The biological activity of the wine anthocyanins delphinidin and petunidin is mediated through Msn2 and Msn4 in *Saccharomyces cerevisiae*.

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Abstract

Polyphenols are considered to be responsible for some of the health benefits derived from the consumption of red wine. These protective effects might probably be explained in the context of the xenohormesis theory that considers plant metabolites as interespecific chemical signals. However, the complexity of the polyphenolic constituents of different wines makes it difficult to clarify the specific contribution of polyphenols to such effects. In the present work we fractionated the polyphenols of a red wine and evaluated the effect of each polyphenolic fraction on the growth pattern of the yeast *Saccharomyces cerevisiae*. We observed a different contribution of the phenolic fractions to the xenohormetic response of *S. cerevisiae*, the fractions that were enriched with red pigments being the most protective against oxidative insults. Moreover, we found that red wine phenolic fractions exert their biological activity through the activation of the Yap1 and Msn2 stress-responsive regulators. Above all, the anthocyanins delphinidin 3-glucoside and petunidin 3-glucoside were found to improve significantly the growth rate of *S. cerevisiae* in a Msn2, Msn4-dependent manner, indicating that the stress regulators Msn2 and Msn4 participate in the xenohormetic activity of the wine polyphenols delphinidin and petunidin.

Introduction

Red wine polyphenols has been historically considered as potent antioxidants, with a number of benefits for human health (Sun*, et al.*, 2002, Corder*, et al.*, 2006). Epidemiological studies have suggested that a moderate consumption of red wine is correlated with a reduction in the risk of cardiovascular disease and carcinogenesis (German & Walzem, 2000).

Polyphenols are plant secondary metabolites with diverse physiological functions, such as plant colour, anti-parasite functions, and antioxidant protection (Stevenson & Hurst, 2007). Polyphenols also exert important functions through the activation of cellular antioxidant enzymes and signalling pathways (Garcia-Alonso*, et al.*, 2004, Stevenson & Hurst, 2007). In addition, plant polyphenols has been recently associated with xenohormesis, a hypothesis that proposes the plant stress signalling molecules as interspecific chemicals that are able to regulate different physiological processes in ways that confer health benefits (Howitz & Sinclair, 2008). Therefore, polyphenols might exert important functions in mammals through the modulation of stress-response pathways rather than acting as direct antioxidant molecules. However, the molecular mechanisms underlying such biological activities have not yet been fully elucidated.

The unicellular yeast *Saccharomyces cerevisiae* is an excellent eukaryotic model for the study of the molecular mechanisms of novel drugs and natural compounds, including polyphenols. Thus, studies with yeast have served to uncover novel genes and regulatory mechanisms involved in important processes such as eukaryotic cell division, proliferation, and cell death (Mager & Winderickx, 2005, Lisa-Santamaria*, et al.*, 2009). Also, the use of genome-wide screenings with collections of yeast deletion mutants has helped to identify novel targets of different chemicals and pharmaceutical compounds (Tucker & Fields, 2004). In particular, *S. cerevisiae* has been extensively used for the study of oxidative stress, which

has been proposed to be a key regulator of cellular physiology and is associated with several human pathologies, including cancer, neurological disorders, and cardiovascular diseases (Ikner & Shiozaki, 2005, Temple*, et al.*, 2005). These studies have provided important insights into the mechanisms of cellular sensing and responses to reactive oxygen species and other forms of stress.

In *S. cerevisiae*, Yap1 and Msn2/Msn4 have been suggested to be the most important stress-responsive transcription factors, participating in the sensing of oxidative and nutritional stress (Temple*, et al.*, 2005). The transcriptional activity of both Yap1 and Msn2/Msn4 is mainly modulated through their nuclear accumulation. Yap1 is activated by multi-step disulphide-bonding in the nuclear export signal at the C-terminus. Thus, oxidized Yap1 does not bind to the nuclear exportin Crm1, which constitutively determines its cytosolic localization, resulting in the nuclear accumulation of Yap1 and subsequent transcriptional activation of target genes (Okazaki*, et al.*, 2007). In contrast, the nuclear localization of Msn2/Msn4 is regulated by phosphorylation. Both the Ras/PKA and TOR pathways are involved in the modulation of the nuclear translocation of Msn2/Msn4 (Medvedik*, et al.*, 2007). Low levels of protein kinase A activity lead to the nuclear import of Msn2, whereas an increase in PKA activity promotes nuclear export (Gorner*, et al.*, 1998, Lee*, et al.*, 2008). Additionally, the inhibition of the TOR signaling pathway triggers dephosphorylation, and hence the nuclear accumulation of Msn2 (Wei*, et al.*, 2008). Msn2/Msn4 has recently been proposed as a link between caloric restriction and increased life-span through the transcriptional activation of sirtuins and antioxidant enzymes (Medvedik*, et al.*, 2007).

The principal aim of this study is to analyze the biological activity of red wine anthocyanins in the context of xenohormesis. Here we evaluated the protective effect of fractionated red wine polyphenols in a set of deletion mutants from the knock-out collection of *S. cerevisiae*. We also examined the ability of both the anthocyanin-enriched fractions and also that of the anthocyanins delphinidin 3-glucoside and petunidin 3-glucoside to induce a hormetic response through Msn2, Msn4 in *S. cerevisiae*.

Materials and methods

Winemaking. Vitis vinifera cv. Tempranillo fresh grapes from the Spanish Rioja Appellation were processed in the 2005 vintage by Bodegas RODA (Haro, La Rioja, Spain). The maceration and fermentation steps were carried out in 1200-L steel tanks. Post-fermentative maceration was maintained for 6 days, after which wine samples were taken.

Sample fractionation. 180-mL samples of wine were fractionated by gel permeation chromatography with a Toyopearl HW-40S column, using the method described by Alcalde-Eon et al., 2006 (Alcalde-Eon*, et al.*, 2006). Nine fractions were obtained, which were subsequently freeze-dried and weighed. Solutions of 0.5 mg/mL in acidic water (HCl, pH 0.5) were prepared for further chromatographic analysis of phenolic compounds.

HPLC–DAD–MS analysis

Analysis of anthocyanins and flavonols. Solutions corresponding to the nine fractions were filtered through a 0.45 µm Millex® syringe-driven filter unit (Millipore Corporation) and analysed using the HPLC–DAD–MS technique. This was performed on a Hewlett-Packard 1100 series liquid chromatograph. An AQUAC18® reverse phase, 5µm, 150mm×4.6mm column (Phenomenex,Torrance, CA, USA) thermostatted at 35 ºC was used for the simultaneous separation of anthocyanins and flavonols. The solvents used were: (A) an aqueous solution (0.1%) of trifluoroacetic acid (TFA) and (B) 100% HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B for 3 min; from 10 to 15% B for 12 min; isocratic 15% B for 5 min; from 15 to 18% B for 5 min, and from 18 to 30% B for 20 min, and from 30 to 35% B for 5 min, at a flow rate of 0.5 mL/min. Detection was carried out at 520 nm (anthocyanins) and 360 nm (flavonols). Spectra were recorded in the 220-600 nm range.

118 *Analysis of flavanols and phenolic acids.* With a view to eliminating the anthocyanins,
119 2 mL of each sample was placed in Oasis MCX cartridges previously conditioned with 2 mL
120 of methanol and 2 mL of water. After a wash in 4 mL of ultrapure water, the flavan-3-ols and
121 phenolic acids were eluted with 8 mL of methanol, the anthocyanins and the flavonols being
122 retained in the cartridge. A small volume of water was added to the eluate and concentrated
123 under vacuum at a temperature of less than 30 °C until complete elimination of the methanol.
124 The volume of the aqueous residue was adjusted to 500 µL with ultrapure water (MilliQ),
125 filtered, and analysed using an HPLC–DAD–MS system with a Waters Spherisorb® S3 ODS-
126 2 reverse phase, 3 µm, 150mm×4.6mm column (Waters, Ireland) thermostatted at 25 °C. The
127 mobile phase was (A) 2.5% acetic acid, (B) acetic acid/acetonitrile (90:10, v:v), and (C)
128 HPLC-grade acetonitrile. The elution gradient established ranged from 0 to 100% B for 5 min;
129 from 0 to 15% C for 25 min; from 15 to 50% C for 5 min, and isocratic 50% C for 5 min, at a
130 flow rate of 0.5 mL/min. Detection was carried out at 280 nm (flavanols) and 330 nm
131 (phenolic acids). Spectra were recorded from 220 to 600 nm.
132
133 *MS conditions*. Mass analyses were performed using a Finnigan^{TM} LCQ ion-trap
134 instrument (Thermoquest, San Jose, CA, USA) equipped with an electrospray ionisation
135 (ESI) interface. The LC system was connected to the probe of the mass spectrometer *via* the
136 UV cell outlet. Both the sheath gas and the auxiliary gas were a mixture of nitrogen and
137 helium. The sheath gas flow was 1.2 L/min, and the auxiliary gas flow was 6 L/min. The
138 capillary voltage was 26 V for anthocyanins and flavonols; 11 V for flavanols, and 10 V for
139 phenolic acids and flavanols. The source voltage was 4.5 kV for anthocyanins, flavonols and
140 flavanols, and 2.5 kV for phenolic acids. The capillary temperature was 195 ºC. Spectra were
141 recorded in positive-ion mode between 120 and 1500*m*/*z*. The mass spectrometer was
142 programmed to perform a series of three consecutive scans: a full mass; an MS^2 scan of the
143 most abundant ion in the full mass, and an MS^3 of the most abundant ion in the MS^2. The
144 normalised collision energy was 45%.

Quantification. For quantitative analyses, calibration curves were obtained using standards of anthocyanin 3-glucosides (delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside and malvidin 3-glucoside); flavonols (myricetin, quercetin and kaempferol); flavanols ((+)-catechin, (-)-epigallocatechin, the dimer B2, and the trimer epicatechin-4,8-epicatechin-4,8-catechin) and phenolic acids (3,4-dyhydroxybenzoic acid and 4-hydroxycinnamic acid) (Gonzalez-Manzano*, et al.*, 2006). Anthocyanins were purchased from Polyphenols Labs., Sandnes, Norway. Myricetin, kaempferol, and (-)-epigallocatechin were purchased from Extrasynthèse, Genay, France. Quercetin, (+)-catechin, 3,4-dyhydroxybenzoic acid and 4-hydroxycinnamic acid were purchased from Sigma, Steinheim, Germany. Dimer and trimer procyanidins were obtained at our laboratory.

Yeast strains and growth conditions. The non-essential haploid *MAT*a yeast deletion strains derived from the parent strain BY4741 (*MAT*a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) were from Euroscarf (Frankfurt, Germany; http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). Double mutants Δ*msn2,* Δ*msn4* and Δ*msn2,* Δ*pnc1* were obtained by a PCR-based gene-replacement strategy (see Table S1 for primer sequences) using a loxP-NatMX-loxP replacement cassette flanked by recombinogenic sequences for the corresponding target gene (Guldener*, et al.*, 1996). This method enables to eliminate and subsequently reuse the selection marker by expressing a Cre recombinase as described (Guldener*, et al.*, 1996). The triple mutant Δ*msn2,* Δ*msn4,* Δ*pnc1* was obtained using the above strategy in a Δ*msn2,* Δ*pnc1* genetic background.

Cells were routinely grown at 28ºC in either standard rich YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose) or synthetic complete medium lacking leucine (SC-Leu), containing 2% glucose as the carbon source. Growth on liquid cultures was monitored spectrophotometrically at $O.D._{600}$ nm. The antibiotics G418 (150 mg/L; Gibco-BRL) and cloNAT (100 mg/L; Werner Bioagents) were added when indicated.

172

173 *Phenotypic screening of S. cerevisiae deletion mutants.* A subset of 44 mutant strains
174 in either the BY4741 or BY4743 background was screened for their ability to grow in the
175 presence or absence of nine red wine fractions (Table S2). Solutions containing 0.2 mg/mL of
176 each lyophilized fraction were prepared in wine-like solution (12% ethanol (v/v) containing 5
177 g/L of tartaric acid buffered with 1 M NaOH at pH 3.6). All assays, including the negative
178 controls without polyphenols, contained the same volume of wine –like solution.

179 The concentration of the phenolic fractions was fixed at 150 µg/mL, which has been
180 shown to be effective in previous studies (Garcia-Alonso*, et al.*, 2004). The chemically
181 undefined F1 fraction was excluded from the analysis. The analyses were repeated four
182 times and some variability was observed between the experiments.

183 Analyses of the wine fractions and standards were carried out in the presence or
184 absence of 1mM hydrogen peroxide or 0.5-1mM t-BOOH. Screening was performed as
185 previously described (Botet*, et al.*, 2008). Briefly, strains were pinned from 96-well frozen
186 stock plates using a stainless steel 96-pin replicator (Nalgene Nunc International) into 96-well
187 plates containing 150 µL of liquid YPD medium supplemented with G418 (150 mg/L; Gibco-
188 BRL). When the cultures had reached stationary phase (approx. 24 hours), the plates were
189 pin-replicated either onto liquid YPD 96-well plates containing wine-like solution or YPD
190 plates containing the wine fractions, or YPD plates containing both the wine fractions and the
191 oxidants. The plates were incubated at 28°C and growth was scored quantitatively every 12 h
192 over a period of 4 days by recording optical density at 600 nm, using a microplate reader
193 spectrophotometer (model 550, Bio-Rad Laboratories). The effect of the red wine fractions on
194 yeast growth was scored on the basis of the relative growth of each mutant in every
195 treatment against that of the mutants on the control YPD plates.

196 Additionally, a number of mutants (see below fig.2 and fig.4) were selected to analyze
197 the effect of either wine fractions or pure anthocyanins on the growth rate in the presence or
198 absence of oxidants. For these experiments 2-5 mL cultures of either YPD or SC media

supplemented with the indicated chemicals (i. e. polyphenols and oxidants) were used and the growth of the cultures were scored by recording optical density at 600 nm at the indicated time-points. All the experiments were carried out including the corresponding volumes of wine-like solution in the negative controls to exclude the effect of ethanol in the results.

Yap1 and Msn2 nuclear translocation analyses. The *S. cerevisiae* strains used in this analysis were Δ*yap1* and Δ*msn2* transformed with either YCplac111/YAP1-GFP or YCplac111/MSN2-GFP, respectively. The plasmids for YAP1-GFP and MSN2-GFP were a gift from Y. Inoue and C. Schüller, respectively (Gorner*, et al.*, 1998, Maeta*, et al.*, 2007). In this assay, we examined the intracellular localization of both MSN2-GFP and YAP1-GFP after the addition of different phenolic compounds to the media: either the F5 red wine fraction, or the F6 red wine fraction, or delphinidin 3-glucoside or petunidin 3-glucoside (concentrations ranging from 12.5 µg/mL to 100 µg/mL). The localization of the GFP-fusion proteins in yeast cells was observed at different times using a Nikon Eclipse 90i fluorescence microscope. Excitation and emission wavelengths were 480 nm and 530 nm, respectively.

Transcription analysis by quantitative real-time PCR. Quantitative real-time PCR was performed with a LightCyler 480 real-time PCR instrument (Roche), using SYBR green I master mix (Roche) and following manufacturer's instructions. Total RNA samples were obtained as previously decribed (Jimenez*, et al.*, 2007) and the cDNA samples were prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Primer sequences are indicated in Table S1. All real-time PCR reactions were performed in duplicate and in at least two independent experiments. Quantitative analyses were carried out using the LightCycler 480 software.

Results

Phenolic composition of the wine fractions

A red wine was fractionated into nine different fractions (F1 to F9) as described above; the phenolic compositions of the resulting fractions are shown in Table 1. While fractions F1, F2, F8 and F9 contained very low amounts of phenolic compounds (0.3 to 4.3 µg/mL), the F5 and F6 fractions had an important proportion of phenolics, with more than 200 µg/mL and 100 µg/mL, respectively. Additionally, more than 50% of the phenolic compounds present in the F5 and F6 fractions were identified as red pigments.

Thirty-seven red pigments were identified among the wine fractions, including monoglucosylated anthocyanins, monoglucosylated anthocyanins esterified with acetic acid, coumaric acid or caffeic acid, and other anthocyanin-derived pigments (pyranoanthocyanins and direct flavanol–anthocyanin condensation pigments). Monoglucosylated anthocyanins were represented in all fractions (Table 2), but the F5 and F6 fractions contained the highest amounts of these compounds. In the F5 fraction, delphinidin 3-glucoside and petunidin 3-glucoside represented 33% and 42%, respectively. In the F6 fraction, delphinidin 3-glucoside was the most abundant anthocyanin, accounting for 72% of all the monoglucosylated anthocyanins (Table 3).

Phenotypic screening of red wine fractions with a set of *S. cerevisiae* deletion mutants

The existence of a complete set of *S. cerevisiae* deletion mutants permits phenotypic genome-wide screenings to be carried out to identify all the nonessential genes that could affect the cellular response to a specific treatment (Giaever*, et al.*, 2002). In this study, we wished to evaluate the biological effect of red wine polyphenols obtained after the chemical fractionation described above. The limited availability of our polyphenolic fractions made it

249 impossible to carry out a phenotypic screening of the genome-wide set of *S. cerevisiae*
250 knock-out mutants, and hence we chose a subset of 44 mutants lacking genes involved in
251 redox metabolism and stress defence to assess the effect of the red wine fractions (Table
252 S2).

253 Screening of the wine fractions was performed in both the presence and absence of
254 the pro-oxidants H_2O_2 and t-BOOH. Our results revealed marked differences among the
255 phenolic fractions when their effect on the growth rate of the *S. cerevisiae* mutants was
256 analyzed (Fig. 1 and Supporting Information, Figure S1). Fractions F6, F8 and F9 increased
257 the growth rate of about 50% of the mutants in the absence of pro-oxidants. Additionally,
258 fractions F2, F3 and F6 were able to alleviate the cytotoxic effect of hydrogen peroxide in a
259 high number of mutants. Finally, fraction F5 improved the growth rate of most of the mutants
260 in the presence of t-BOOH to a considerable extent (Fig. 1). Thus, anthocyanin-enriched
261 fractions (especially fractions F5 and F6) were able to improve the growth rate of a higher
262 number of mutants than the other fractions.

263

264 **Anthocyanin-enriched fractions diminish oxidative-induced toxicity in *S.***
265 ***cerevisiae***

266 In order to gain further insight into the protective effect of fractions F5 and F6, we
267 analyzed their ability to increase the growth rate of three selected mutant strains that have
268 been proposed to be adequate models for analyses of oxidative stress; namely, *grx5*, *ahp1*
269 and *tsa2*. In this sense, the mutant *grx5*, which lacks a mitochondrial glutaredoxin, is
270 hypersensitive to hydrogen peroxide while the *ahp1* mutant is unable to grow in the presence
271 of alkyl hydroperoxides such as t-BOOH. The mutant *tsa2* lacks a thioredoxin peroxidase
272 with ROS-scavenging activity. The growth kinetics of the liquid cultures was obtained for
273 each yeast strain and the results are shown in the Figure 2. In the absence of pro-oxidants,
274 the four strains analyzed did not undergo any significant rise in their growth rates after the
275 addition of the F5 and F6 fractions to the cultures (data not shown). However, both the F5

and the F6 fractions contributed to reduce the toxicity of hydrogen peroxide in the *grx5* mutant. Additionally, the *grx5*, *tsa2* mutants and the BY4741 parental strain displayed reduced sensitivity to t-BOOH after the addition of both the F5 and F6 phenolic fractions to the culture medium. The *ahp1* mutant, which lacks an alkyl hydroperoxide reductase, was unable to grow when t-BOOH was present in the culture medium. Overall, our results indicate that red wine anthocyanins may elicit a cellular response contributing to alleviate oxidative stress in *S. cerevisiae*.

Anthocyanin-enriched fractions induce the nuclear accumulation of Yap1 and Msn2

Our results suggested that the addition of anthocyanin-enriched fractions might trigger an antioxidant response in *S. cerevisiae*. Yap1 and Msn2/Msn4 are stress-responsive factors that induce the expression of genes that participate in cellular defence against oxidative stress. Accordingly, we decided to investigate whether our phenolic fractions might affect the localization of Yap1 and Msn2, which determines their activity as transcriptional activators.

We used GFP-tagged Yap1 and Msn2 to analyze their localization after the addition of the F5 and F6 red wine fractions by epifluorescence microscopy. Fraction F5 induced a reversible nuclear translocation of both Yap1-GFP and Msn2-GFP (Fig. 3). The translocation of Msn2-GFP was much faster than that of Yap1-GFP, which occurred approximately 1 hour after the addition of the F5 fraction to the culture. In contrast, the F6 fraction was only able to induce the nuclear translocation of Msn2-GFP, which appeared in the nucleus immediately after the addition of that fraction to the culture (Fig. 3). In this case, Msn2-GFP moved out to the cytosol after 1 hour but was again localized to the nucleus after two hours. Nuclear localization of both Yap1-GFP and Msn2-GFP were confirmed by DAPI staining of the corresponding samples (Fig. S2).

Delphinidin 3-glucoside and petunidin 3-glucoside improve fitness of *S. cerevisiae* in a Msn2, Msn4 dependent manner.

The phenolic fractions F5 and F6 used in our previous analyses indicated a possible mode of action regarding the subcellular localization of both Yap1-GFP and Msn2-GFP. However, to better understand the molecular mechanisms underlying such effects it is important to determine the specific compounds that are responsible of those biological effects. As evidenced by our HPLC analyses, the F5 fraction was mainly composed of petunidin 3-glucoside (Pt3g) and delphinidin 3-glucoside (Dp3g), whereas the F6 fraction was essentially constituted by Dp3g. Consequently, we analyzed the effect of these two phenolic standards over the growth rate of *S. cerevisiae* in the presence and absence of t-BOOH. We used the parental strain BY4741 and several single, double and triple mutants lacking genes encoding the stress regulators Msn2 and Msn4 (Fig. 4A)

Dp3g increased fitness of *S. cerevisiae* in SC medium and it clearly alleviated the toxic effect of t-BOOH (Fig. 4B-C). Moreover, the protective effect of Dp3g seems to be dependent on the presence of both Msn2 and Msn4 because neither the double *msn2, msn4* nor the triple *msn2, msn4, pnc1* mutants improved their growth rates after Dp3g treatment (Fig. 4). Indeed, only those mutants lacking both *MSN2* and *MSN4* were not able to reach a growth rate similar to the BY4741 parental strain when t-BOOH was added to the culture. The same results were obtained when Pt3g was analyzed (data not shown), indicating that both anthocyans may exert identical effects.

Delphinidin 3-glucoside and petunidin 3-glucoside affect the nucleocytoplasmic trafficking of Msn2

Our previous data indicate that stress-responsive factors participate in the biological activity of the wine anthocyanins Dp3g and Pt3g. Therefore we examined the effect of pure Dp3g on the nuclear accumulation of both Yap1-GFP and Msn2-GFP by epifluorescence microscopy (Fig. 5). Our results revealed that Dp3g was unable to induce the nuclear

translocation of Yap1-GFP. However, Msn2-GFP was rapidly localized to the nucleus after the addition of Dp3g to the culture medium. As occurred when the F6 fraction was used, Msn2-GFP again relocalized to the cytosol after 1 hour, and it appeared once again in the nucleus after two hours. These results indicate that the effect of the F6 fraction on the nucleocytoplasmic trafficking of Msn2-GFP could be attributed to the anthocyanin Dp3g.

We also investigated the effect of Pt3g on the subcellular localization of both Yap1-GFP and Msn2-GFP. In this case, we used either pure Pt3g (Fig. 6) or Pt3g combined with Dp3g at the same proportion as observed in the F5 fraction (data not shown), obtaining the same results. Pt3g was also unable to induce the nuclear localization of Yap1-GFP. However, the dynamics of Msn2-GFP resembled those observed when the F5 fraction was used. Thus, Pt3g, either combined with Dp3g or alone, elicited a rapid translocation of Msn2-GFP from the cytosol to the nucleus, but the fluorescence was seen in the cytosol after only one hour. In contrast to what was observed with Dp3g, Pt3g did not induce a second nuclear relocalization of Msn2-GFP.

Transcriptional activation of Msn2 targets induced by Delphinidin-3-glucoside.

The nuclear localization of Msn2-GFP upon either Dp3g or Pt3g treatments suggests that the putative target genes of the transcription factor Msn2 should be activated. Therefore, we analyzed the transciption levels of different Msn2 target genes (*PNC1*, *AHP1*, *TRX2*, *CTT1*) after Dp3g treatment. *TRX2* and *CTT1*, and to a lesser extent *AHP1*, showed a significant increase in their transcription after the addition of Dp3g to the cultures (Fig. 7). However, we did not detect a significant variation in the transcription of *PNC1*. The mRNA levels of the antioxidant genes *AHP1*, *TRX2* and *CTT1* increased immediately after the addition of the anthocyanins and decreased rapidly after 30-60 minutes (Fig. 7).

Discussion

Understanding the molecular mechanisms underlying the "French paradox" has contributed to the growing interest in investigating the biological activity of the polyphenols of red wine. Thus, recent studies have shown that many of the constituents of red wine, such as resveratrol and its derivatives, may serve as nutraceuticals (Baur*, et al.*, 2006, Feige*, et al.*, 2008, Pearson*, et al.*, 2008). In addition, the idea that stress-derived plant molecules may act as chemical vaccines against stress, activating certain signalling protective pathways, in heterotrophs organisms (i. e. xenohormesis) is being widely accepted (Lamming*, et al.*, 2004, Howitz & Sinclair, 2008).

Relatively little is known about the nature of absorption and *in vivo* metabolism of polyphenols in humans. Indeed, the apparent bioavailability of polyphenols has been shown to be very low. However, recent studies have reported that, after oral intake, a high proportion of anthocyanins could pass the small intestine unmetabolized and reach the colon, where the anthocyanins are absorbed (Kahle*, et al.*, 2006, Neto, 2007, Knaup*, et al.*, 2009). Furthermore, it has been described that some flavonoids including delphinidin exert some of their activities through their binding to receptors at the plasma membrane and activating important signaling pathways without entering the cell (Fridrich*, et al.*, 2008, Kwon*, et al.*, 2009, Teller*, et al.*, 2009).

To gain further insight into the cellular effects of such phenolic compounds at the molecular level, here we wished to investigate whether the coloured phenolic fractions of red wine might trigger a xenohormetic response in eukaryotic cells. We therefore decided to perform a phenotypic screening with HPLC-fractionated polyphenols in a set of *S. cerevisiae* deletion mutants lacking the genes involved in redox metabolism.

A Tempranillo red wine was fractionated after post-fermentative maceration, and anthocyanin-derived pigments, which are formed during the later steps of the maturation

process, were found in a very low proportion. In contrast, monoglucosylated anthocyanins were the red pigments with the highest concentrations in the samples obtained.

The effect of the phenolic fractions on the growth of *S. cerevisiae* mutants was assessed under both non-oxidative and oxidative conditions (using H_2O_2 or t-BOOH). Fractions F6, F2, F3, and to a lesser extent F7, were the most active fractions as regards the increase in the growth rate of the mutants when H_2O_2 was included in the analysis; however, among them, only F6 was also able to reduce the cytotoxicity of t-BOOH in a significant number of strains. Surprisingly, fraction F5 abolished the toxicity of t-BOOH in 80% of the mutants analyzed, and was only seen to be inactive in hypersensitive strains such as *ahp1*. An additional analysis of the F5 and F6 fractions, which were enriched in red pigments, was carried out on the wild-type BY4741 and the *ahp1*, *grx5* and *tsa2* mutants of *S. cerevisiae*, which have been shown to be hypersensitive to oxidants and thus have a constitutive level of oxidative stress. Our results showed that both fractions F5 and F6 conferred protection against the oxidative stress in the mutant strains and did not reveal significant differences between the treatments. Thus, it could be speculated that the molecular mechanism underlying the effect of the red pigments in fractions F5 and F6 may be comparable. The mutant *ahp1* is extremely sensitive to hydroperoxides and thus unable to grow in the presence of t-BOOH.

Cells adapt to environmental changes by modifying their gene expression patterns, mainly through the activation of transcription factors. Several flavonoids such as resveratrol or curcumin has been proposed to regulate signalling pathways involved in cellular energy supply or stress response in mammals (Howitz & Sinclair, 2008). Previous studies have shown that green tea polyphenols are able to activate oxidative-stress-responsive transcription factors in yeast (Takatsume*, et al.*, 2005, Maeta*, et al.*, 2007). Yap1 and Msn2/4 are stress-responsive transcription factors that are localized to the nucleus during oxidative stress or under other starving conditions (Temple*, et al.*, 2005). We hypothesized that red wine pigments might also promote the nuclear accumulation of those transcription factors,

405 which in turn might trigger the induction of stress-responsive genes and consequently
406 alleviate the effect of oxidative stress. Accordingly, GFP-fusion proteins were used to analyze
407 the subcellular localization of Yap1 and Msn2 upon treatment with fractions F5 and F6. It is
408 remarkable that significant differences were observed between the treatments with fractions
409 F5 and F6; these could be attributed to the different compositions of the phenolic fractions.
410 The effect of the F5 and F6 fractions on Msn2 can certainly be assigned to the
411 monoglucosylated anthocyanins Pt3g and Dp3g. These anthocyanins represent more than
412 75% of the total monoglucosylated anthocyanins in fractions F5 and F6. Indeed, our analyses
413 using pure Pt3g or Dp3g demonstrated that both compounds were able to induce the nuclear
414 import of Msn2-GFP. This effect was completely reversible with Pt3g, as also reported for
415 catechins and green tea extracts (Maeta*, et al.*, 2007); however when Dp3g was used, the
416 nuclear localization of Msn2-GFP was recurrent after two hours. The structural difference
417 among the anthocyanins tested is the substitution in the B ring. Dp3g contains a
418 gallocatechol structure (3'-, 4'-, and 5'-hydroxyl groups) whereas Pt3g contains two hydroxyl
419 groups and one methoxyl group. This variation might be relevant for explaining the behaviour
420 of the two anthocyanins examined here. In fact, in a previous study the authors proposed that
421 it is mainly the presence of the gallocatechol group that determines the prominent *in vitro*
422 antioxidant potential of Dp3g (Garcia-Alonso*, et al.*, 2004) and it might also be responsible for
423 the antiangiogenic properties of delphinidin (Lamy*, et al.*, 2006). Therefore, the structure of
424 Dp3g provides a chemical reducing potential, which might be responsible of the periodicity in
425 the nucleocytoplasmic trafficking of Msn2-GFP when either F6 or Dp3g was used.

426 Both Dp3g and Pt3g improved fitness of *S. cerevisiae* and induced a significant
427 resistance to oxidative stress. Moreover, this effect is completely abolished in mutant strains
428 lacking both *MSN2* and *MSN4* genes, indicating that both transcription factors are involved in
429 the biological activity of these wine anthocyanins. This suggests that both Dp3g and Pt3g
430 might induce a xenohormetic response in *S. cerevisiae* that is related to the environmental
431 stress response (Gasch*, et al.*, 2000, Causton*, et al.*, 2001). In this sense, it has been

described that the nuclear accumulation of Msn2 induces the expression of *PNC1*, a nicotinamidase gene that regulates sirtuin activity and extends life-span (Medvedik*, et al.*, 2007). However, we found that Dp3g did not cause a significant change in the transcription level of *PNC1* after 1 hour of treatment, although Msn2-GFP is effectively localized to the nucleus. Again, this suggests that Dp3g triggers a specific xenohormetic response through Msn2/Msn4, that was mainly related to the antioxidant defence as evidenced by the increased transcription of the antioxidant genes *TRX2*, *AHP1* and *CTT1,* which are also Msn2 targets (Schmitt & McEntee, 1996). Indeed, the induction of *AHP1* expression after Dp3g treatment is consistent with the hypersensitivity to hydroperoxide of the Δ*ahp1* strain and the ineffectiveness of the polyphenols to rescue the growth capacity of this mutant. The activation gene expression kinetics resembles that of the environmental stress response and it might be speculated that these plant polyphenols could activate an adaptive response in *S. cerevisiae* as described for the acquired stress resistance (Berry & Gasch, 2008).

The effect of the F5 fraction on Yap1 cannot be attributed to either Pt3g or Dp3g. In this case, other constituents of the F5 fraction such as flavonols, which represent 35% of the total phenolic composition, could be responsible for the relocalization of Yap1. In this sense, previous studies have reported a pro-oxidant activity of catechins and other flavanols present in green tea, which may trigger the intramolecular disulphide bonding of Yap1 and hence the nuclear localization of Yap1 (Maeta*, et al.*, 2007, Wang*, et al.*, 2009). Nonetheless, synergistic interactions between different components of the fraction F5 might also be responsible for the Yap1 relocalization.

The chemical features of Pt3g and Dp3g as natural colorants together with their bioactivity seem to offer a good possibility of using these compounds as nutraceutical food additives. Anthocyanins have been proposed to possess beneficial effects against different human diseases (Hou, 2003, Leifert & Abeywardena, 2008), but further studies using animal models would be required to determine whether monoglucosylated anthocyanins are indeed beneficial to human health.

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574
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Figure legends

Figure 1. Phenotypic analysis of the phenolic fractions of red wine. Bar diagram representing the number of *S. cerevisiae* mutants whose growth rate was improved after the addition either of fractions of red wine (F2-F9) or pro-oxidants alone (zero strains). YPD rich media, no pro-oxidant was added; H_2O_2 or t-BOOH (1mM) was added when indicated.

Figure 2. Growth kinetics of the anthocyanin-enriched fractions. The *S. cerevisiae* strains BY4741, *grx5*, *ahp1* and *tsa2* were cultured in 2 mL of YPD rich media with either the F5 or the F6 fraction, in the presence or absence of either H_2O_2 (1mM) or t-BOOH (0.5mM). The cell growth was scored by measuring the O.D. $_{600nm}$ of the cultures over a period of 56-64 hours. The cultures were carried out in duplicate and in at least three independent experiments.

Figure 3. Anthocyanin-enriched fractions trigger Yap1 and Msn2 nuclear translocation. BY4741 cells transformed with either YAP1-GFP or MSN2-GFP plasmids were incubated with either the F5 or the F6 fraction and the distribution of the GFP fluorescence was visualized at different time-points after the addition of the polyphenols. The concentration of the phenolic fractions was 12.5 µg/mL in all the assays. The corresponding volume of wine-like solution was also added to the negative controls.

Figure 4. Dp3g protects *S. cerevisiae* against oxidative stress in a *MSN2* and *MSN4* dependent manner. A, plate assay of BY4741 and Δ*msn2*, Δ*msn4*, Δ*pnc1* mutants in the presence of 0.5mM t-BOOH. B, microtiter plate of duplicate liquid cultures of BY4741 and Δ*msn2*, Δ*msn4*, Δ*pnc1* mutants after 24 hours. Dp3g (25 µg/mL) and/or t-BOOH (0.5mM) was added when indicated. Wells corresponding to the double or triple Δ*msn2*, Δ*msn4 or*

602 Δ*msn2*, Δ*msn4,* Δ*pnc1* without cellular growth are marked with a red square. C, growth
603 kinetics of BY4741 and Δ*msn2*, Δ*msn4*, Δ*pnc1* mutants in either SC medium or SC media
604 containing Dp3g (25 µg/mL) and/or t-BOOH (0.5mM). In all assays, the corresponding
605 volume of wine-like solution was also added to the negative controls.
606
607 **Figure 5.** Effect of Dp3g on the intracellular localization of Yap1 and Msn2. BY4741 cells
608 transformed with either YAP1-GFP or MSN2-GFP plasmids were treated with Dp3g (12.5
609 µg/mL) and the distribution of the GFP fluorescence was visualized at different time-points.
610
611 **Figure 6.** Effect of Pt-3g on the intracellular localization of Yap1 and Msn2. BY4741 cells
612 transformed with either YAP1-GFP or MSN2-GFP plasmids were treated with Pt3g (20
613 µg/mL) and the distribution of the GFP fluorescence was visualized at different time-points.
614
615 **Figure 7.** Relative transcription levels of *PNC1*, *TRX2*, *AHP1* and *CTT1* genes after various
616 Dp3g treatments. Total mRNA was obtained from BY4741 cells at different time points (0, 15,
617 30 and 60 minutes) after the addition of Dp3g (25 µg/mL). Transcription levels are normalized
618 using the *ACT1* gene as a reference. Quantitative analyses were performed using the LightCycler
619 480 software. The results are the average of two independent experiments and are
620 expressed as a ratio of the cDNA abundance of the target genes in the cultures with Dp3g
621 with respect to that without Dp3g. Error bars indicate the standard deviation.

Tables

Table 1. Phenolic composition of the fractions obtained from Tempranillo red wine (µg/mL).

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Red pigments*	n.d.	2.51	6.39	27.5	131	56.50	9.46	1.51	1.12
Flavanols	0.23	0.45	5.70	3.66	2.66	5.59	4.53	0.60	n.d.
Phenolic acids	0.11	0.20	1.80	3.25	3.11	3.18	1.37	0.18	0.39
Flavonols	n.d.	1.14	25.60	11.30	75.70	37.50	5.98	n.d.	n.d.
TOTAL	**0.34**	**4.30**	**39.50**	**45.70**	**212.47**	**102.77**	**21.30**	**2.29**	**1.50**

*Anthocyanins and anthocyanin-derived pigments; n. d., not detected.

Table 2. Content of red pigments in the fractions obtained from Tempranillo red wine (μg/mL)

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Anthocyanin monoglucosides	n.d.	0.79	3.87	19.90	70.50	26.30	2.91	1.51	1.12
Acylated Anthocyanins	n.d.	0.97	n.d.	4.11	30.0	10.80	3.27	n.d.	n.d.
Anthocyanin-derived pigments	n.d.	0.31	2.52	2.13	22.30	13.10	2.69	n.d.	n.d.
TOTAL	n.d.	**2.07**	**6.39**	**26.14**	**122.80**	**50.20**	**8.87**	**1.51**	**1.12**

n. d., not detected.

Table 3. Content of the main anthocyanin monoglucosides in the fractions obtained from Tempranillo red wine (µg/mL)

	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Dp-3-glc	n.d.	0.20	2.02	1.31	22.10	18.20	0.81	0.85	0.65
Cy-3-glc	n.d.	0.17	n.d.	0.35	4.98	2.11	0.54	n.d.	n.d.
Pt-3-glc	n.d.	0.20	n.d.	1.42	28.2	2.59	0.59	n.d.	n.d.
Pn-3-glc	n.d.	0.05	n.d.	1.04	2.98	0.54	0.19	n.d.	n.d.
Mv-3-glc	n.d.	0.17	1.85	15.60	8.27	1.76	0.79	0.65	0.47
TOTAL	n.d.	**0.79**	**3.87**	**19.70**	**66.53**	**25.20**	**2.91**	**1.51**	**1.12**

n. d., not detected. Dp-3-glc, delphinidin-3-glucoside; Cy-3-glc, cyaniding-3-glucoside; Pt-3-glc, petunidin-3-glucoside; Pn-3-glc, peonidin-3-glucoside; Mv-3-glc, malvidin-3-glucoside.

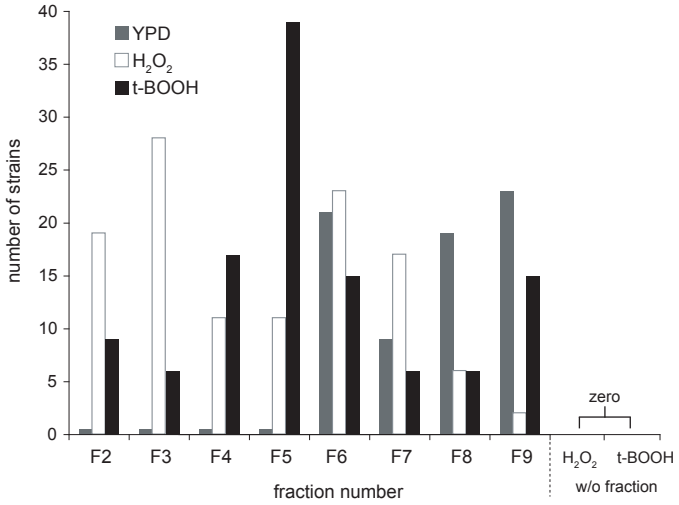

Figure 1, Jiménez et al., 2010

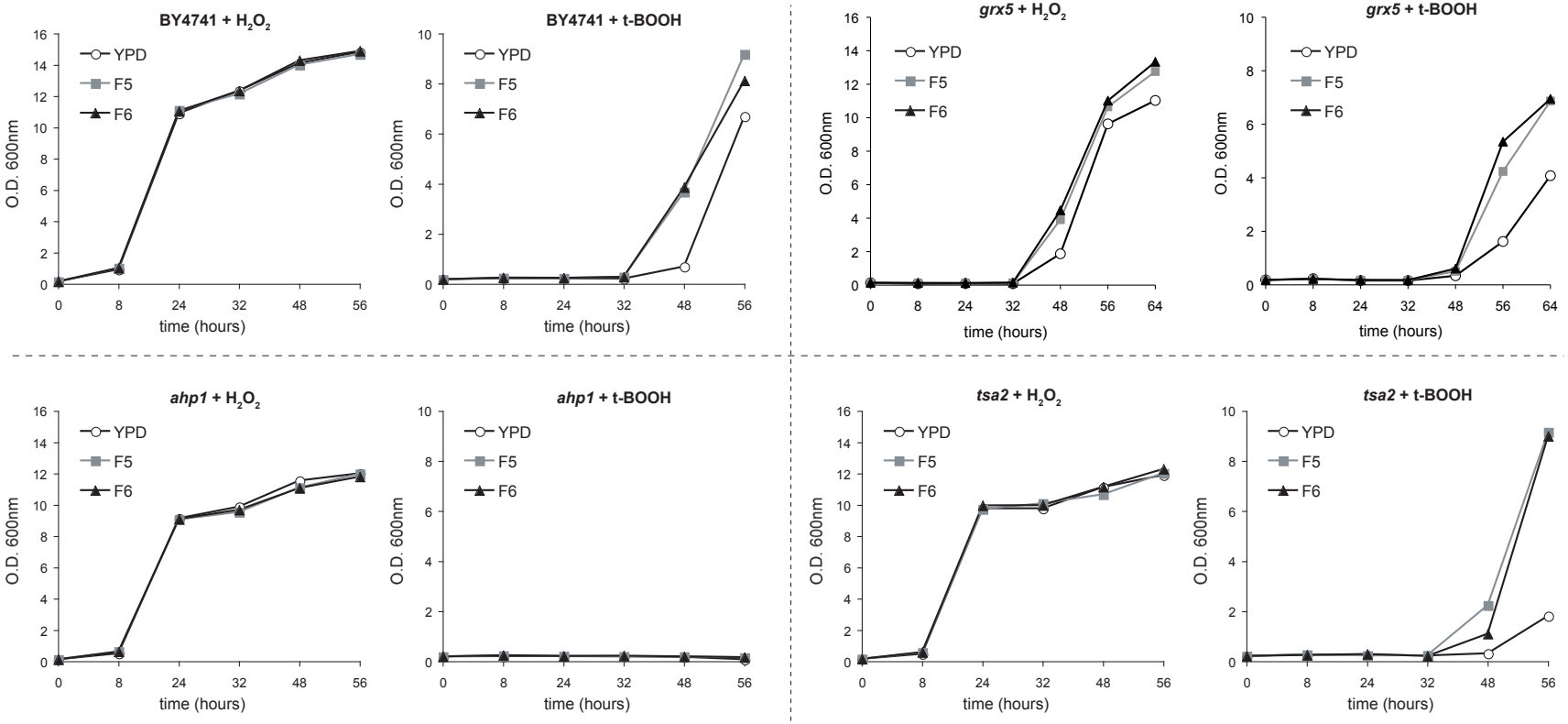

Figure 2, Jiménez et al., 2010

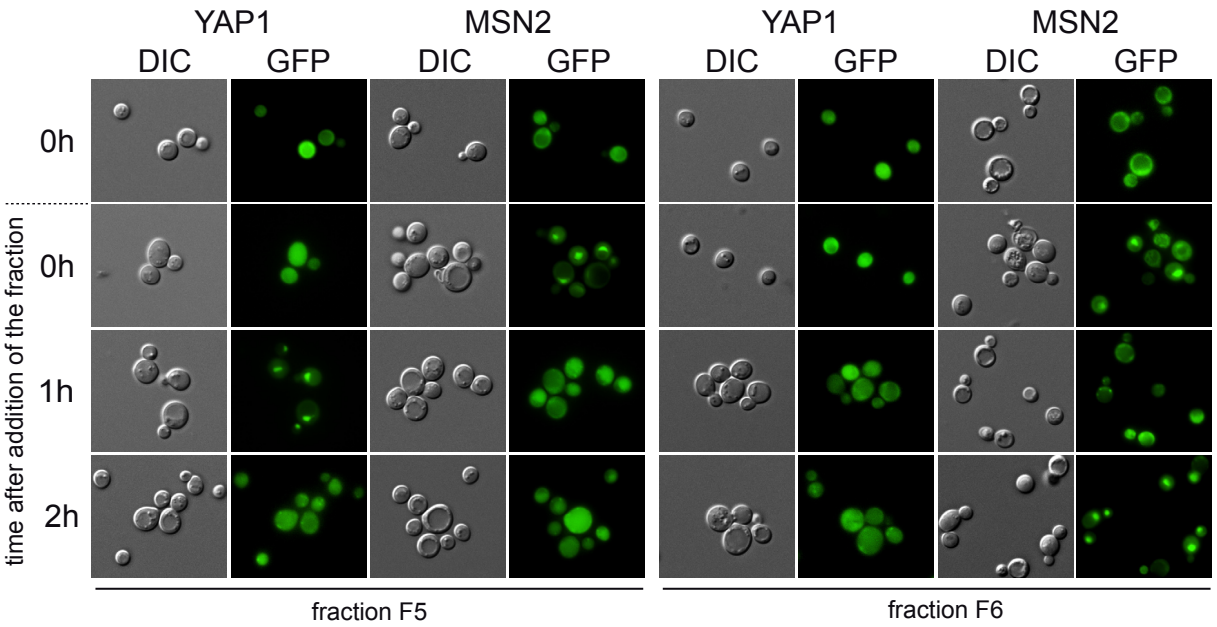

Figure 3, Jiménez et al., 2010

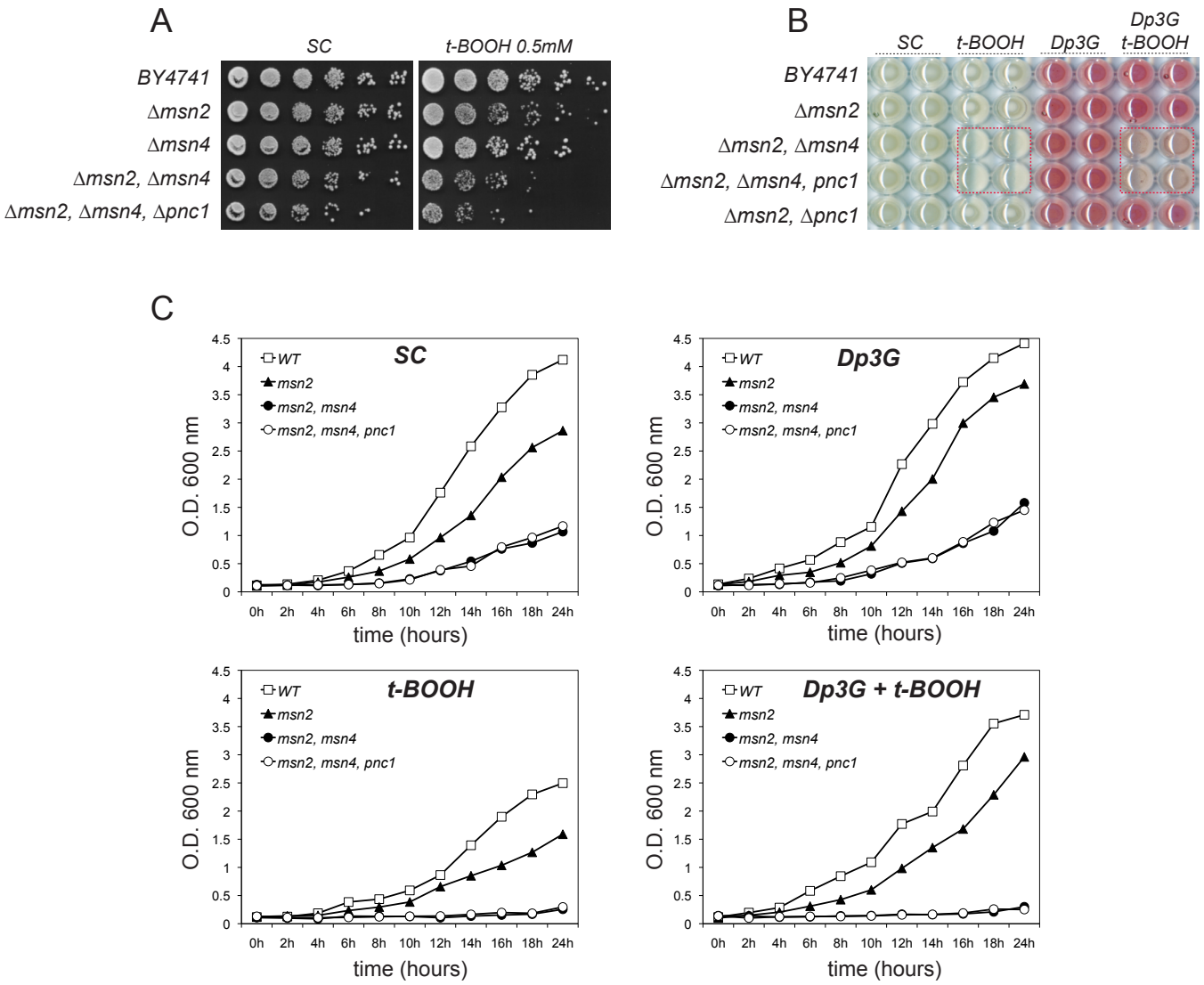

Figure 4, Jiménez et al., 2010

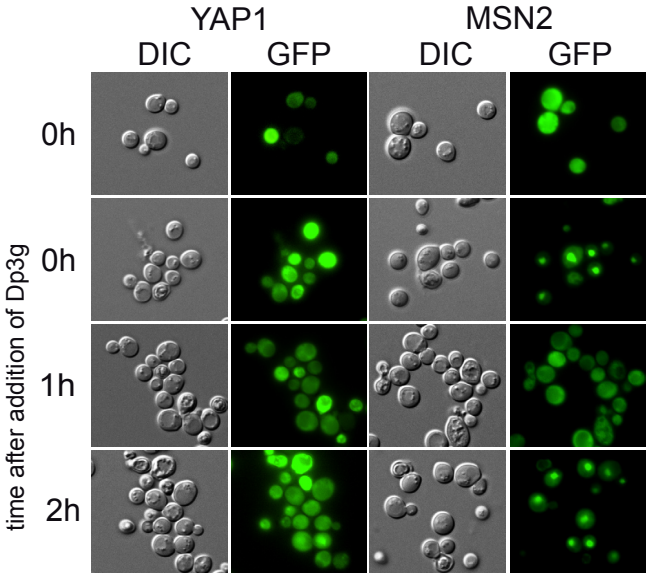

Figure 5, Jiménez et al., 2010

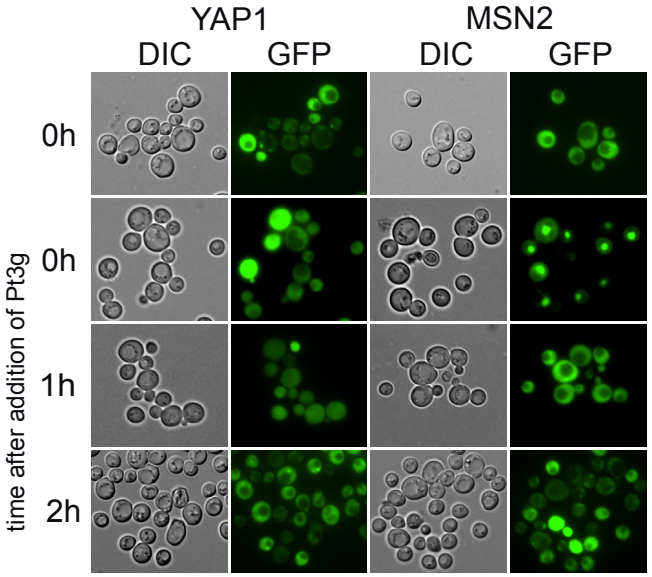

Figure 6, Jiménez et al., 2010

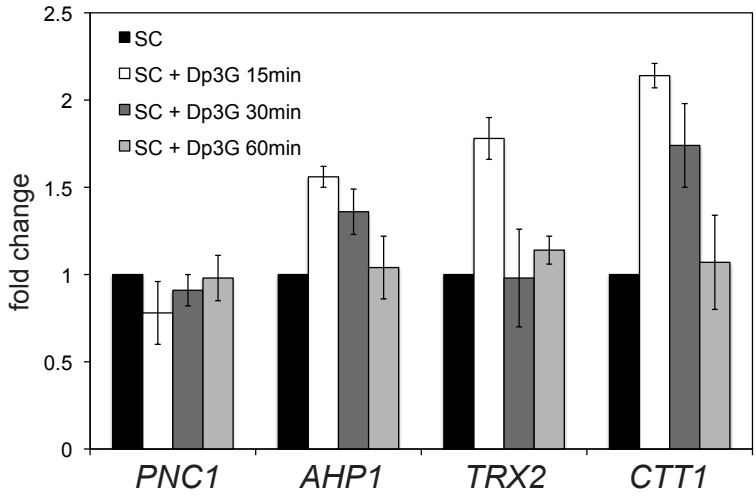

Figure 7, Jiménez et al., 2010