfr017p and not due to some other genomic alteration during the construction of the null mutant strain.

Gdb1p is a catabolic enzyme; it works in conjunction with glycogen phosphorylase to convert polymeric glycogen into monomeric glucose. Cells lacking Yfr017p appear to be defective in storing glycogen, although under fermentation conditions, these cells can achieve the same maximum glycogen level. Therefore, we suggest that Yfr017p serves as an inhibitor of Gdb1p and that cells lacking Yfr017p are overactive in their glycogen catabolism.

Yfr017p inhibits Gdb1p activity *in vitro*

Our results from experiments with *yfr017c* null mutant strains of M2 wine yeast suggest a role for this protein in inhibiting glycogen catabolism, mediated through the glycogen debranching enzyme. Therefore, we tested whether Yfr017p could directly inhibit Gdb1p activity *in vitro*. Both proteins were expressed in *E. coli* and purified to near homogeneity, and then used in a glycogen debranching assay. This assay involved the debranching of limit dextrin and measurement of the glucose released. As expected, adding purified Gdb1p to limit dextrin drastically increased the rate of glucose production (Fig. 3). However, the further addition of Yfr017p in a 1 : 1 molar ratio drastically decreased the rate of glucose production, demonstrating an inhibitory function for this protein on Gdp1p activity. In the absence of Gdb1p, Yfr017p had little effect on the rate of glucose production, suggesting that this protein has no catalytic activity towards glycogen on its own. These results confirm an inhibitory role for Yfr017p in Gdb1p activity.

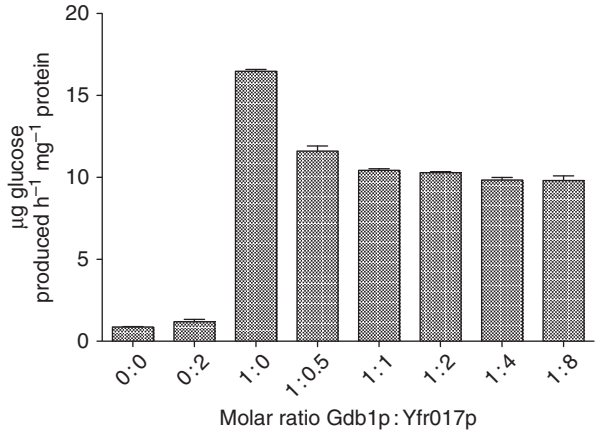

Fig. 3. Yfr017p is an inhibitor of Gdb1p activity. Glycogen debranching activity was assayed by glucose release, as described in Materials and methods. As expected, the addition of purified Gdb1p produced significant debranching activity. The addition of Yfr017p in increasing doses significantly attenuated this activity, indicating that it significantly inhibits Gdb1p activity. Error bars represent the SE, $n = 3$.

Discussion

Although the basic steps of wine fermentation by *S. cerevisiae* are well known, important questions remain. Most notably, the mechanisms that yeast cells use to survive the harsh environment of fermentation are poorly understood. As a first step towards elucidating these processes, our laboratory has determined how the cells respond to the stresses of wine-making at the transcriptional level. We identified a significant number of genes whose expression is stimulated during fermentation (FSR), but whose cellular function is unknown. Grape must fermentations, which are much more stressful than standard laboratory growth conditions, should reveal a role for these nonannotated genes (Marks *et al.*, 2008).

Our initial experiments involved tagging the FSR genes with a GFP cassette, which produces proteins tagged at the C-terminus with GFP. Wine yeast strains carrying these tagged genes were fermented in grape juice for 6 days, allowing significant expression of the FSR proteins. Next, the cells were lysed and the lysates were immunoprecipitated with anti-GFP beads. Our goal was to identify coimmunoprecipitating proteins, with the notion that the identification of interaction partners would provide clues to the function of the original FSR protein.

This strategy was successful for the FSR protein Yfr017p. This 21.8-kDa cytoplasmic protein appears unique to *Saccharomyces*, without homologs in other species. Also, it does not contain any conserved domains or motifs. We were able to identify and confirm a strong and specific interaction between the GFP-tagged Yfr017p and the glycogen debranching enzyme (Gdb1p) (Fig. 1a and b). No other interactions were detected, suggesting that these proteins are acting as a heterodimer. Previous large-scale screening experiments had suggested such an interaction, but not during fermentation (Ito *et al.*, 2000; Krogan *et al.*, 2006). Gdb1p is a catabolic enzyme; in conjunction with glycogen phosphorylase, it is responsible for breaking down stored glycogen to produce glucose for use by the cell. Glycogen phosphorylase catalyzes the hydrolysis of the backbone α-1,4 glycosidic bonds of the glycogen molecule to within two or three glucose units of an α-1,6 branch point. Glycogen debranching enzyme transfers one or two of these glucose residues to the end of a nearby chain, and then cleaves the remaining glucose unit linked to the glycogen backbone by

an α-1,6 glycosidic bond. Therefore, Gdb1p contains two catalytic activities: an oligo-1,4 → 1,4 glucanotransferase activity and α-1,6 glucosidase activity. Large-scale phosphoprotein screening has suggested that Gdb1p is phosphorylated in the mitochondria (Reinders *et al.*, 2007). This modification is unlikely to play a role in the interaction with Yfr017p, because there is no indication from GFP localization studies that this protein is present in this organelle (data not shown and Huh *et al.*, 2003).

Given the interaction between Yfr017p and Gdb1p, we were interested in determining whether Yfr017p protein played a role in glycogen metabolism. We found that the absence of Yfr017p resulted in lower levels of intracellular glycogen, both under normal laboratory growth conditions and during wine fermentation (Fig. 2a and b). This result is novel, because a global screen of genes that affected glycogen metabolism did not reveal a role for Yfr017p (Wilson *et al.*, 2002). Therefore, *yfr017c* null cells were either defective in the synthesis of glycogen or overactive in glycogen breakdown. Given that Yfr017p interacts with the catabolic enzyme Gdb1p, we hypothesized that the latter explanation was the correct one. Consistent with this hypothesis, the absence of Yfr017p results in higher than normal glycogen breakdown and glycogen levels can be restored by removal of Gdb1p in a *yfr017c* null strain (Fig. 2d). Therefore, we suspected that Yfr017p acts as an inhibitor of Gdb1p. We tested this hypothesis and found that the addition of Yfr017p attenuates Gdb1p activity using an assay that measures both glucanotransferase and glucosidase activity (Fig. 3). Interestingly, only a partial inhibition to about 50% of the maximum activity is observed. Preliminary experiments testing the overexpression of Yfr017p did not produce a decrease in the rate of glycogen breakdown (data not shown), suggesting that the ability of this protein to inhibit Gdb1p is limited. It is also possible that additional proteins or modifications to Yfr017p are missing from our *in vitro* assay, preventing full inhibition. Notably, mass spectroscopic analysis of Yfr017p revealed at least one phosphorylated peptide (Table 2, Band A, last line). This serine residue has recently been identified as a potential Yfr017p phosphorylation site from a high-throughput study (Bodenmiller *et al.*, 2010). Whether or not the phosphorylation status of Yfr017p affects its ability to interact with or inhibit Gdb1p awaits further study. Based on these results, we propose that the YFR017C ORF be given the gene name *IGD1*, for inhibitor of glycogen debranching.

Acknowledgements

We wish to thank Calvin Adams and Brandon Chau for expert and enthusiastic technical assistance. Mass spectrometric analysis was carried out by Darryl Hardie under the supervision of Derek Smith at the University of Victoria – Genome British Columbia Proteomics Centre, Victoria. We thank Genome Canada and Genome British Columbia for platform funding and support. This work was funded by Grant # 071WIN to H.J.J.v.V. and Steven Lund from Genome British Columbia.

References

Adams C & van Vuuren HJJ (2010) Effect of timing of diammonium phosphate addition to fermenting grape must on the production of ethyl carbamate in wine. *Am J Enol Viticult* **61**: 125–129.

Bodenmiller B, Wanka S, Kraft C *et al.* (2010) Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. *Sci Signal* **3**: rs4.

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES & Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell* **12**: 323–337.

Chen YT, He JK, Ding JH & Brown BI (1987) Glycogen debranching enzyme: purification, antibody characterization, and immunoblot analyses of type III glycogen storage disease. *Am J Hum Genet* **41**: 1002–1015.

De Antoni A & Gallwitz D (2000) A novel multi-purpose cassette for repeated integrative epitope tagging of genes in *Saccharomyces cerevisiae*. *Gene* **246**: 179–185.

Deed NK, van Vuuren HJJ & Gardner RC (2011) Effects of nitrogen catabolite repression and di-ammonium phosphate addition during wine fermentation by a commercial strain of *S. cerevisiae*. *Appl Microbiol Biot* **89**: 1537–1549.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D & Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* **11**: 4241–4257.

Guldener U, Heck S, Fielder T, Beinhauer J & Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24**: 2519–2524.

Hansen J, Felding T, Johannesen PF, Piskur J, Christensen CL & Olesen K (2003) Further development of the cassette-based pYC plasmid system by incorporation of the dominant *hph*, *nat* and *AUR1-C* gene markers and the *lacZ* reporter system. *FEMS Yeast Res* **4**: 323–327.

Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS & O'Shea EK (2003) Global analysis of protein localization in budding yeast. *Nature* **425**: 686–691.

Ito T, Tashiro K, Muta S, Ozawa R, Chiba T, Nishizawa M, Yamamoto K, Kuhara S & Sakaki Y (2000) Toward a protein–protein interaction map of the budding yeast: a comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *P Natl Acad Sci USA* **97**: 1143–1147.

Krogan NJ, Cagney G, Yu H *et al.* (2006) Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* **440**: 637–643.

Lee EY, Carter JH, Nielsen LD & Fischer EH (1970) Purification and properties of yeast amylo-1,6-glucosidase–oligo-1,4 leads to 1,4-glucantransferase. *Biochemistry* **9**: 2347–2355.

Lillie SH & Pringle JR (1980) Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. *J Bacteriol* **143**: 1384–1394.

Marks VD, Ho Sui SJ, Erasmus D, van der Merwe GK, Brumm J, Wasserman WW, Bryan J & van Vuuren HJJ (2008) Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. *FEMS Yeast Res* **8**: 35–52.

Olesen K, Franke Johannesen P, Hoffmann L, Bech Sorensen S, Gjermansen C & Hansen J (2000) The pYC plasmids, a series of cassette-based yeast plasmid vectors providing means of counter-selection. *Yeast* **16**: 1035–1043.

Parker CE, Warren MR, Loiselle DR, Dicheva NN, Scarlett CO & Borchers CH (2005) Identification of components of protein complexes. *Methods Mol Biol* **301**: 117–151.

Parrou JL & Francois J (1997) A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. *Anal Biochem* **248**: 186–188.

Reinders J, Wagner K, Zahedi RP, Stojanovski D, Eyrich B, van der Laan M, Rehling P, Sickmann A, Pfanner N & Meisinger C (2007) Profiling phosphoproteins of yeast mitochondria reveals a role of phosphorylation in assembly of the ATP synthase. *Mol Cell Proteomics* **6**: 1896–1906.

Richards KD, Goddard MR & Gardner RC (2009) A database of microsatellite genotypes for *Saccharomyces cerevisiae*. *Antonie van Leeuwenhoek* **96**: 355–359.

Rothbauer U, Zolghadr K, Muyldermans S, Schepers A, Cardoso MC & Leonhardt H (2008) A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Mol Cell Proteomics* **7**: 282–289.

Teste MA, Enjalbert B, Parrou JL & Francois JM (2000) The *Saccharomyces cerevisiae YPR184w* gene encodes the glycogen debranching enzyme. *FEMS Microbiol Lett* **193**: 105–110.

Trinkle-Mulcahy L, Boulon S, Lam YW *et al.* (2008) Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *J Cell Biol* **183**: 223–239.

Wilson WA, Wang Z & Roach PJ (2002) Systematic identification of the genes affecting glycogen storage in the yeast *Saccharomyces cerevisiae*: implication of the vacuole as a determinant of glycogen level. *Mol Cell Proteomics* **1**: 232–242.

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. Elimination of YFR017C gene does not affect fermentation.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.