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The Hog1 Mitogen-Activated Protein Kinase Mediates a Hypoxic Response in *Saccharomyces cerevisiae*

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ABSTRACT

We have studied hypoxic induction of transcription by studying the seripauperin (*PAU*) genes of *Saccharomyces cerevisiae*. Previous studies showed that *PAU* induction requires the depletion of heme and is dependent upon the transcription factor Upc2. We have now identified additional factors required for *PAU* induction during hypoxia, including Hog1, a mitogen-activated protein kinase (MAPK) whose signaling pathway originates at the membrane. Our results have led to a model in which heme and ergosterol depletion alters membrane fluidity, thereby activating Hog1 for hypoxic induction. Hypoxic activation of Hog1 is distinct from its previously characterized response to osmotic stress, as the two conditions cause different transcriptional consequences. Furthermore, Hog1-dependent hypoxic activation is independent of the *S. cerevisiae* general stress response. In addition to Hog1, specific components of the SAGA coactivator complex, including Spt20 and Sgf73, are also required for *PAU* induction. Interestingly, the mammalian ortholog of Spt20, p38IP, has been previously shown to interact with the mammalian ortholog of Hog1, p38. Taken together, our results have uncovered a previously unknown hypoxic-response pathway that may be conserved throughout eukaryotes.

CHANGES in the environmental level of molecular oxygen can have profound effects on the growth of most organisms. Oxygen is required as an electron receptor in aerobic respiration, as well as for the biosynthesis of sterols, unsaturated fatty acids (UFAs), and heme, all of which are essential cellular components (Rosenfeld and Beauvoit 2003). In contrast, oxygen can also have negative consequences when it is metabolized into reactive oxygen species that can damage cellular components (Jamieson 1998). To adapt to altered levels of oxygen in the environment, most organisms, from bacteria to humans, respond to changes in oxygen levels by extensive changes in transcription (Bunn and Poyton 1996).

Several distinct mechanisms govern how cells respond to low levels of oxygen (hypoxia) in both metazoans and microorganisms, and four have been well described. First, in metazoans, many genes are induced during hypoxia by the transcription factor, hypoxia-inducible factor (HIF) (Kaelin 2005). In the presence of oxygen, HIF is inhibited by an oxygen-dependent hydroxylation that targets HIF for degradation; however, when oxygen levels are low, this hydroxylation cannot occur and HIF accumulates to activate transcription. Second, in Rhizobia and other bacterial species, the FixJ transcription factor promotes transcription during hypoxia (Rodgers 1999; Delgado-Nixon *et al.* 2000). Oxygen, when present, binds to a heme molecule attached to FixL, a histidine kinase, thereby preventing it from phosphorylating and activating FixJ. Third, in the yeast *Schizosaccharomyces pombe*, the transcription factor, sterol regulatory element-binding protein (SREBP) is activated during hypoxia (Hughes *et al.* 2005). This activation is caused by a decreased level of sterols, whose biosynthesis is oxygen dependent. Like its human ortholog, SREBP is tethered to a cellular membrane and is released to the nucleus when sterol levels are low (Brown and Goldstein 1997; Delgado-Nixon *et al.* 2000). While there is no SREBP ortholog in *Saccharomyces cerevisiae*, the Upc2 transcription factor is considered a functional homolog (Vik and Rine 2001) and is one focus of this work. Finally, *S. cerevisiae* contains an oxygen-responsive transcription factor, Hap1, that is described below.

In *S. cerevisiae*, ~400 genes respond to changes in oxygen levels. Several studies have shown that Hap1 plays a prominent role in this regulation, under both hypoxic and aerobic conditions (Zitomer and Lowry 1992; Ter Linde *et al.* 1999; Becerra *et al.* 2002; Kwast *et al.* 2002; Lai *et al.* 2005, 2006; Hickman and Winston 2007). Hap1 directly regulates many aerobic genes through activation in the presence of oxygen and repression in hypoxia (Zitomer and Lowry 1992; Hickman and Winston 2007). This switch between repression and activation is

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regulated through the binding of heme, which, like sterols, requires oxygen for its biosynthesis and is thus absent from hypoxic cells (Hon *et al.* 2003; Hickman and Winston 2007). Hap1 also regulates several hypoxic genes and does so indirectly through aerobic induction of *MOT3* and *ROX1*, which encode transcriptional repressors of these hypoxic genes (Sertil *et al.* 2003; Lai *et al.* 2006). However, there is likely at least one other oxygen-sensing pathway in *S. cerevisiae* since many genes respond to oxygen levels in the absence of Hap1 (Becerra *et al.* 2002; Hickman and Winston 2007).

In this work, we have studied hypoxic induction in *S. cerevisiae* by analysis of the hypoxia-induced seripauperin (*PAU*) genes. The *PAU* genes comprise a family of 24 genes that encode proteins related to fungal cell wall proteins and are thought to be important in remodeling the cell wall during certain types of stress, including hypoxic stress (Viswanathan *et al.* 1994; Rachidi *et al.* 2000; Abramova *et al.* 2001; Ai *et al.* 2002; Luo and van Vuuren 2009). Most *PAU* genes are subtelomeric and previous studies have shown that they are subject to regulation both by the Sir complex and by stresses that phosphorylate Sir3 (Ai *et al.* 2002; Radman-Livaja *et al.* 2011). With respect to hypoxic induction, the focus of our studies, one study has shown that adding heme to hypoxic cells prevents *PAU* induction (Rachidi *et al.* 2000), suggesting that the *PAU* genes are regulated through the known heme sensor, Hap1. However, the finding that *PAU* induction is Rox1 independent (Rachidi *et al.* 2000) and data from our previous microarray experiments (Hickman and Winston 2007) suggested that the hypoxic induction of *PAU* genes is Hap1 independent, and we confirm that in this study. Other work has shown that the *PAU* genes are induced by the transcription factor, Upc2, known to activate many other hypoxic genes through an unknown mechanism (Abramova *et al.* 2001; Kwast *et al.* 2002; Wilcox *et al.* 2002; Lai *et al.* 2006; Luo and van Vuuren 2009).

Here, we have identified additional factors and conditions required for the Upc2-dependent hypoxic induction of *PAU* genes, including the depletion of both sterols and heme, and the Hog1 mitogen-activated protein kinase (MAPK) pathway. Microarray analysis shows that, in addition to *PAU* genes, several other genes involved in maintaining the cell membrane or cell wall are induced by this pathway, demonstrating that the Hog1 pathway contributes to the maintenance of cell integrity during hypoxic growth. Extensive work has shown that Hog1 is required for the response to osmotic stress (Hohmann *et al.* 2007; de Nadal and Posas 2010); our results are the first demonstration that Hog1 is required as part of the response to hypoxic growth. Finally, we show that the SAGA transcriptional coactivator complex is also required for the expression of *PAU* genes. Our data, taken together, suggest the existence of a hypoxic-response regulatory system that may be conserved throughout eukaryotes.

MATERIALS AND METHODS

Yeast strains: All *S. cerevisiae* strains are listed in Table 1 and are isogenic with a *GAL2*$^+$ derivative of S288C containing a repaired *HAP1* allele (Winston *et al.* 1995; Hickman and Winston 2007). Strains were constructed by standard methods, either by crosses or by transformation (Ausubel *et al.* 1991). The deletion alleles were created by replacing the respective ORF with the *KanMX, URA3, LEU2, HIS3* (Brachmann *et al.* 1998), or *NatMX* marker (Goldstein and McCusker 1999). All primers used for strain construction are listed in supporting information, Table S1. Strains containing the *hem1Δ::KanMX* allele were grown on 200 μg/ml δ-ala, except where indicated. The *KanMX::GAL1*$_{pr}$*-ERG25* allele was constructed by placing the *KanMX* marker and the *GAL1* promoter upstream of the *ERG25* ORF (Longtine *et al.* 1998); strains containing this allele of the essential *ERG25* gene were maintained on YP galactose medium, except where indicated. The *HOG1-13X-myc::KanMX* allele was created by inserting 13 copies of the myc epitope tag at the C-terminal end while adding a *KanMX* marker (Longtine *et al.* 1998); this allele did not affect hypoxic *PAU* induction (data not shown).

Media and growth conditions: Culture and hypoxic methods were as described (Hickman and Winston 2007), except where noted below. For all experiments, cells were grown at 30° in YP (1% yeast extract and 2% peptone) supplemented with 2% glucose or, where indicated, 2% galactose. For RNA and protein analyses, overnight saturated cultures were diluted and grown aerobically for approximately four generations to mid-log ($1–2 \times 10^7$ cells/ml). For hypoxic growth, these aerobic mid-log cultures were diluted appropriately and grown again in 250-ml flasks continuously sparged with ultra-high-purity nitrogen gas at ~3 liters/min. For analysis of mRNA levels or proteins during hypoxic growth, cells were grown for 5 hr after the shift to nitrogen unless otherwise noted. Cells were chilled on ice for 5 min before removing from nitrogen gas. Heme, in the form of hemin (BioChemika), was made up at 50 mg/ml in 1:1 (ethanol):(water with 0.1 m NaOH). Ergosterol (>95%) (Sigma) was made up at 2 mg/ml in 1:1 Tween 80:ethanol. δ-Aminolevulinate (δ-ala; Sigma) was dissolved in water at 20 mg/ml and added to the media at the indicated concentration. Importantly, except where indicated, ergosterol was not included in the media for our hypoxic experiments because it has an effect on expression of some genes, as described elsewhere in this report. For the short time points used, there was no decrease in cell viability even though the ergosterol solution is essential for long-term growth in hypoxia. The appropriate amount of the stock solution was added to cells to achieve the desired final concentration, and an equal volume of the solvent was used as a solvent-only control. For the heme and ergosterol depletion experiments in Figure 1, cells were grown for 12 hr.

Expression microarray, Northern blot analysis, and real-time PCR: The mRNA expression of all yeast genes was analyzed by hybridization to an Agilent yeast array as described (Brauer *et al.* 2008). The 24 *PAU* genes are >95% identical in nucleotide sequence (Luo and van Vuuren 2009), and hence probes designed for one *PAU* gene will cross-hybridize with all the other genes. Thus, we averaged all the expression values for *PAU* genes into one value that represents the average *PAU* expression. The microarray data (Table S2) have been deposited in the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) database and are accessible through GEO series accession no. GSE26593.

Northern blot analysis was performed as described (Ausubel *et al.* 1991), using PCR-amplified and random-primed ^{32}P-

TABLE 1

***Saccharomyces cerevisiae* strains**

Strain	Genotype	Reference
FY2609	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1*	Hickman and Winston (2007)
FY2637	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 hem1Δ0::KanMX*	Hickman and Winston (2007)
FY2611	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ hap1Δ0::KanMX*	Hickman and Winston (2007)
FY2867	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 hap2Δ0::LEU2*	This study
FY2868	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ hap1Δ0::KanMX hap2Δ0::LEU2*	This study
FY2869	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 upc2Δ0::HIS3*	This study
FY2870	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 KanMX-*$GAL1_{pr}$*-ERG25*	This study
FY2871	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 KanMX-*$GAL1_{pr}$*-ERG25 upc2Δ0::HIS3*	This study
FY2872	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 hem1Δ0::KanMX upc2Δ0::HIS3*	This study
FY2873	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 hog1Δ0::LEU2*	This study
FY2874	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 HOG1-13X-myc::KanMX*	This study
FY2875	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 ste11Δ0::KanMX*	This study
FY2876	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 hog1Δ0::LEU2 ste11Δ0::KanMX*	This study
FY2877	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 pbs2Δ0::HIS3*	This study
FY2878	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 ssk1Δ1::HIS3*	This study
FY2879	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 ssk1Δ1::HIS3 ste11Δ0::KanMX*	This study
FY2880	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 mga2Δ0::NatMX*	This study
FY2881	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 spt23Δ0::URA3*	This study
FY2882	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 mga2Δ0::NatMX spt23Δ0::URA3*	This study
FY2883	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 spt20Δ0::URA3*	This study
FY2884	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 gcn5Δ::URA3*	Roberts and Winston (1997) and this study
FY2885	*MAT***a** *ura3-52 HAP1 spt3Δ-202*	Winston and Minehart (1986) and this study
FY2886	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 sgf73Δ0::KanMX*	Martens *et al.* (2005) and this study
FY2887	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 sus1Δ::HIS3*	This study
FY2888	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 sgf11Δ::KanMX*	This study
FY2889	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 ubp8Δ::URA3*	This study
FY2890	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 sus1Δ::HIS3 sgf11Δ::KanMX ubp8Δ::URA3*	This study
FY2891	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 sgf73Δ::KanMX sus1Δ::HIS3 sgf11Δ::KanMX ubp8Δ::URA3*	This study
FY2892	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 nsg1Δ::HIS3*	This study
FY2893	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 nsg2Δ::LEU2*	This study
FY2894	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 nsg1Δ::HIS3 nsg2Δ::LEU2*	This study
FY2895	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 msn2Δ::URA3*	This study
FY2896	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 msn4Δ::LEU2*	This study
FY2897	*MAT***a** *ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 msn2Δ::URA3 msn4Δ::LEU2*	This study
FY2898	*MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-128δ HAP1 PAU5-URA3::NatMX can1Δ::*$MFA1_{pr}$*-HIS3*	Tong *et al.* (2001) and this study

labeled probes. The primers for PCR are listed in Table S1. The Northern probe for *PAU* genes also detected a weak band and we showed that it was the homologous *DAN1* gene by Northern analysis of a *dan1Δ* strain (data not shown). For all panels in all figures, a representative of at least three independent experiments is shown. *SNR190* encodes a small nucleolar RNA and serves as a loading control.

For real-time PCR, RNA was isolated from cells as described above and then reverse transcribed using a poly(T) primer. The resulting cDNA was quantitated on the Stratagene (La Jolla, CA) MX3000P. Primers used are listed in Table S1. Shown are the mean and standard deviation for at least three independent experiments. Detection of *UPC2* mRNA by Northern analysis was hampered because the *UPC2* mRNA overlaps with a ribosomal band. Thus, real-time PCR was used to quantify *UPC2* mRNA levels.

Western analysis: Whole-cell extracts were prepared by bead lysis. Protein concentrations were measured by Bradford assay and equal amounts of extract were separated by SDS–PAGE and transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membrane was incubated overnight at 4° with primary antibodies to myc (1:1000; clone 9E10; BD Pharmingen no. 51-1485GR), total Hog1 (1:1000; polyclonal yC-20; Santa Cruz no. sc-6815), phospho-Hog1 (1:1000; clone 3D7; Cell Signaling no. 9215S), and Mbp1 [1:5000; polyclonal; New England BioLabs (Beverly, MA) no. E8030S]. The membrane was then incubated for 1 hr at room temperature with HRP-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories) and treated with Lumigen HRP substrate (GE Healthcare).

Deletion set screen for mutants unable to induce *PAU* genes: To screen for mutants with altered regulation of *PAU*

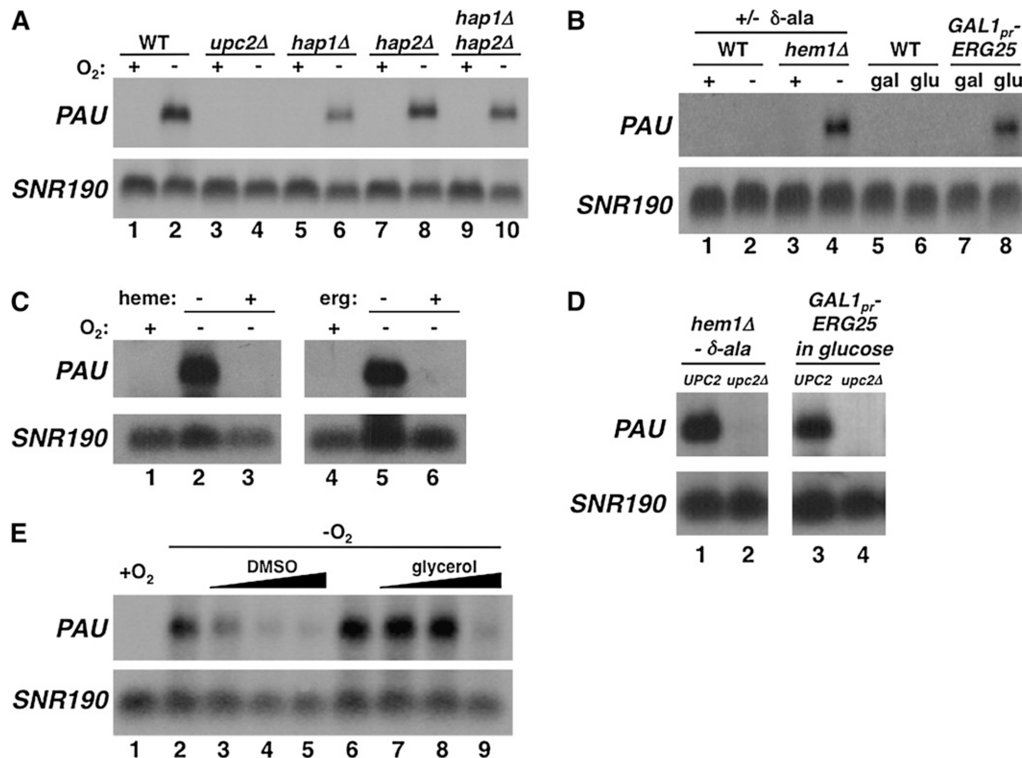


Figure 1.—Factors and conditions that control *PAU* induction. *PAU* mRNA levels were measured by Northern analysis, with *SNR190* serving as the loading control. The experiments were done as described in materials and methods. (A) Upc2 is required for *PAU* induction. Wild-type (FY2609), *upc2*Δ (FY2869), *hap1*Δ (FY2611), *hap2*Δ (FY2867), and *hap1*Δ *hap2*Δ (FY2868) strains were grown in the presence (+) or absence (−) of oxygen for 5 hr. (B) Depletion of heme or ergosterol during aerobic growth leads to *PAU* induction. To test the effect of heme depletion, wild-type (lanes 1 and 2, FY2609) and *hem1*Δ (lanes 3 and 4, FY2637) strains were grown with or without 280 μg/ml δ-ala for 12 hr and *PAU* mRNA levels were measured. To test the effect of ergosterol depletion, wild-type (lanes 5 and 6, FY2609) and *GAL1$_{pr}$-ERG25* (lanes 7 and 8, FY2870) strains were grown for 12 hr with 2% galactose or 2% glucose and *PAU* mRNA levels were measured. (C) Addition of heme or ergosterol represses the hypoxic induction of *PAU* genes. A wild-type (FY2609) strain was grown aerobically (lanes 1 and 4), for 4 hr hypoxically (lanes 2 and 3), or for 8 hr hypoxically (lanes 5 and 6). Heme (at 500 μg/ml, lane 3) or ergosterol (at 20 μg/ml, lane 6) was added to the media 40 min before shifting to hypoxia. For the −heme (lane 2) and −erg (lane 5) controls, an equal volume of the solvent was added as described in materials and methods. (D) Upc2 is required for *PAU* induction during heme or ergosterol depletion. To test whether Upc2 is required during heme depletion, *hem1*Δ (FY2637, lane 1) and *hem1*Δ *upc2*Δ (FY2872, lane 2) strains were grown in the absence of δ-ala for 12 hr and *PAU* mRNA levels were measured. To test whether Upc2 is required during ergosterol depletion, *GAL1$_{pr}$-ERG25* (FY2870, lane 3) and *GAL1$_{pr}$-ERG25* upc2Δ (FY2871, lane 4) were each grown in glucose for 12 hr and *PAU* mRNA levels were measured. (E) Manipulating membrane fluidity with DMSO or glycerol affects hypoxic *PAU* induction. Northern analysis is shown of wild-type cells (FY2609) grown in the presence (+O_2) or absence (−O_2) of oxygen for 4 hr. DMSO (5%, 10%, or 15% v/v; lanes 3–5) or glycerol (5%, 10%, or 15% v/v; lanes 7–9) was added to the medium immediately before shifting cells to hypoxic growth.

genes, we constructed a strain in which the *PAU5* ORF was replaced with that of *URA3*, and a NatMX cassette was integrated 152 nucleotides downstream of the *PAU5* stop codon to provide a closely linked selectable marker. This strain has a Ura$^-$ phenotype during aerobic growth and a Ura$^+$ phenotype during hypoxic growth; it also showed the expected *URA3* expression by Northern analysis (data not shown). The strain also contains the *can1*Δ*::MFA1$_{pr}$-HIS3* marker, to allow a screen of the *S. cerevisiae* deletion set (Tong *et al.* 2001). After crossing by the deletion set on YPD plates, diploids were selected on YPD plates containing the drugs nourseothricin (to select for the NatMX marker) and G418 (to select for the KanMX marker). The diploids were sporulated and the *MAT***a** haploids were selected on synthetic complete media lacking histidine and arginine and containing the drug canavanine. The haploids were then tested for hypoxic growth in a BBL GasPak chamber (Becton, Dickinson) on the same media containing nourseothricin, G418, and 5-fluoroorotic acid (5FOA). Under these conditions, most strains exhibit very weak growth on 5FOA due to the hypoxic expression of *PAU5*. Those strains that grew better were considered to be candidates for defective *PAU5* expression and were further tested by making fresh deletions and testing *PAU* expression by Northern analysis.

RESULTS

***PAU* genes are strongly regulated by Upc2 but not by Hap1 or Hap2:** Previous studies have demonstrated that *PAU* genes are tightly regulated by oxygen levels. During aerobic growth, *PAU* transcription is undetectable; however, upon a shift to hypoxic conditions, *PAU* transcription is strongly induced (Rachidi *et al.* 2000; Abramova *et al.* 2001). Our previous microarray experiments (Hickman and Winston 2007) suggested that hypoxic induction of *PAU* genes can occur independently of Hap1 and other studies have shown that the induction is dependent upon Upc2 (Abramova *et al.* 2001; Kwast *et al.* 2002). To test these results in our strains, we performed Northern analysis, also testing the requirement for Hap2, another heme-responsive transcription factor that regulates many of the same genes as Hap1 (Pinkham and Guarente 1985; Lai *et al.* 2006). Our results show a modest requirement for Hap1 and no requirement for Hap2 for *PAU* activation

during hypoxic growth (Figure 1A). The modest requirement for Hap1 is likely due to its requirement as a repressor of heme and ergosterol biosynthetic genes under hypoxic conditions (Hickman and Winston 2007 and see below). Further, Hap1 and Hap2 play no role in the aerobic repression of *PAU* genes (Figure 1A). In contrast to Hap1 and Hap2 independence, our results show a strong dependence upon Upc2 for the hypoxic induction of *PAU* (Figure 1A). This induction is likely direct, as there is a Upc2 DNA-binding site found in almost all *PAU* gene promoters (Cohen *et al.* 2001; Kwast *et al.* 2002; Luo and van Vuuren 2009). Thus, hypoxic induction of *PAU* expression is controlled by a Upc2-dependent pathway that is independent of Hap1 and Hap2.

***PAU* transcription is regulated by heme and ergosterol levels:** A previous study suggested that aerobic repression of *PAU* transcription requires heme (Rachidi *et al.* 2000). As heme is required for ergosterol biosynthesis in *S. cerevisiae* (Gollub *et al.* 1977; Lorenz and Parks 1991), we tested the roles of both heme and ergosterol in *PAU* regulation. First, we depleted either heme or ergosterol during aerobic growth to test the effect on aerobic repression of *PAU* transcription. To deplete heme during aerobic growth, we used a strain with a deletion of *HEM1*, required for the production of δ-ala in the first committed step of heme biosynthesis (Gollub *et al.* 1977). When *hem1*Δ cells were grown without δ-ala (and thus could not produce heme), the *PAU* genes were induced (Figure 1B). To deplete cells of ergosterol, we constructed a strain containing the essential *ERG25* gene, required for ergosterol biosynthesis, under the control of the *GAL1* promoter and shifted it from galactose, which induces the gene, to glucose, which represses the gene. Similar to heme depletion, when *ERG25* is repressed, *PAU* transcription is induced (Figure 1B). Thus, depletion of either heme or ergosterol during aerobic growth abolishes repression of *PAU* transcription. As heme is required for ergosterol biosynthesis (Gollub *et al.* 1977; Lorenz and Parks 1991), heme depletion might induce *PAU* genes because it leads to ergosterol depletion. To address this possibility, we added heme to ergosterol-depleted cells during aerobic growth and found that the addition of heme blocked *PAU* induction (Figure S1A). Similarly, adding ergosterol to heme-depleted cells dramatically reduces *PAU* induction (Figure S1B). These results suggest that heme and ergosterol each are required to regulate *PAU* transcription. As the depletion experiments suggest that decreased levels of heme and ergosterol are necessary during hypoxic growth for *PAU* induction, we added exogenous heme or ergosterol to hypoxic cells. In both cases, we found that they repressed *PAU* induction (Figure 1C). Finally, to determine the relationship of heme or ergosterol depletion to activation by Upc2, we repeated each depletion in a *upc2*Δ mutant background. Our results show that neither heme nor ergosterol depletion leads to *PAU* induction in a *upc2*Δ mutant (Figure 1D), suggesting that Upc2 activation occurs downstream of the signal from heme or ergosterol depletion. Taken together, these results show that heme and ergosterol levels each play critical roles downstream of oxygen levels and upstream of Upc2 in mediating the regulation of *PAU*.

Membrane fluidity, but not sterol sensing, is important for *PAU* regulation: Our finding that ergosterol depletion mimics hypoxic induction of *PAU* genes suggested that *S. cerevisiae* might employ a sterol-sensing system, similar to what has been described in *S. pombe* and mammals (Yang *et al.* 2002; Hughes *et al.* 2005). However, the results described in the previous section suggest that this is not the case as heme itself plays a role. Second, we did not find a significant role for putative sterol sensors in hypoxic *PAU* induction. While *S. cerevisiae* does not have an SREBP ortholog, it does contain two orthologs of the *S. pombe* and human INSIG protein, required for sterol-sensing and SREBP regulation (Yang *et al.* 2002; Hughes *et al.* 2005). We found that neither of the *S. cerevisiae* orthologs, Nsg1 and Nsg2 (Flury *et al.* 2005), plays a significant role in *PAU* regulation (Figure S2). In addition, Ncr1, predicted to be a sterol sensor (Malathi *et al.* 2004), is not required for *PAU* regulation (data not shown). These results suggest that direct sterol sensing, as occurs in *S. pombe* and humans, does not play a role in *PAU* induction in *S. cerevisiae.*

On the basis of these results, we speculated that the *PAU* genes may be induced in response to a more general effect on membranes, as both heme and sterols are known to decrease membrane fluidity (Lees *et al.* 1979; Shviro *et al.* 1982; Ginsburg and Demel 1984; Schmitt *et al.* 1993; Berg *et al.* 2002; Abe *et al.* 2009). To test directly whether changing membrane fluidity contributes to *PAU* induction, we employed dimethyl sulfoxide (DMSO) and glycerol, both of which are known to decrease membrane fluidity (Surewicz 1984; Lewis *et al.* 1994; Gurtovenko and Anwar 2007). Indeed, we found that increased levels of DMSO or glycerol repressed *PAU* transcription during hypoxia (Figure 1E) or sterol depletion (data not shown). This result suggests that during the hypoxic response, the increased membrane fluidity that occurs as a consequence of decreased heme and ergosterol levels is required for *PAU* induction.

The Hog1 MAP kinase is activated by hypoxia and is required for *PAU* induction: The Hog1 MAP kinase is important for the response to osmotic stress, as well as other stresses that may affect membranes (Hohmann *et al.* 2007). We wondered whether Hog1 is also involved in the response to hypoxia, given our evidence that membrane changes are a key aspect of *PAU* induction. Indeed, we found that Hog1 plays a major role in the hypoxic induction of *PAU* expression, as a *hog1*Δ

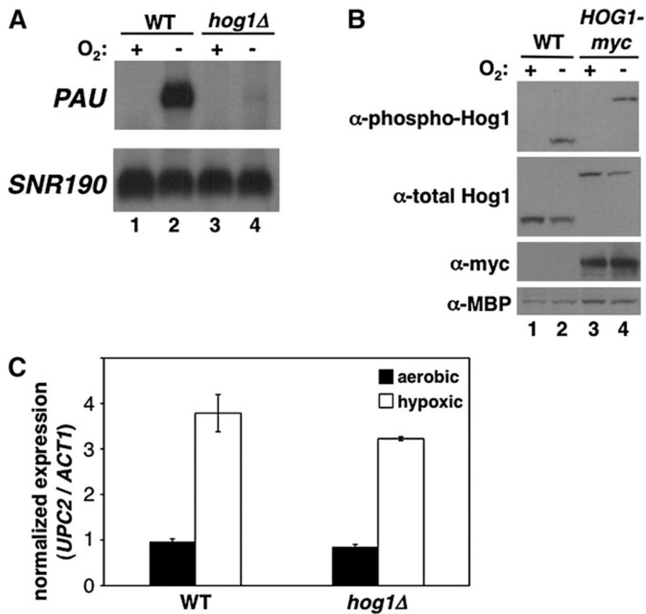


Figure 2.—The role of the Hog1 MAP kinase in *PAU* and *UPC2* induction. (A) *PAU* hypoxic induction is impaired in a *hog1*Δ mutant. *PAU* expression was monitored by Northern blot analysis of wild-type (FY2609) or *hog1*Δ (FY2873) cells grown in the presence (+) or absence (−) of O_2 for 5 hr. (B) Hog1 becomes phosphorylated after a shift to hypoxic growth. The expression and phosphorylation of Hog1 protein were determined by Western blot analysis of wild-type (FY2609) or *HOG1-myc* (FY2874) cells grown in the presence (+) or absence (−) of O_2 for 5 hr, using the indicated antibodies. (C) *UPC2* mRNA levels are not affected by *hog1*Δ. *UPC2* mRNA levels were monitored by real-time PCR in wild-type (FY2609) or *hog1*Δ (FY2873) cells in the presence (aerobic) or absence (hypoxic) of oxygen for 5 hr.

mutant exhibited little induction compared to wild type (Figure 2A). Hog1 is activated in response to osmotic stress by phosphorylation of two threonine residues (Brewster *et al.* 1993; Saito and Tatebayashi 2004). Using a phospho-specific antisera and Western blot analysis, we found that Hog1 is also phosphorylated under hypoxic conditions (Figure 2B). We verified the identity of Hog1 in our Western blots by showing that a myc epitope tag added to Hog1 caused a band shift to a higher molecular weight (Figure 2B).

The *UPC2* gene is transcriptionally induced under hypoxia (Abramova *et al.* 2001) and we wanted to test whether this induction is Hog1 dependent. To do this, we measured *UPC2* mRNA levels by real-time PCR. While we did confirm that *UPC2* is induced by hypoxia, we found that Hog1 is not required for the induction (Figure 2C). Thus, Hog1 does not induce the *PAU* genes through regulation of *UPC2* mRNA levels.

Other members of the Hog1 MAPK pathway are required for the hypoxic response: The Hog1 pathway contains two independent branches upstream of Hog1, both of which are used during osmotic stress (Saito and Tatebayashi 2004; de Nadal and Posas 2010). One pathway uses two redundant MAPKKKs, Ssk2 and Ssk22, while the other pathway uses a different MAPKKK, Ste11. Both of these pathways activate the MAPKK, Pbs2, which then phosphorylates Hog1 (Brewster *et al.* 1993; Saito and Tatebayashi 2004), although some evidence suggests that the Ssk2/Ssk22 pathway plays a more prominent role (O'Rourke and Herskowitz 2004). To determine whether either of these pathways is required during the hypoxic response, we measured *PAU* induction in mutants that are defective for each pathway. To test the Ssk2/Ssk22 pathway, we used an *ssk1*Δ mutant. Ssk1 is an upstream kinase that phosphorylates and activates both Ssk2 and Ssk22; an *ssk1*Δ allele thus abrogates activation of both of these MAPKKKs (Maeda *et al.* 1994). Our results show that an *ssk1*Δ mutant exhibited the same low level of *PAU* induction as a *hog1*Δ mutant (Figure 3A), suggesting that this pathway is activated during hypoxia. In contrast, a *ste11*Δ mutation did not have any effect on hypoxic *PAU* induction (Figure 3A and Figure S3), suggesting that this pathway does not play a role in this hypoxic response. As Ste11 is also the MAPKKK for the filamentous/invasive growth and pheromone response pathways (Harris *et al.* 2001), our results suggest that these other pathways that signal from the cell membrane do not participate in hypoxic *PAU* induction. In addition, an *ssk1*Δ *ste11*Δ double mutant had the same defect as an *ssk1*Δ single mutant, again suggesting that Ste11 plays no role in the hypoxic activation of Hog1 (Figure 3A). As expected, a *pbs2*Δ mutant had the same defect in *PAU* induction as a *hog1*Δ strain (Figure 3B). These data show that the Ssk2/Ssk22 branch of the Hog1 pathway, but not the Ste11 branch, is required for *PAU* induction.

The Hog1-mediated hypoxic response does not require the general stress response factors Msn2 and Msn4: Previous studies have suggested that the Hog1 pathway interacts with the general stress response pathway (Martinez-Pastor *et al.* 1996; Gasch *et al.* 2000; Rep *et al.* 2000; Capaldi *et al.* 2008). The general stress response pathway is activated by a number of environmental changes, including osmotic shock, oxidative stress, heat shock, and nutritional starvation (Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996; Gorner *et al.* 1998). Upon any one of these events, the transcription factors Msn2/Msn4 are activated and induce a transcriptional response. We tested whether Msn2 and Msn4 are required for the hypoxic induction of *PAU* transcription and found that in *msn2*Δ, *msn4*Δ, and *msn2*Δ *msn4*Δ mutants the hypoxic induction of *PAU* transcription was normal (Figure 3C). Thus, the Hog1-mediated hypoxic response is independent of the general stress response.

The ER-membrane protein, Mga2, is required for *PAU* induction: The transcription factor Mga2 has been previously shown to be required for the hypoxic induction of the *OLE1* gene (Zhang *et al.* 1999; Jiang *et al.* 2001, 2002). Additional experiments have shown

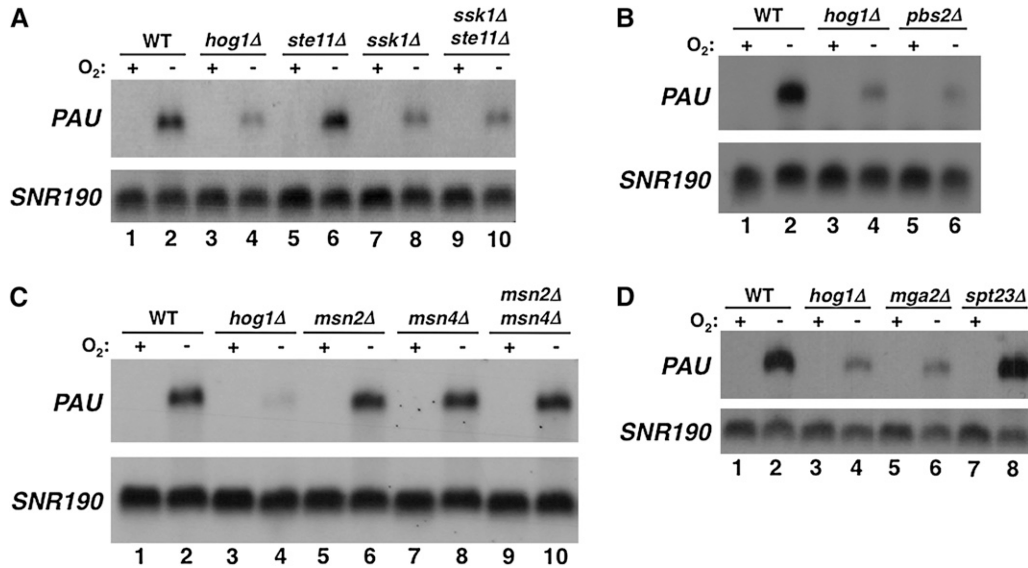


FIGURE 3.—Role of the Hog1 MAPK cascade in *PAU* induction. (A) *PAU* hypoxic induction requires Ssk1 but not Ste11. Northern analysis is shown of *PAU* expression in wild-type (FY2609), *hog1*Δ (FY2873), *ste11*Δ (FY2875), *ssk1*Δ (FY2878), and *ssk1*Δ *ste11*Δ (FY2879) strains grown in the presence (+) or absence (−) of O_2 for 5 hr. (B) *PAU* hypoxic induction requires Pbs2. Northern analysis is shown of *PAU* expression in wild-type (FY2609), *hog1*Δ (FY2873), and *pbs2*Δ (FY2877) strains grown in the presence (+) or absence (−) of O_2 for 5 hr. (C) *PAU* hypoxic induction does not require Msn2 or Msn4. Northern analysis is shown of wild-type (FY2609), *hog1*Δ (FY2873), *msn2*Δ (FY2895), *msn4*Δ (FY2896), and *msn2*Δ *msn4*Δ (FY2897) strains grown in the presence (+) or absence (−) of O_2 for 5 hr. (D) *PAU* hypoxic induction requires Mga2 but not Spt23. Northern analysis is shown of wild-type (FY2609), *hog1*Δ (FY2873), *mga2*Δ (FY2880), and *spt23*Δ (FY2881) strains grown in the presence (+) or absence (−) of O_2 for 5 hr.

that Mga2 is normally associated with the endoplasmic reticulum, but can be activated by ubiquitin-dependent proteolysis, under a set of conditions that includes low levels of unsaturated fatty acids (UFAs) and hypoxia (Hoppe *et al.* 2000; Jiang *et al.* 2002). UFA biosynthesis, like that of heme and sterols, is dependent on oxygen and is therefore impaired in hypoxic cells. We wanted to test whether the Mga2 pathway plays a role in hypoxic induction of *PAU* genes. Indeed, we have found that in an *mga2*Δ mutant, *PAU* induction is defective, equivalent to the defect in a *hog1*Δ mutant (Figure 3D). As *MGA2* is partially redundant with the *SPT23* gene, we also tested an *spt23*Δ mutant, but found that it did not impair *PAU* induction (Figure 3D). These results are similar to induction of *OLE1*, which requires Mga2 but not Spt23 (Jiang *et al.* 2001).

Hog1 distinguishes between the hypoxic and osmotic stress signals: The demonstration that Hog1 is required for a normal hypoxic response and that this pathway requires Pbs2 and Ssk1 raised the question of whether the hypoxic pathway is distinct from that for osmotic stress, the pathway best characterized for Hog1. To examine this, we assayed the regulation of *GRE2*, a gene that responds to osmotic stress (Garay-Arroyo and Covarrubias 1999), and *PAU* genes, which respond to hypoxic conditions. Our results show that each response is specific, as *GRE2* is induced only upon osmotic stress, and *PAU* genes are induced only upon hypoxic induction, all occurring in a Hog1-dependent manner (Figure 4A).

We then compared the kinetics of Hog1 phosphorylation during osmotic stress and hypoxic induction. We found that osmotic stress causes a dramatic peak of Hog1 phosphorylation within minutes of treatment (Figure 4B, lanes 1–5), as previously reported (Maeda *et al.* 1995). We also found a second peak of Hog1 that reproducibly appeared 2 hr after treatment (Figure 4B). In contrast, hypoxia led to a gradual increase in Hog1 phosphorylation, reaching a peak at 5 hr (Figure

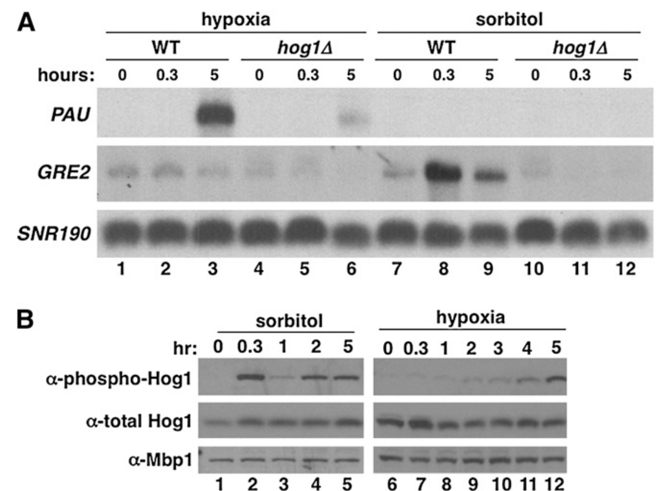
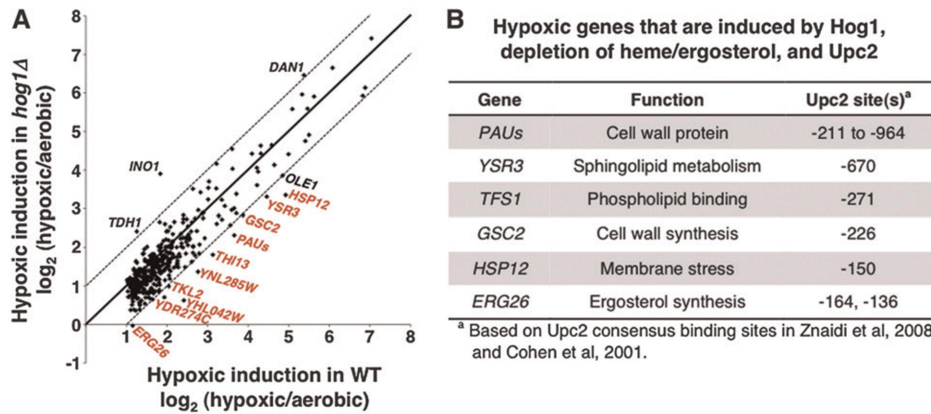


FIGURE 4.—Hog1 distinguishes between the hypoxic and osmotic stress signals. (A) Expression of *PAU* mRNA during hypoxic growth and during osmotic stress. Northern analysis is shown of *PAU* and *GRE2* mRNA levels in wild-type (FY2609) and *hog1*Δ (FY2873) strains. Cells were grown for the indicated times in hypoxia (lanes 1–6) or 1 M sorbitol (lanes 7–12). (B) Hog1 is activated with different kinetics under osmotic stress and during hypoxic induction. Western analysis is shown of Hog1 phosphorylation in a wild-type (FY2609) strain grown in 1 M sorbitol (lanes 1–5) or hypoxia (lanes 6–12) for the indicated times.



Hypoxic genes that are induced by Hog1, depletion of heme/ergosterol, and Upc2

Gene	Function	Upc2 site(s)[a]
PAUs	Cell wall protein	-211 to -964
YSR3	Sphingolipid metabolism	-670
TFS1	Phospholipid binding	-271
GSC2	Cell wall synthesis	-226
HSP12	Membrane stress	-150
ERG26	Ergosterol synthesis	-164, -136

[a] Based on Upc2 consensus binding sites in Znaidi et al, 2008 and Cohen et al, 2001.

Figure 5.—Several hypoxic genes are dependent upon Hog1. (A) Expression microarray analysis showing hypoxic induction of genes in wild-type (FY2609, *x*-axis) and *hog1*Δ (FY2873, *y*-axis) strains. Only the genes hypoxically induced more than twofold in two of two experiments are shown on the plot. The solid line represents the same induction in wild-type and *hog1*Δ strains, while the dotted lines represent a twofold difference in induction. Genes shown in red have mRNA levels decreased by twofold or more in the *hog1*Δ mutant. The data point labeled "*PAUs*" represents the average expression of all 24 *PAU* genes. (B) Genes in the sterol/heme/Hog1/Upc2 pathway are listed with their function and conserved Upc2 binding site(s). The list of genes includes only those that meet three criteria: (1) regulated by O_2 and Hog1 (Figure 5A), (2) induced by depletion of sterols and depletion of heme (Figure S4), and (3) contain a conserved Upc2 binding site in the promoter, on the basis of the previously determined consensus (TCGTATA or TCGTTYAG) (Cohen *et al.* 2001; Znaidi *et al.* 2008).

4B, lanes 6–12 and data not shown). The kinetics of Hog1 phosphorylation in response to each treatment are consistent with the kinetics of gene induction seen in Figure 4A, strongly supporting the idea that Hog1 responds differently to hypoxia and osmotic stress.

Hog1 plays a role in the induction of several hypoxic genes: As Hog1 regulates the transcription of 50–200 genes in response to osmotic stress (Posas *et al.* 2000; Rep *et al.* 2000), we wanted to determine how many genes are regulated by Hog1 in response to hypoxia. To test this, we used expression microarrays to compare the hypoxic induction of genes in wild-type and *hog1*Δ strains. In addition to *PAU* genes, there were 9 other hypoxic genes that exhibited a more than twofold decrease in mRNA levels in a *hog1*Δ mutant compared to wild type under hypoxic conditions (Figure 5A, gene names in red). There were also three hypoxic genes (*DAN1, INO1*, and *TDH1*) that had increased mRNA levels in a *hog1*Δ mutant under hypoxic conditions; these have not yet been analyzed further.

To test if the nine Hog1-dependent, hypoxia-induced genes that we identified are also regulated by ergosterol and heme levels, we examined a previous data set of gene expression changes upon heme and ergosterol depletion (Mnaimneh *et al.* 2004). Of the nine genes, five were induced in both heme and ergosterol depletion, similar to *PAU* genes (Figure S4). All five of these genes contain at least one Upc2 consensus binding site in the promoter region (TCGTATA or TCGTTYAG) (Cohen *et al.* 2001; Znaidi *et al.* 2008). These five genes, listed in Figure 5B, encode proteins that are involved in either cell membrane (*YSR3, TFS1, HSP12*, and *ERG26*) or cell wall (*PAU* and *GSC2*) functions, and three of them have been previously shown to be hypoxia induced: *PAU* (Rachidi *et al.* 2000; Abramova *et al.* 2001; Hickman and Winston 2007), *GSC2* (Lai *et al.* 2005), and *HSP12* (Becerra *et al.* 2002; Hickman and Winston 2007). Thus, there are several genes regulated by the Hog1-mediated hypoxic response that are likely to be important in maintaining the cell wall and cell membrane when oxygen is not present. Interestingly, part of the osmotic stress response involves decreased expression of some *ERG* genes (Montanes *et al.* 2011), perhaps to maintain proper membrane fluidity during this different form of membrane stress.

The SAGA coactivator complex is required for *PAU* and *UPC2* induction: To identify other regulators of *PAU* expression, we screened the *S. cerevisiae* deletion set for mutants unable to express a *PAU* reporter under hypoxic conditions. This screen identified several components of the SAGA transcriptional coactivator complex, including Ada2, Gcn5, and Sgf29. We then directly tested the requirement for most nonessential SAGA genes by Northern analysis and found that the SAGA mutants with the strongest defects in *PAU* induction occurred in four classes of SAGA genes (Figure 6A and data not shown): those encoding core components (*ADA1, SPT7*, and *SPT20*), those required for histone acetyltransferase activity (*GCN5, ADA2*, and *ADA3*), a component required for TBP recruitment (*SPT3*), and a component required for the assembly of the histone deubiquitination (DUB) module of SAGA (*SGF73*) (see Koutelou *et al.* 2010 for a review).

We were particularly interested in the strong requirement for Sgf73 in *PAU* induction. While Sgf73 is clearly important in humans (Helmlinger *et al.* 2004), only a few studies have identified phenotypes for *sgf73* mutants in *S. cerevisiae* (Shukla *et al.* 2006; Jordan *et al.* 2007; Gresham *et al.* 2008). Sgf73 was recently found to anchor the DUB module of SAGA within the complex (Lee *et al.* 2009; Rodriguez-Navarro 2009; Kohler *et al.* 2010; Samara *et al.* 2010). The DUB module contains three proteins that are required for histone deubiquitination: (1) Ubp8, the deubiquitinase; (2) Sus1, also involved in SAGA-mediated mRNA export; and (3) Sgf11, a possible structural protein. To determine

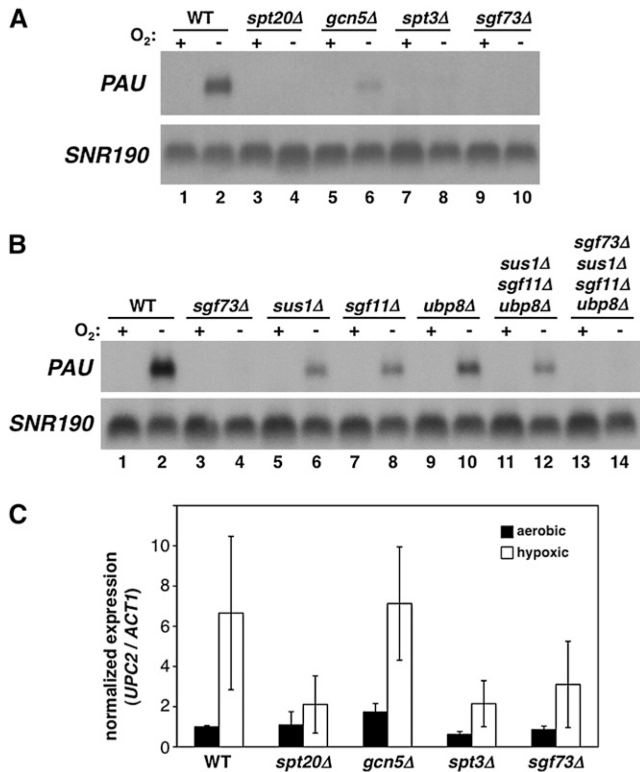


FIGURE 6.—The SAGA coactivator complex is required for *PAU* induction. (A) Northern analysis of *PAU* mRNA levels in wild-type (FY2609), *spt20*Δ (FY2883), *gcn5*Δ (FY2884), *spt3*Δ (FY2885), and *sgf73*Δ (FY2886) strains grown in the presence (+) or absence (−) of O_2 for 5 hr. (B) Northern analysis of *PAU* mRNA levels in wild-type (FY2609), *sgf73*Δ (FY2886), *sus1*Δ (FY2887), *sgf11*Δ (FY2888), *ubp8*Δ (FY2889), *sus1*Δ *sgf11*Δ *ubp8*Δ (FY2890), and *sgf73*Δ *sus1*Δ *sgf11*Δ *ubp8*Δ (FY2891) strains grown in the presence (+) or absence (−) of O_2 for 5 hr. (C) Real-time PCR analysis of *UPC2* mRNA levels in wild-type (FY2609), *spt20*Δ (FY2883), *gcn5*Δ (FY2884), *spt3*Δ (FY2885), and *sgf73*Δ (FY2886) strains grown in the presence (aerobic) or absence (hypoxic) of oxygen for 5 hr. The high standard deviation for some samples is due to an outlier experiment that showed the same relative levels for all of the mutants.

whether Sgf73 plays a role in *PAU* expression via the DUB module, we deleted the genes encoding each of the DUB components and compared their effects, singly and in combination, to *sgf73*Δ's. Our results show that even in a *sus1*Δ *sgf11*Δ *ubp8*Δ triple mutant, there is still significant *PAU* induction, albeit reduced (Figure 6B, lanes 11 and 12). In contrast, in the *sgf73*Δ single mutant, or when *sgf73*Δ is combined with the other three mutations, there is no detectable *PAU* induction. These results show that Sgf73 plays a prominent role in the hypoxic induction of *PAU* genes and does so, at least partly, in a DUB-independent manner.

To further define the role of SAGA in the hypoxic response, we tested whether SAGA is required for *UPC2* transcription. Our results show that most of the SAGA mutants tested that impair the hypoxic expression of *PAU* also impair the hypoxic expression of *UPC2* (Figure 6C). One notable exception is *gcn5*Δ, which significantly reduces *PAU* mRNA levels but not those for *UPC2*. Thus, SAGA may contribute to *PAU* expression in two ways: (1) by regulating transcription of *UPC2* in an Sgf73- and Spt3-dependent fashion and (2) by directly regulating *PAU* expression via Gcn5.

DISCUSSION

In this work, we have identified several factors that control the hypoxic induction of the *PAU* genes of *S. cerevisiae*. Combined with previous results concerning *PAU* regulation (RACHIDI *et al.* 2000; ABRAMOVA *et al.* 2001; KWAST *et al.* 2002), our studies suggest the existence of a previously unknown hypoxic-response regulatory system that is distinct from the well-studied Hap1-dependent pathway and that requires the Hog1 MAPK pathway. Our microarray analysis has shown that Hog1 is required for the hypoxic induction of several genes in addition to the *PAU* genes, showing that it plays a significant role in the *S. cerevisiae* hypoxic response. As the mammalian Hog1 ortholog, p38, is also activated by hypoxia (SEKO *et al.* 1997; JIN *et al.* 2000; KACIMI *et al.* 2000; BLASCHKE *et al.* 2002; KULISZ *et al.* 2002; ZHU *et al.* 2002; EMERLING *et al.* 2005), this role may be conserved throughout eukaryotes.

Our results suggest a regulatory framework for most of the factors now known to be required for the hypoxic induction of *PAU* genes (Figure 7). In this regulatory pathway, the direct consequence of decreased oxygen levels is the depletion of heme and sterols, which causes an increase in membrane fluidity. This step is supported by our result that chemicals that decrease membrane fluidity (DMSO and glycerol) block the hypoxic induction of *PAU* transcription and that the levels of both ergosterol (LEES *et al.* 1979; BERG *et al.* 2002; ABE *et al.* 2009) and heme (KIRSCHNER-ZILBER *et al.* 1982; SHVIRO *et al.* 1982; GINSBURG and DEMEL 1984; SHAKLAI *et al.* 1985; LIGHT and OLSON 1990; BALLA *et al.* 1991; SCHMITT *et al.* 1993) affect membrane fluidity.

Our results showing that *PAU* activation requires Ssk1 and Pbs2 suggest that the change in membrane fluidity then activates the Hog1 pathway via Sln1, a transmembrane protein that responds to osmotic stress and that is part of the Sln1-Ypd1-Ssk1 phospho-relay complex that activates the Hog1 pathway (MAEDA *et al.* 1994). Sln1 has been proposed to be a sensor of membrane turgor pressure (REISER *et al.* 2003), but Sln1 may more directly be a sensor of membrane fluidity, since membrane turgor influences membrane fluidity (YAMAZAKI *et al.* 1989; LAROCHE *et al.* 2001; HAYASHI and MAEDA 2006; PANADERO *et al.* 2006). Indeed, studies have shown that treatments that affect membrane fluidity, such as DMSO and low temperature, influence Hog1 signaling via the Sln1 pathway (HAYASHI and MAEDA 2006; PANADERO *et al.* 2006). Once activated by changing membrane fluidity, Sln1 would initiate the MAPK cascade that leads to activation

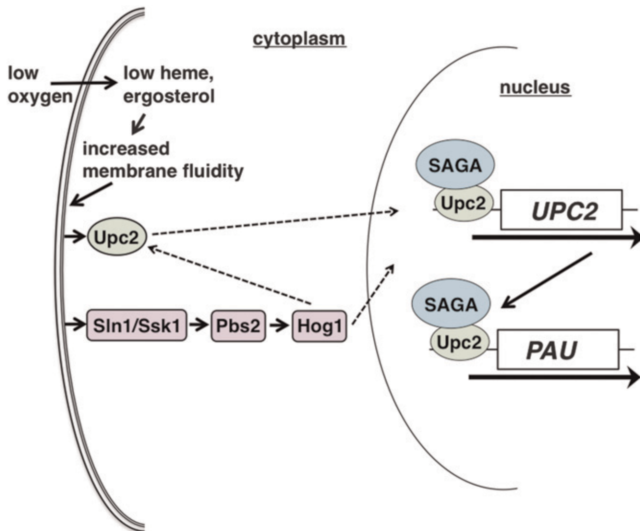


Figure 7.—A model for hypoxic induction of *PAU* gene expression. During hypoxic growth, when heme and ergosterol levels are low, membrane fluidity is altered. This change is proposed to activate the Hog1 MAPK cascade via the Sln1/Ssk1 pathway. In turn, activated Hog1 may induce *PAU* transcription directly, by translocating to the nucleus and activating transcription factors, or indirectly, by influencing signaling events in the cytoplasm, such as activation of Upc2. In addition, a SAGA-dependent, Hog1-independent pathway induces *UPC2* transcription. The induction of *PAU* and other genes by this pathway is important in maintaining the cell wall and membrane in the absence of oxygen. Solid arrows indicate parts of the model strongly supported by our results; dashed arrows represent speculative consequences of Hog1 activation. We have left Mga2 out of the current model, pending further information on its relationship to the factors shown.

of Pbs2 and Hog1. Our results have also shown that another pathway to activate Hog1, via Sho1 and Ste11, is not involved in the hypoxic response. Past results have suggested that the Sln1 branch plays a more prominent role in Hog1 activation under certain conditions, including low-temperature activation, and the Sho1/Ste11 branch plays a distinct role in pseudohyphal growth (O'Rourke and Herskowitz 1998, 2004; Maeta *et al.* 2005; Hayashi and Maeda 2006; Panadero *et al.* 2006). Our results have provided a further distinction between these two pathways.

Once activated, Hog1 may have multiple functions in hypoxic induction and our results have not yet distinguished among these possibilities. In the osmotic stress response, Hog1 functions in several ways, with both transcriptional and cytoplasmic roles (Proft and Struhl 2004; Mettetal *et al.* 2008; Westfall *et al.* 2008; de Nadal and Posas 2010). Interestingly, the cytoplasmic roles of Hog1 are sufficient for the osmotic stress response (Westfall *et al.* 2008). In the hypoxic response, one obvious possibility is that Hog1 activates Upc2. While we have shown that Hog1 is not required for *UPC2* transcriptional induction under hypoxic conditions, previous studies have suggested that Upc2 requires an activation step beyond transcriptional induction (Kwast *et al.* 2002; Davies and Rine 2006) and that it relocalizes from intracellular membranes to the nucleus upon sterol depletion (Marie *et al.* 2008). Thus, Hog1 may control Upc2 localization or activity by phosphorylation, the latter possibility similar to its role in activation of Sko1 during osmotic stress (Proft *et al.* 2001). Chromatin immunoprecipitation experiments have not yet been able to detect either Hog1 or Upc2 association with *PAU* promoters. However, all *PAU* promoters have consensus Upc2 binding sites (Kwast *et al.* 2002; Luo and van Vuuren 2009), suggesting that Upc2 acts directly on *PAU* induction and that the lack of a ChIP signal is due to the technical complication of multiple copies of *PAU* promoters. Future experiments will address the levels at which Hog1 controls the hypoxic response.

The role of the SAGA transcriptional coactivator in hypoxic induction may involve both Hog1-independent and Hog1-dependent activities. One role of SAGA must be independent of Hog1 activity, as SAGA is required for *UPC2* transcriptional induction, while Hog1 is not. However, SAGA may also function directly at *PAU* promoters, possibly recruited by Upc2, where SAGA components could be regulated more directly by Hog1 in response to changing membrane fluidity. Indeed, there is strong evidence for connections between stress, Hog1, and SAGA: (1) SAGA-dependent genes are mainly those regulated by stress (Huisinga and Pugh 2004), (2) SAGA is required for Hog1-dependent gene expression in response to osmotic stress (Proft and Struhl 2002; Zapater *et al.* 2007), and (3) during mouse development, the SAGA subunit Spt20 interacts with p38, the mammalian Hog1 ortholog (Zohn *et al.* 2006; Wang *et al.* 2008; Nagy *et al.* 2009). Whatever the exact role of SAGA in hypoxic induction, it is of interest that its activity during hypoxic induction is strongly dependent upon the SAGA component, Sgf73. This role of Sgf73 is at least partially independent of its well-characterized requirement for the assembly and activity of the histone deubiquitylase activity of SAGA (Lee *et al.* 2009; Rodriguez-Navarro 2009; Kohler *et al.* 2010; Samara *et al.* 2010). Other studies have shown that Sgf73 is also required for preinitiation complex formation at some SAGA-dependent promoters (Shukla *et al.* 2006) and that Sgf73 can bind to nucleosomes (Bonnet *et al.* 2010). Further analysis of Sgf73 in the hypoxic response may reveal whether these or additional Sgf73 functions are important during the hypoxic response.

While both osmotic stress and hypoxic induction require Hog1 activation, there are clear differences in these responses. There is a large difference in the kinetics of Hog1 activation with respect to osmotic stress and hypoxic induction. This is likely due to the distinct ways that these environmental changes affect membrane fluidity. Osmotic stress rapidly changes the membrane turgor pressure and therefore likely changes the

fluidity almost immediately. In contrast, hypoxia causes sterol and heme depletion, with a change in membrane fluidity probably occurring more slowly, as the dilution of heme and ergosterol is required to change membrane fluidity. The kinetics of Hog1 activation correlate with the induction of the respective transcriptional programs. In addition, although both pathways depend upon Hog1, they have distinct transcriptional outputs, as the *PAU* genes respond solely to hypoxia while *GRE2* responds solely to osmotic stress. This result strongly suggests that there are additional components that are distinctly required for each response.

To conclude, our results have shown that the Hog1 MAPK pathway, SAGA, and other factors are required for the hypoxic induction of several genes in *S. cerevisiae*. These genes likely help cells adapt to an environment with little or no oxygen, where they are unable to make heme and sterols and hence unable to properly maintain the membrane. Most of these genes were previously shown to be induced by different stresses, such as changing temperature, that cause damage to membranes and are all involved in protecting and maintaining cell membrane and cell wall components (Viswanathan *et al.* 1994; Mazur *et al.* 1995; Mao *et al.* 1997; Mandala *et al.* 1998; Rachidi *et al.* 2000; Sales *et al.* 2000; Abramova *et al.* 2001; Swain *et al.* 2002; Caesar and Blomberg 2004; Serrano *et al.* 2006; Pacheco *et al.* 2009). In addition to conservation of heme, sterols, the Hog1 pathway, and SAGA, at least three of the induced genes (*YSR3*, *ERG26*, and *TFS1*) are conserved in humans and in most eukaryotes. We thus speculate that parts of this pathway also function in metazoans and play important roles in the adaptation of cells and tissues to changing oxygen levels.

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Supporting Information
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The Hog1 Mitogen-Activated Protein Kinase Mediates a Hypoxic Response in *Saccharomyces cerevisiae*

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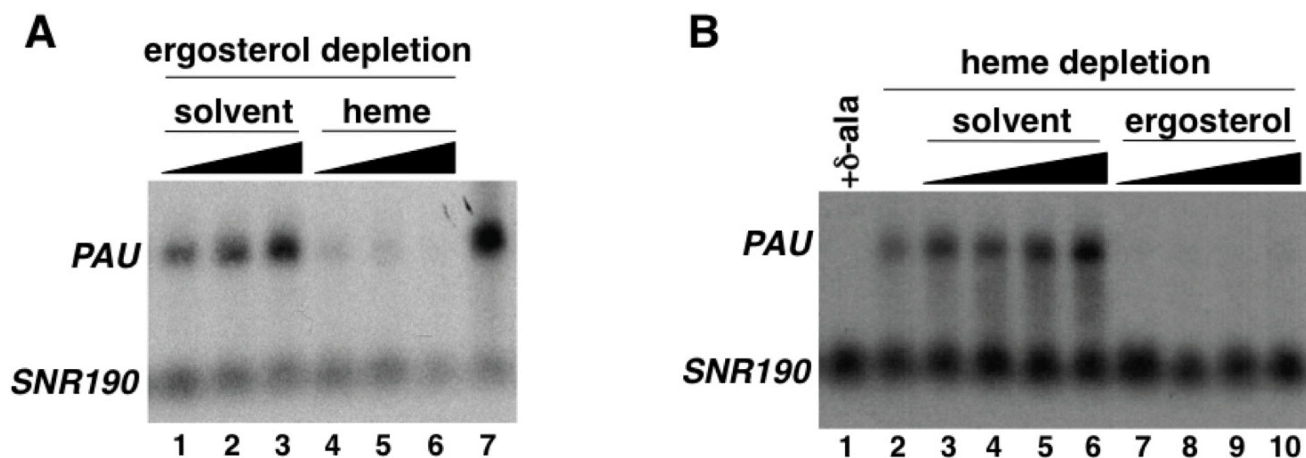

FIGURE S1.—Heme suppresses ergosterol depletion, and ergosterol suppresses heme depletion. A. Northern analysis of a *GAL1PR-ERG25* strain (FY2870) grown for 12 hours under aerobic conditions with 2% glucose, in the absence (lanes 1-3) or presence (lanes 4-6) of heme. Lane 7 contains a sample grown under hypoxic conditions as a positive control for *PAU* expression. B. Northern analysis of *hem1Δ* strain (FY2637) grown for 12 hours under aerobic conditions without δ-ala (lanes 2-10), in the absence (lanes 3-6) or presence (lanes 7-10) of ergosterol. Lane 1 contains a sample with δ-ala added and lane 2 contains a heme-depleted sample without solvent or ergosterol.

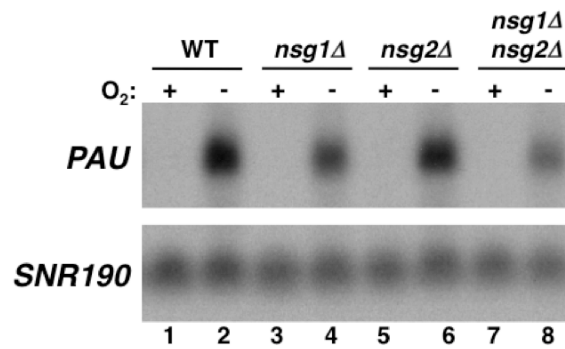

FIGURE S2.—Nsg1 and Nsg2 do not play a major role in *PAU* oxygen regulation. The reduction of hypoxic *PAU* expression in the *nsg1Δ* single and the *nsg1Δnsg2Δ* double mutants is consistently modest in comparison to that seen in other mutants, like *hog1Δ*. Northern analysis of wild-type (FY2609), *nsg1Δ* (FY2892), *nsg2Δ* (FY2893) or *nsg1Δ nsg2Δ* (FY2894) strains grown in the presence (+) or absence (-) of oxygen for 5 hours.

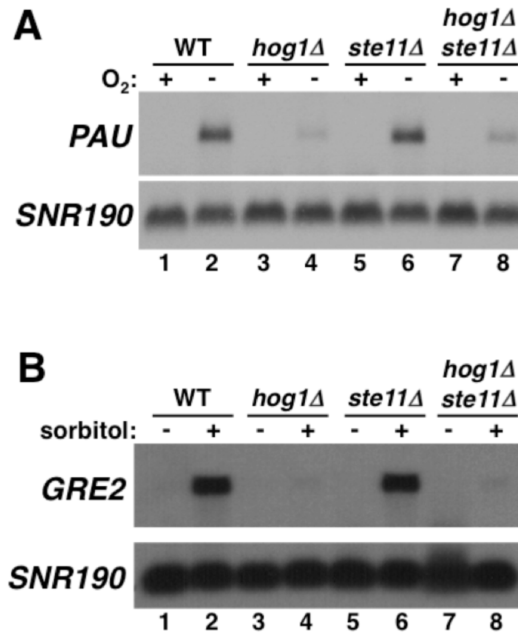

FIGURE S3.—The MAPKKK, Ste11, is not required for hypoxic induction of *PAUs* or osmostress induction of *GRE2*, even in the absence of Hog1. O'Rourke and Herskowitz (Genes Dev 12: 2874-2886 (1998)) reported that there is signaling cross-talk in a *hog1Δ* mutant that can be eliminated by simultaneously deleting *STE11*. We found that this is not the case for *PAUs* or *GRE2*. A. Northern analysis was used to monitor *PAU* expression in cells grown in the presence (+) or absence (-) of oxygen for 5 hours. Wild-type (FY2609), *hog1Δ* (FY2873), *ste11Δ* (FY2875), and *hog1Δste11Δ* (FY2876) strains were used in both panels. B. Northern analysis was used to monitor *GRE2* expression in cells grown in the presence of 1M sorbitol (+) or an equal volume of solvent (-) for 20 minutes.

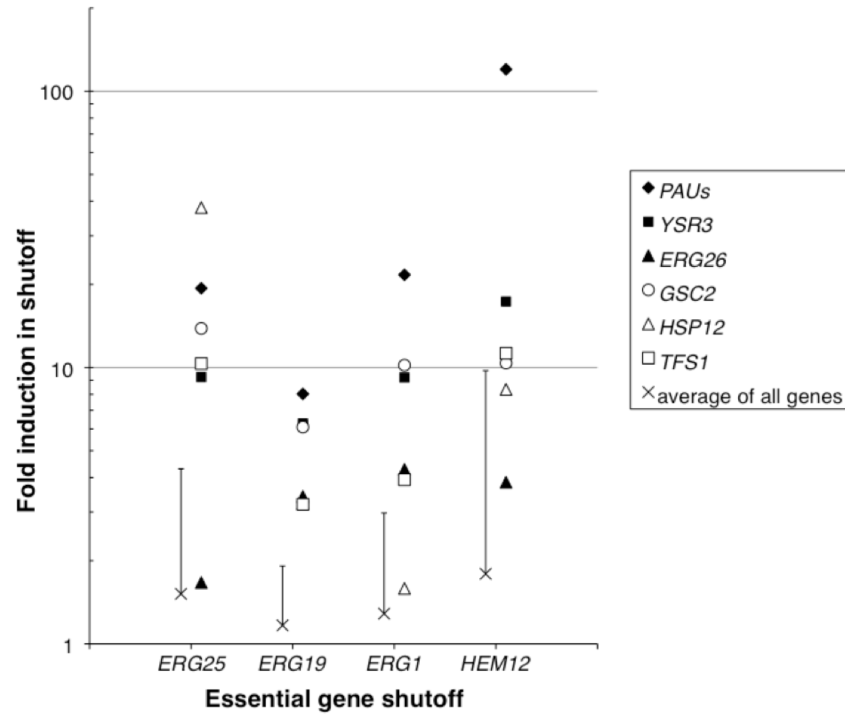

FIGURE S4.—Heme and ergosterol depletion induces a subset of the Hog1-dependent hypoxic genes. Genes identified in Figure 5A as regulated by Hog1 that also contain consensus Upc2 binding sites in their promoters, were analyzed for their fold-induction when heme or ergosterol biosynthetic genes are repressed. The data shown here were obtained from a previous study that employed microarrays to monitor expression of all yeast genes 12 hours after turning off individual essential genes (Mnaimneh S, Davierwala AP, Haynes J, Moffat J, Peng WT, et al. Cell 118: 31-44 (2004)). Fold-induction was determined by comparing the expression of each gene to a wild-type strain grown under the same conditions. The bar labeled "average of all genes" represents the average induction of all ~6300 genes for that particular essential gene shutoff, and the standard deviation. The symbol labeled "*PAUs*" represents the average expression of all 24 *PAU* genes.

TABLE S1

Primers used in this study

Primer #	Location[1]	Purpose	Sequence (5' to 3')
FO7527	*UPC2* +690	Northern probe, forward	GCAGCAATCTCAACCACAGA
FO7528	*UPC2* +1381	Northern probe, reverse	CTGCACGTTCATGATGCTCT
FO2934	*PAU13* +13	Northern probe, forward	ACTTCAATCGCCGCTGGTGT
FO2935	*PAU13* +356	Northern probe, reverse	GTGATAGTGTAGATACCGTC
FO1324	*SNR190* +1	Northern probe, forward	GGCCCTGATGATAATG
FO1325	*SNR190* +190	Northern probe, reverse	GGCTCAGATCTGCATG
FO6549	*GRE2* +17	Northern probe, forward	CAGGTGCTAACGGGTTCATT
FO6550	*GRE2* +903	Northern probe, reverse	ATGGGTAGCACCAGAACCTG
FO7977	*UPC2* +754	Real-time PCR, forward	CATTCGATCTTGCGGGTAGT
FO7978	*UPC2* +900	Real-time PCR, reverse	GCAGCTGCTGGTGTAATTGA
FO961	*ACT1* +532	Real-time PCR, forward	TGTCACCAACTGGGACGATA
FO962	*ACT1* +721	Real-time PCR, reverse	GGCTTGGATGGAAACGTAGA
FO3137	*PAU5* -40	Replace *PAU5* ORF with *URA3* ORF, forward	TATCTCCACTAAGCAACAACCCAAAAAAC AACAAATACAATGTCGAAAGCTACATATA A
FO3138	*PAU5* +409	Replace *PAU5* ORF with *URA3* ORF, reverse	TATTCTCTAATTCGTATCACATTTTATCC CTAAGGGAATTTAGTTTTGCTGGCCGCAT C
FO3225	*PAU5* +521	Insert NatMX marker downstream of *PAU5*, forward	TTGAAGCCGTAAGTAATATGCAAATTTGT TATGACTTCTTCAGCTGAAGCTTCGTACG C
FO3226	*PAU5* +599	Insert NatMX marker downstream of *PAU5*, reverse	GTTGGAAGTTAAATCCCAACATTTGCTGC AAGAATAGGCATAGGCCACTAGTGGATCT G
FO2647	*HAP2* -40	Replace *HAP2* ORF with *LEU2* marker, forward	TGGAAAAGCATTTCTTTTTGGAAGAGGAA CAAGAACGCCAGATTGTACTGAGAGTGCA C
FO2648	*HAP2* +839	Replace *HAP2* ORF with *LEU2* marker, reverse	TTCTTTTAGGAATGATATTAACATTGGAA TATTACAAAACTGTGCGGTATTTCACACC G
FO2965	*UPC2* -40	Replace *UPC2* ORF with *HIS3* marker, forward	TCAAAAAAAGTTAAGTACAAATATTTACAG TTCAGCAGTAGATTGTACTGAGAGTGCAC
FO2966	*UPC2* +2782	Replace *UPC2* ORF with *HIS3* marker, reverse	GGAATCTATTTCGAATATTCTGCACTTTT AAATTTTCTACTGTGCGGTATTTCACACC G
FO5707	*ERG25* -90	Insert KanMX marker and $GAL1_{pr}$ upstream of *ERG25* ORF, forward	AATCTTTATATTAGTTGTAACTTTTTCTC TTTAGATAGTAGAATTCGAGCTCGTTTAA AC
FO5708	*ERG25* +4	Insert KanMX marker and	GGACTAGACCTGAAAGGGTAGCGTTGTT

		$GAL1_{pr}$ upstream of *ERG25* ORF, reverse	GAAAACGGCAGACATTTTGAGATCCGGGTTTT
FO6385	*HOG1* -40	Replace *HOG1* ORF with *LEU2* marker, forward	AAAGGGAAAACAGGGAAAACTACAACTATCGTATATAATAAGATTGTACTGAGAGTGCAC
FO6386	*HOG1* +1348	Replace *HOG1* ORF with *LEU2* marker, reverse	GAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAACACGTCTGTGCGGTATTTCACACCG
FO6672	*HOG1* +1266	Insert 13 copies of myc and KanMX marker downstream of *HOG1*, forward	CGGTAACCAGGCCATACAGTACGCTAATGAGTTCCAACAGCGGATCCCCGGGTTAATTAA
FO6673	*HOG1* +1348	Insert 13 copies of myc and KanMX marker downstream of *HOG1*, reverse	GAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAACACGTGAATTCGAGCTCGTTTAAAC
FO6531	*STE11* -40	Replace *STE11* ORF with KanMX marker, forward	TAAAGCTAGTATGATAAGATCACCGGTAGACGAAATATACAGATTGTACTGAGAGTGCAC
FO6532	*STE11* +2194	Replace *STE11* ORF with KanMX marker, reverse	CACTTTAGTGCCATAAAAAGAATTAATAAGTAGCCCTTTTCTGTGCGGTATTTCACACCG
FO6624	*PBS2* -40	Replace *PBS2* ORF with *HIS3* marker, forward	ATTATTATATTAAGCAGATCGAGACGTTAATTTCTCAAAGAGATTGTACTGAGAGTGCAC
FO6625	*PBS2* +2047	Replace *PBS2* ORF with *HIS3* marker, reverse	TATATTCACGTGCCTGTTTGCTTTTATTTGGATATTAACGCTGTGCGGTATTTCACACCG
FO6682	*SSK1* -40	Replace all but the last 40 nulceotides of the *SSK1* ORF with *HIS3* marker, forward	ATGCTCAATTCTGCGTTACTGTGGAAGGTTTGGCTACGAAAGATTGTACTGAGAGTGCAC
FO6683	*SSK1* +2139	Replace all but the last 40 nulceotides of the *SSK1* ORF with *HIS3* marker, reverse	TCACAATTCTATTTGAGTGGGCGAGAGGTTTGAATTTTTTCTGTGCGGTATTTCACACCG
FO3125	*MGA2* -40	Replace *MGA2* ORF with NatMX marker, forward	CACTTATTGAAGGTCATTTTGGCGAACAGAACATTTCGTTCAGCTGAAGCTTCGTACGC
FO3126	*MGA2* +3382	Replace *MGA2* ORF with NatMX marker, reverse	ACACACATATATATATATATATATACGTAAAAAAGCAGAGCATAGGCCACTAGTGGATCTG
FO7979	*SPT23* -40	Replace *SPT23* ORF with *URA3* marker, forward	CCTCTAAACGACTAATCACAACAGTAGTACACCACTGAAAAGATTGTACTGAGAGTGCAC
FO7980	*SPT23* +3289	Replace *SPT23* ORF with *URA3* marker, reverse	ATCTATATAGTGTAAGGATTATGTAGCTAGAAAAATGTCTCTGTGCGGTATTTCACAC

			CG
FO3998	*SGF73* -101	Replace *SGF73* ORF with KanMX marker, PCR of *sgf73D0*::KanMX allele from FY2475 in (Martens et al, 2005), forward	TCCAGCATCGCTCATTAGAG
FO4000	*SGF73* +2062	Replace *SGF73* ORF with KanMX marker, PCR of *sgf73D0*::KanMX allele from FY2475 in (Martens et al, 2005), reverse	CCACAAACATAGACGTGTAC
FO4785	*SUS1* -40	Replace *SUS1* ORF with *HIS3* marker, forward	CATGCGACAAAATCAGAAGTAACAATTCT GGCCTTCACTCCAAGATTGTACTGAGAGT GCAC
FO4786	*SUS1* +481	Replace *SUS1* ORF with *HIS3* marker, reverse	TATGTAATAATATTGGGAATTAAGGTGCA TTTTCGTATCCTCTGTGCGGTATTTCACA CCG
FO4434	*SGF11* -514	Replace *SGF11* ORF with KanMX marker, PCR of *sgf11D0*::KanMX allele from deletion set (Giaever et al, 2002), forward	GTTGGTGCAGTTATGGTTAGGGGCC
FO4435	*SGF11* +848	Replace *SGF11* ORF with KanMX marker, PCR of *sgf11D0*::KanMX allele from deletion set (Giaever et al, 2002), reverse	GCTCAAGCAGTTTGACTCGCATATTC
FO2201	*UBP8* -40	Replace *UBP8* ORF with *URA3* marker, forward	TACTTGAAACCCTGCTTTTTTTATTTGTT ATTAATAATTCTGTGCGGTATTTCACACC G
FO2202	*UBP8* +1456	Replace *UBP8* ORF with *URA3* marker, reverse	TTTTTGTTTTATTATTATTGTTGAATGCT ATTTGCTGAAAGATTGTACTGAGAGTGCA C
FO6350	*NSG1* -40	Replace *NSG1* ORF with *HIS3* marker, forward	GCAAGTGAGAAAAAAAAAATTTAAACAGA GGAGGTTACTAAGATTGTACTGAGAGTGC AC
FO6351	*NSG1* +916	Replace *NSG1* ORF with *HIS3* marker, reverse	ACACATCGATACTAATCATTGAACGCCCC TATGGGAACACCTGTGCGGTATTTCACAC CG
FO6354	*NSG2* -40	Replace *NSG2* ORF with *LEU2* marker, forward	ATAAACGACAAAGTATTTCTCAAAGAAAA CAGCATACAGAAGATTGTACTGAGAGTGC AC
FO6355	*NSG2* +940	Replace *NSG2* ORF with *LEU2* marker, reverse	TACTTCTAATTAATAATATTTACTCGTCA GAATTTCGACTCTGTGCGGTATTTCACAC CG
FO7014	*MSN2* -40	Replace *MSN2* ORF with *URA3* marker, forward	TTTTTCAACTTTTATTGCTCATAGAAGAA CTAGATCTAAAAGATTGTACTGAGAGTGC

			AC
FO7015	*MSN2* +2145	Replace *MSN2* ORF with *URA3* marker, reverse	TTATGAAGAAAGATCTATCGAATTAAAAA AATGGGGTCTACTGTGCGGTATTTCACAC CG
FO7018	*MSN4* -40	Replace *MSN4* ORF with *LEU2* marker, forward	TTCGGCTTTTTTTTTCTTTTCTTCTTATTA AAAACAATATAAGATTGTACTGAGAGTGC AC
FO7019	*MSN4* +1933	Replace *MSN4* ORF with *LEU2* marker, reverse	TAGCTTGTCTTGCTTTTATTTGCTTTTGA CCTTATTTTTTCTGTGCGGTATTTCACAC CG

[1] Location shown relative to the ATG start codon.

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Martens JA, Wu PY, Winston F (2005) Regulation of an intergenic transcript controls adjacent gene transcription in Saccharomyces cerevisiae. *Genes Dev* **19**(22)**:** 2695-2704

TABLE S2

Microarray Data

Table S2 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.111.128322/DC1.