Increased heme synthesis in yeast induces a metabolic switch from fermentation to respiration even under conditions of glucose repression

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Regulation of mitochondrial biogenesis and respiration is a complex process that involves several signaling pathways and transcription factors as well as communication between the nuclear and mitochondrial genomes. Under aerobic conditions, the budding yeast *Saccharomyces cerevisiae* metabolizes glucose predominantly by glycolysis and fermentation. We have recently shown that altered chromatin structure in yeast induces respiration by a mechanism that requires transport and metabolism of pyruvate in mitochondria. However, how pyruvate controls the transcriptional responses underlying the metabolic switch from fermentation to respiration is unknown. Here, we report that this pyruvate effect involves heme. We found that heme induces transcription of *HAP4*, the transcriptional activation subunit of the Hap2/3/4/5p complex, required for growth on nonfermentable carbon sources, in a Hap1p- and Hap2/3/4/5p-dependent manner. Increasing cellular heme levels by inactivating *ROX1*, which encodes a repressor of many hypoxic genes, or by overexpressing *HEM3* or *HEM12* induced respiration and elevated ATP levels. Increased heme synthesis, even under conditions of glucose repression, activated Hap1p and the Hap2/3/4/5p complex and induced transcription of *HAP4* and genes required for the tricarboxylic acid (TCA) cycle, electron transport chain, and oxidative phosphorylation, leading to a switch from fermentation to respiration. Conversely, inhibiting metabolic flux into the TCA cycle reduced cellular heme levels and *HAP4* transcription. Together, our results indicate that the glucose-mediated repression of respiration in budding yeast is at least partly due to the low cellular heme level.

The yeast *Saccharomyces cerevisiae*, even under aerobic conditions, metabolizes glucose predominantly by glycolysis and fermentation, producing ethanol and carbon dioxide (1–4). This metabolic specialty of the budding yeast is partly due to the high activity of pyruvate decarboxylase Pdc1p (5, 6), which leads to cytoplasmic conversion of pyruvate to acetaldehyde and subsequently to ethanol. Because the majority of the glycolytically produced pyruvate in glucose-grown cells is converted to acetaldehyde, only a small fraction of pyruvate is translocated into mitochondria and used for the tricarboxylic acid (TCA)[2] cycle (7–9) (see Fig. 1). Under these conditions of low metabolic flux into the TCA cycle, the expression of genes encoding enzymes of the TCA cycle, electron transport chain (ETC), and oxidative phosphorylation (OXPHOS) is low. After glucose is exhausted during diauxic shift, yeast cells activate TCA cycle, ETC, and OXPHOS genes and switch metabolism from fermentation to respiration, utilizing the produced ethanol as a carbon source (3, 10–12).

The transition from fermentation to respiration is controlled by PKA, target of rapamycin (TOR), Sch9p, Snf1p, and Mec1p/Rad53p signaling pathways and requires several transcription factors, including Hap1p, Hap2/3/4/5p, and Rtg1/3p (10, 13–19). However, despite the central position of glycolysis, the TCA cycle, ETC, and OXPHOS in cell metabolism and physiology, the signaling mechanisms and transcriptional regulation by which these pathways are coordinated and aligned with nutritional conditions are not well understood.

The expression of TCA cycle, ETC, and OXPHOS genes is regulated by the heterotetrameric transcription complex Hap2/3/4/5p (heme-activated protein (HAP) complex) independently of PKA and SNF1, suggesting that the HAP complex provides an additional, separate mechanism of transcriptional regulation of mitochondrial respiration (4, 20) (Fig. 1). The HAP complex binds to DNA through the Hap2, -3, and -5 subunits, which are constitutively expressed (21). The activation domain of the complex is contained within the Hap4p subunit (22). *HAP4* expression increases upon glucose depletion, and overexpression of *HAP4* induces respiration even in a glucose-repressed state, resulting in an extension of replicative life span (23–26). The HAP complex is activated by heme and is required for growth on non-fermentable carbon sources (27, 28). The human homolog of the HAP complex is the heterotrimeric NF-Y complex (29). Interestingly, the NF-Y complex is also regulated by heme (30).

Heme stimulates the transcriptional activity of Hap1p, and as such, Hap1p acts as the key sensor of heme (20, 31). Heme synthesis in yeast is regulated by the availability of oxygen, and heme serves as an intermediate in the signaling mechanism for

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[2] The abbreviations used are: TCA, tricarboxylic acid; ETC, electron transport chain; OXPHOS, oxidative phosphorylation; HAP, heme-activated protein; ALA, 5-aminolevulinic acid; NF-Y, nuclear transcription factor Y; YPD, yeast extract-peptone-dextrose.

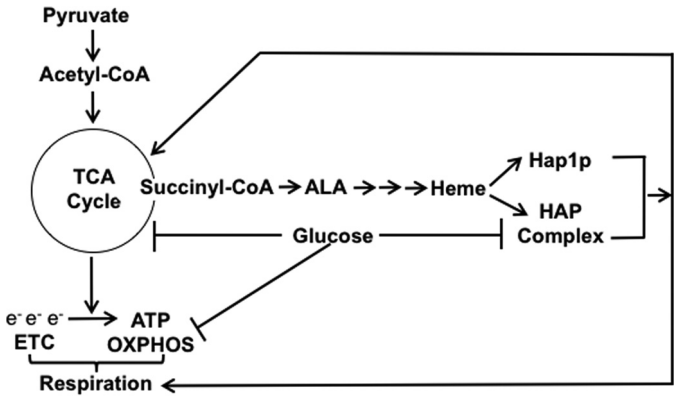

Figure 1. A model depicting the role of heme and glucose in regulation of respiration. Synthesis of heme requires succinyl-CoA, an intermediate of the TCA cycle. Heme activates Hap1p and the HAP complex, both of which activate genes of the TCA cycle, ETC, and OXPHOS. Glucose represses transcription of genes encoding enzymes of the TCA cycle, ETC, and OXPHOS. Glucose also down-regulates the HAP complex by repressing transcription of *HAP4*. When cells are grown on glucose, only a small fraction of pyruvate is transported into mitochondria and converted to acetyl-CoA.

sensing oxygen levels (32, 33). Thus, under hypoxic conditions, when heme is not synthesized, Hap1p functions as a repressor (34). In addition, Hap1p can repress its own transcription independently of the heme level (35). The Hap1p-regulated genes include genes required for respiration, such as *CYC1*, *CYC7*, and *CYT1* as well as *ROX1*, which encodes repressor of many hypoxic genes (20, 32).

Our previous work has shown that altered chromatin structure in *S. cerevisiae* induces transcription of *HAP4* and respiration (36). On the basis of nucleosomal chromatin architecture, yeast genes can be classified into two broad groups: growth genes and stress genes (37). Growth genes are typically expressed at high levels and feature a nucleosome-free region upstream of the coding region where transcription factors can bind in an unobstructed way. The stress genes are generally expressed at lower levels, and their promoters are dominated by delocalized nucleosomes rather than by a nucleosome-free region. Consequently, stress genes are regulated by factors that affect the structure of chromatin, including histone level. The respiratory genes in *S. cerevisiae* belong to the stress gene category unlike respiratory genes in higher eukaryotes (38, 39). Altered chromatin structure in *S. cerevisiae* makes promotors of respiratory genes more accessible to transcription factors and allows increased expression of respiratory genes and transition from fermentation to respiration (36). The increased transcription of respiratory genes and induction of respiration require a product of mitochondrial carbon metabolism (36).

In this study, we show that this metabolite is heme. We found that the effect of heme does not require an altered chromatin structure; in cells with normal chromatin architecture, heme regulatestranscriptionof*HAP4*inaHap1p-andHAPcomplex-dependent manner. Perhaps more importantly, increasing synthesis of heme induces a switch of metabolic mode from fermentation to respiration even under conditions of glucose repression. Together, our results indicate that the glucose-mediated repression of respiration in budding yeast is at least partly due to the low metabolic flux into the TCA cycle and low cellular heme level.

Results

Heme regulates HAP4 transcription

We have previously shown that altered chromatin structure activates transcription of *HAP4* and induces respiration by a mechanism that requires transport and metabolism of pyruvate in mitochondria (36). Because many of the TCA cycle and OXPHOS genes are regulated by the heme-activated Hap1p and the HAP complex (27, 28), we speculated that a defect in transport and metabolism of pyruvate in mitochondria would result in diminished synthesis of heme and decreased activities of Hap1p, HAP complex, TCA cycle, and OXPHOS.

Heme biosynthesis starts in mitochondria and requires succinyl-CoA, an intermediate of the TCA cycle, and glycine to produce a key heme intermediate, 5-aminolevulinic acid (ALA) (20, 31). To determine whether the heme level is limiting for *HAP4* transcription in wild-type cells, we added ALA or hemin (a heme derivative used to treat porphyrias) to cells grown in rich media containing glucose or galactose. We found that in cells grown on glucose, addition of ALA or hemin increased *HAP4* expression 3.4- and 5.9-fold, respectively (Fig. 2*A*). In cells grown on galactose, *HAP4* expression was elevated 4.7-fold in comparison with glucose-grown cells; however, addition of ALA did not significantly increase *HAP4* expression, and addition of hemin decreased *HAP4* expression. In glucose-grown cells, addition of ALA and hemin increased the heme level 3.8- and 4.2-fold, respectively (Fig. 2*B*). In cells grown on galactose, the heme level was elevated 2.7-fold in comparison with glucose-grown cells. Addition of ALA or hemin further increased the heme level 4.3- and 5.5-fold in comparison with glucose-grown cells (Fig. 2*B*). The effect of ALA or hemin addition on *HAP4* transcription appears to be more significant in cells grown on glucose where the effect of ALA or hemin addition on the cellular heme level is more pronounced than in cells grown on galactose. These results suggest that *HAP4* transcription is regulated by heme and that the heme synthesis is limiting for *HAP4* expression only in cells grown on glucose.

Heme synthesis and HAP4 transcription are regulated by metabolic flux into the TCA cycle

The lower heme level in glucose-grown cells suggests that heme synthesis is limited by metabolic flux into the TCA cycle and by availability of succinyl-CoA. To test this possibility, we determined the heme level and *HAP4* expression in *pda1*Δ and *mpc1*Δ cells. Pda1p is a subunit of pyruvate dehydrogenase complex that catalyzes the conversion of pyruvate to acetyl-CoA in mitochondria. Mpc1p is a pyruvate transporter localized in the inner mitochondrial membrane; mitochondria isolated from *mpc1*Δ cells have decreased pyruvate uptake (40, 41). Both *HAP4* expression and heme levels in *pda1*Δ and *mpc1*Δ cells were significantly decreased in comparison with wild-type cells both in glucose and galactose media (Fig. 2, *C* and *D*). To extend these findings, we compared heme levels in cells grown to midexponential phase, diauxic shift, and early stationary phase (Fig. 2*E*). The heme level in cells undergoing diauxic shift was elevated almost 2-fold in comparison with midexponential phase cells, indicating that heme synthesis is up-regulated

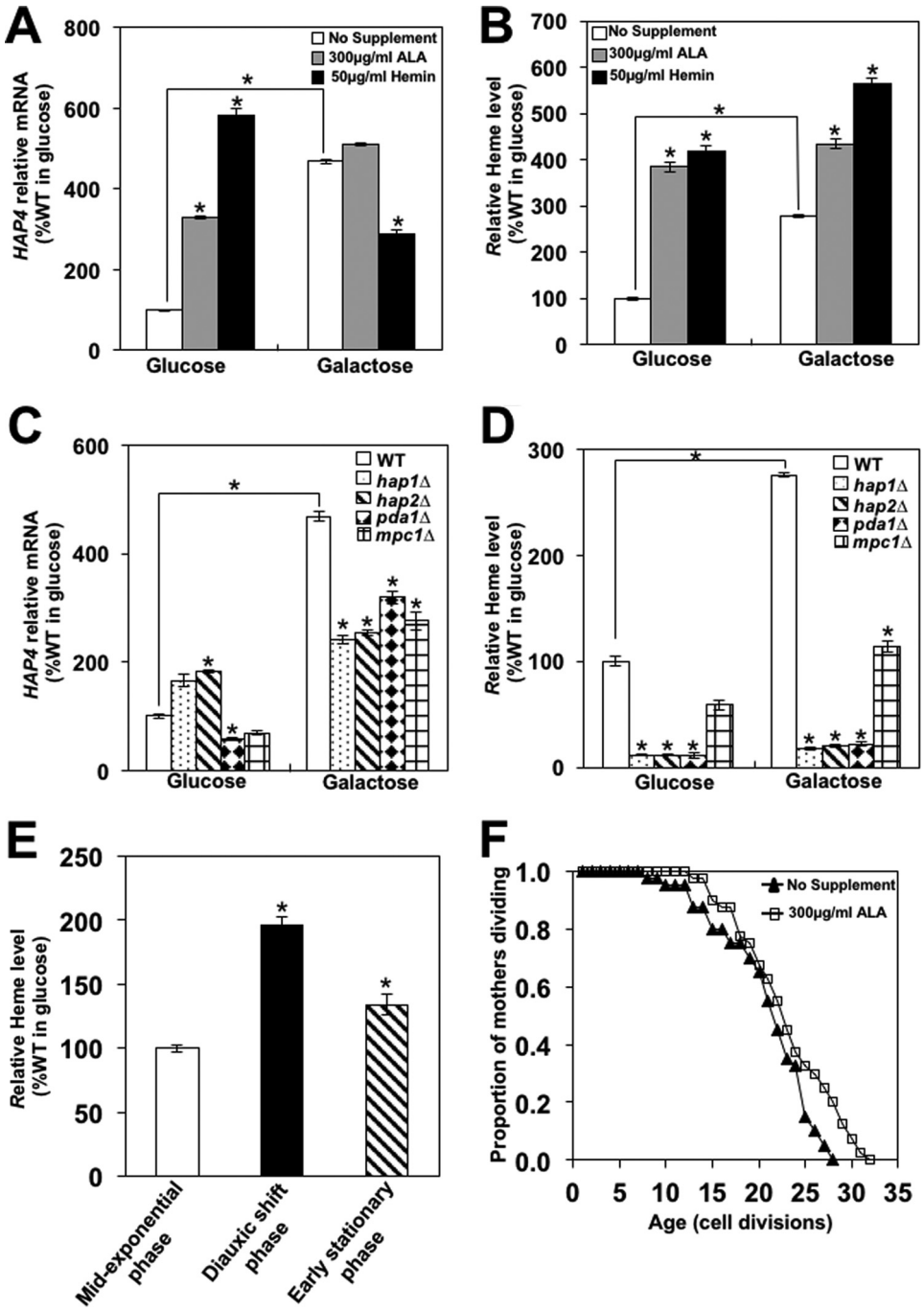

Figure 2. HAP4 transcription is regulated by heme in Hap1p- and HAP complex-dependent manner. *A* and *B*, WT (W303-1a) cells were grown in YEP medium containing 2% glucose or 2% galactose with or without addition of ALA (300 μg/ml) or hemin (50 μg/ml). Relative *HAP4* mRNA levels (*A*) and cellular heme levels (*B*) were determined. The heme level in WT cells grown on glucose was 17.3 pmol/10^{10} cells and was set as 100%. *C* and *D*, WT (W303-1a), *hap1*Δ (TZ766), *hap2*Δ (TZ742), *pda1*Δ (TZ354), and *mpc1*Δ (TZ341) cells were grown in YEP medium containing 2% glucose or 2% galactose. Relative *HAP4* mRNA levels (*C*) and cellular heme levels (*D*) were determined. *E*, WT (W303-1a) cells were grown in YEP medium containing 2% glucose to midexponential phase ($A_{600\ nm}$ of 0.8), diauxic shift ($A_{600\ nm}$ of 5.0), and early stationary phase ($A_{600\ nm}$ of 9.0), and cellular heme levels were determined. *F*, replicative life span was determined for wild-type (W303-1a) cells grown on YEP medium containing 2% glucose (no supplement) or the same medium supplemented with 300 μg/ml ALA. Forty cells were analyzed for each condition. *A–E*, the experiments were repeated three times, and the results are shown as means ± S.D. (*error bars*). Values that are statistically significantly different ($p < 0.05$) from the WT cells grown on the corresponding carbon source are indicated by an *asterisk*. Values that are statistically significantly different ($p < 0.05$) from each other are indicated by a *bracket* and an *asterisk*. The results are expressed relative to the value for the wild-type strain grown in 2% glucose.

when cells transition from utilizing glucose to ethanol. We interpret these results to mean that metabolic flux into the TCA cycle regulates the cellular heme level, most likely by regulating succinyl-CoA synthesis. These results are also consistent with the role of heme in regulation of *HAP4* expression (Fig. 2, *A* and *B*) and with the observed increase of *HAP4* transcription during diauxic shift (23). Overexpression of *HAP4* induces respiration and extends yeast replicative life span (23–26). Because addition of ALA increased *HAP4* expression (Fig. 2*A*), we wanted to determine whether ALA also increases life span. Indeed, we

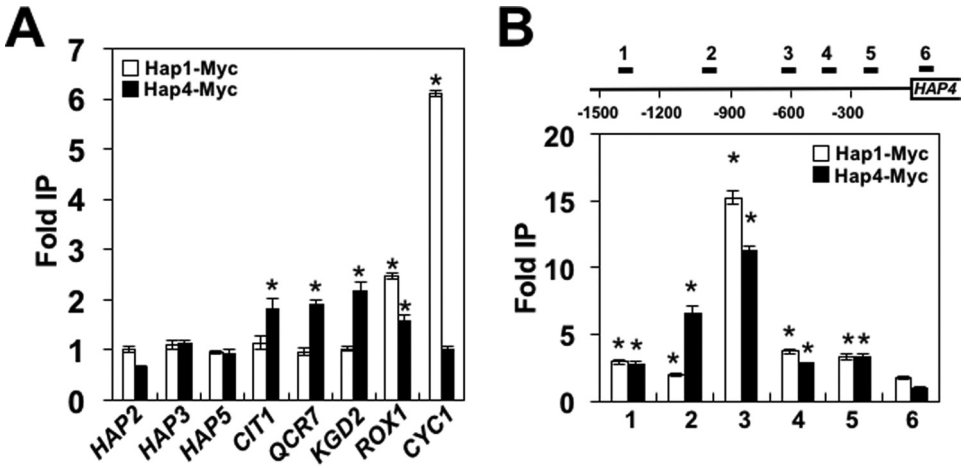

Figure 3. Hap1p and HAP complex directly regulate *HAP4* transcription. *A*, Hap1p and Hap4p are not recruited to *HAP2*, *HAP3*, and *HAP5* promoters. *B*, Hap1p and Hap4p are recruited to *HAP4* promoter. *A* and *B*, ChIP was performed using chromatin from cells expressing myc-Hap1p (FY2612) and Hap4p-myc (TZ909). Each immunoprecipitation (*IP*) was performed at least three times using different chromatin samples, and the occupancy at the indicated genes was calculated using the *POL1* coding sequence as a negative control. The data are presented as -fold occupancy over the *POL1* coding sequence control and represent means ± S.D. (*error bars*). Values that are statistically different ($p < 0.05$) from recruitment of Hap1p or Hap4p to the *POL1* locus are indicated by an *asterisk*.

found that addition of ALA to glucose medium increases the replicative life span of wild-type cells (Fig. 2*F*).

HAP4 transcription is regulated by Hap1p and HAP complex

Because *HAP4* transcription is affected by heme level and Hap1p and the HAP complex are the only known yeast transcription factors regulated by heme, we next determined *HAP4* transcription in wild-type, *hap1*Δ, and *hap2*Δ cells grown on glucose or galactose (Fig. 2*C*). As noted above, *HAP4* expression was elevated 4.7-fold in cells grown on galactose in comparison with glucose-grown cells, but this induction was largely abolished in *hap1*Δ and *hap2*Δ cells (Fig. 2*C*). These results indicate that *HAP4* transcription is regulated by Hap1p and the HAP complex. Alternatively, the *HAP4* transcription might be regulated by another, previously uncharacterized heme-responsive factor. In that case, the roles of Hap1p and the HAP complex would be only indirect, mediated by the cellular heme level. This possibility is supported by the decreased levels of heme in both *hap1*Δ and *hap2*Δ cells (Fig. 2*D*).

To determine whether Hap1p and the HAP complex regulate *HAP4* transcription directly or indirectly, we performed a chromatin immunoprecipitation (ChIP) experiment with myc-tagged Hap1p and Hap4p. As expected, we found that Hap1p was recruited to promoters of *ROX1* and *CYC1* genes, which are known targets of Hap1p (Fig. 3*A* and Ref. 31). Hap4p was recruited to promoters of *CIT1*, *QCR7*, and *KGD2*, known targets of the HAP complex (42). In addition, we detected occupancy of Hap4p at the *ROX1* promoter. Neither Hap1p nor Hap4p was recruited to the constitutively expressed *HAP2*, *HAP3*, or *HAP5* genes. To determine whether Hap1p and the HAP complex directly regulate transcription of *HAP4*, we assayed occupancy of Hap1p and Hap4p within 1.5 kb upstream of the *HAP4* coding region (Fig. 3*B*). Both Hap1p and Hap4p bound to the *HAP4* promoter; the maximum occupancy was found at around 600 bp upstream of the *HAP4* transcriptional start site. Together, these results suggest that *HAP4* transcription is directly regulated by both Hap1p and the HAP complex.

HAP4 transcription correlates with cellular heme level

To test the heme responsiveness of *HAP4* transcription, we grew *hem1*Δ, *hem1*Δ*hap1*Δ, *hem1*Δ*hap2*Δ, and *hem1*Δ*hap1*Δ*hap2*Δ cells in YPD medium containing different concentrations of ALA. *HEM1* encodes ALA synthase, the first enzyme in the heme biosynthetic pathway (43). Yeast with the *hem1*Δ mutation require the addition of ALA to the growth medium, and the cellular heme level can be regulated by the concentration of ALA in the medium (27).

In *hem1*Δ cells grown on glucose, *HAP4* expression increased with increasing concentration of ALA (Fig. 4*A*). In *hem1*Δ*hap1*Δ and *hem1*Δ*hap2*Δ cells, the expression of *HAP4* was decreased in comparison with *hem1*Δ cells but still correlated with the ALA concentration in the medium. The heme responsiveness was completely lost in *hem1*Δ*hap1*Δ*hap2*Δ cells. These results indicate that in cells grown on glucose both Hap1p and the HAP complex mediate the heme responsiveness of *HAP4* expression.

In *hem1*Δ and *hem1*Δ*hap2*Δ cells grown on galactose, *HAP4* expression correlated with the ALA concentration in the medium. However, at high concentrations of ALA, *HAP4* expression was higher in *hem1*Δ than in *hem1*Δ*hap2*Δ or *hem1*Δ*hap1*Δ cells (Fig. 4*B*). The heme responsiveness was lost in *hem1*Δ*hap1*Δ cells and significantly reduced in *hem1*Δ*hap1*Δ*hap2*Δ cells. These results indicate that in cells grown on galactose the heme responsiveness of *HAP4* transcription is mediated primarily by Hap1p. The lack of heme responsiveness in *hem1*Δ*hap1*Δ cells is surprising because it would be expected that the heme activation of the HAP complex would still result in increased *HAP4* transcription, similarly to *hem1*Δ*hap1*Δ cells grown on glucose (Fig. 4*A*). In addition, transcription of *CIT1*, *SDH1*, and *SDH2* in *hem1*Δ*hap1*Δ cells grown on glucose or galactose correlates with the concentration of ALA in the medium, indicating the activation of the HAP complex by heme (Fig. 4, *C–H*). It is possible that full activation of *HAP4* transcription by heme requires a synergistic

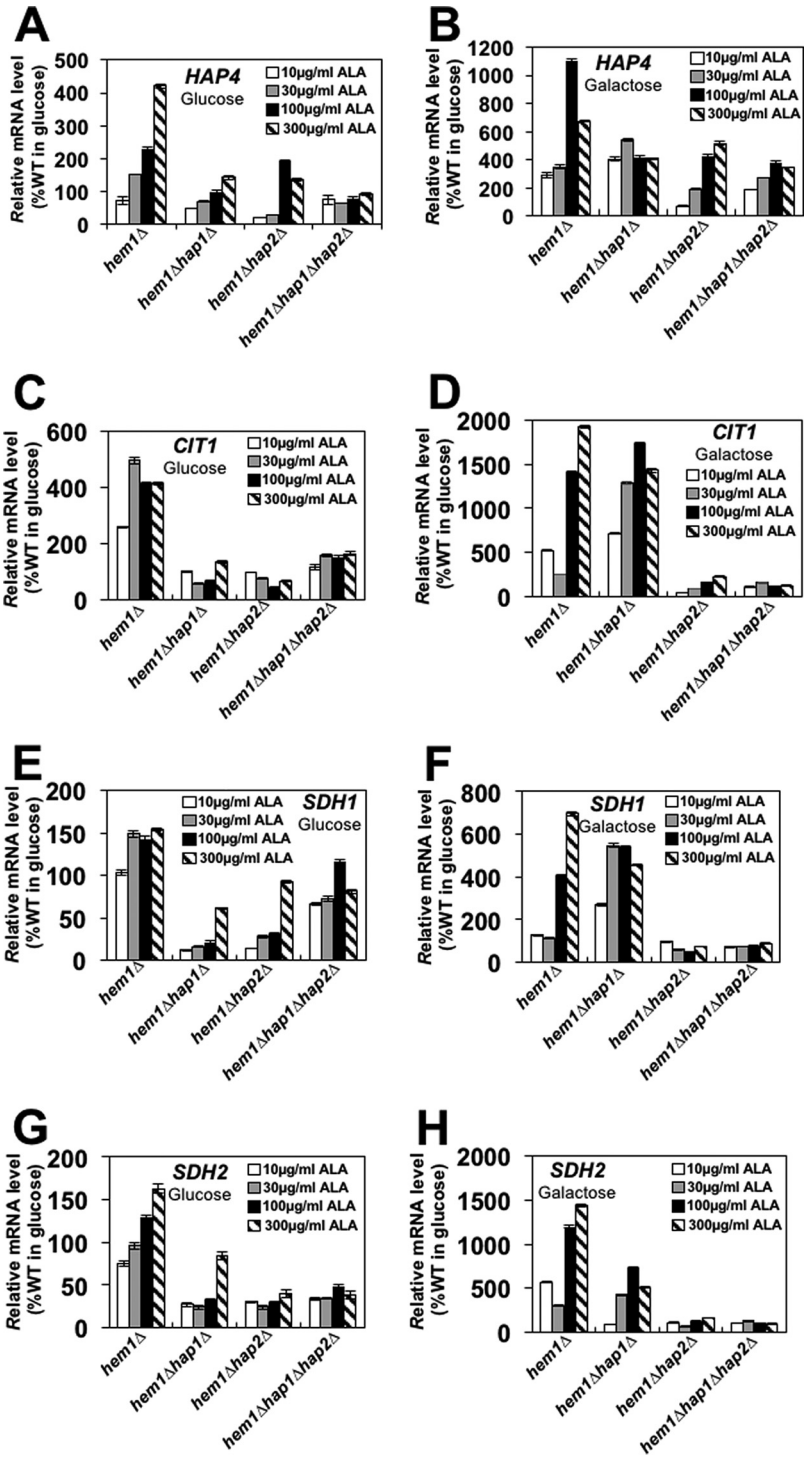

Figure 4. Transcription of *HAP4* and TCA cycle genes correlates with cellular heme level. Strains *hem1*Δ (TZ779), *hem1*Δ*hap1*Δ (TZ864), *hem1*Δ*hap2*Δ (TZ881), and *hem1*Δ*hap1*Δ *hap2*Δ (TZ884) were grown in YEP medium containing 2% glucose (*A*, *C*, *E*, and *G*) or 2% galactose (*B*, *D*, *F*, and *H*) and 10, 30, 100, or 300 μg/ml ALA. Shown are relative mRNA levels for *HAP4* (*A* and *B*), *CIT1* (*C* and *D*), *SDH1* (*E* and *F*), and *SDH2* (*G* and *H*). The experiments were repeated three times, and the results are shown as means ± S.D. (*error bars*). The results are expressed relative to the value for the wild-type strain grown in 2% glucose.

action of both Hap1p and the HAP complex. Similar synergistic regulation was described for genes regulated by repressors Rox1p and Mot3p (44–46). Alternatively, heme-mediated activation of the HAP complex may induce expression of a repressor that, directly or indirectly, counteracts the activation of the HAP complex and down-regulates transcription of *HAP4*. A similar situation occurs at the *HEM1* promoter where positive and negative regulators cancel each other's effects (47). The logical candidate for this repressor in the case of *HAP4* transcription would be Rox1p, which is transcriptionally regulated by heme in a Hap1p-dependent manner (48). However, *HAP4* has not been identified as a target of Rox1p yet (32, 49).

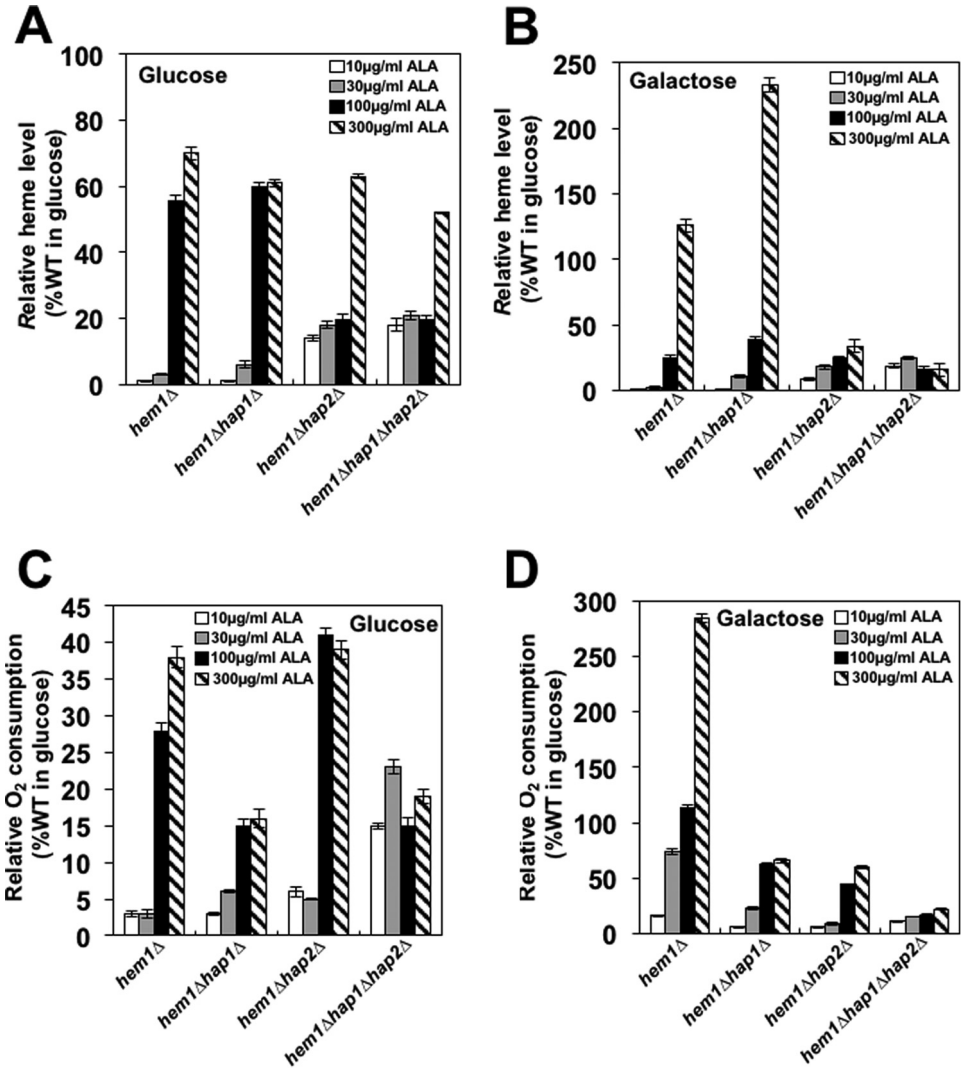

Figure 5. Respiration correlates with cellular heme level. Strains *hem1*Δ (TZ779), *hem1*Δ*hap1*Δ (TZ864), *hem1*Δ*hap2*Δ (TZ881), and *hem1*Δ*hap1*Δ *hap2*Δ (TZ884) were grown in YEP medium containing 2% glucose (*A* and *C*) or 2% galactose (*B* and *D*) and 10, 30, 100, or 300 μg/ml ALA. Shown are cellular heme levels (*A* and *B*) and oxygen consumption rates (*C* and *D*). The oxygen consumption rate in WT cells grown on glucose (YPD medium) was 5.08 pmol/10^6 cells/s and was set as 100%. The experiments were repeated three times, and the results are shown as means ± S.D. (*error bars*). The results are expressed relative to the value for the wild-type strain grown in 2% glucose.

Transcription of TCA cycle genes is regulated by heme

To determine whether the transcription of HAP complex-regulated genes encoding enzymes of the TCA cycle is also regulated by heme, we measured mRNA levels of *CIT1*, *SDH1*, and *SDH2* in *hem1*Δ, *hem1*Δ*hap1*Δ, *hem1*Δ*hap2*Δ, and *hem1*Δ*hap1*Δ*hap2*Δ cells in YEP medium (1% yeast extract, 2% Bacto peptone) containing glucose or galactose and different concentrations of ALA. As expected, the mRNA levels were significantly lower in cells grown on glucose in comparison with galactose-grown cells. In *hem1*Δ cells grown on glucose or galactose, the mRNA levels of the three genes correlated with the ALA concentration (Fig. 4, *C–H*). Both Hap1p and the HAP complex make comparable contributions to transcription of *CIT1*, *SDH1*, and *SDH2* in glucose-grown cells (Fig. 4, *C*, *E*, and *G*). However, the HAP complex appears to play a more dominant role than Hap1p in cells grown on galactose (Fig. 4, *D*, *F*, and *H*). This is likely due to the higher activity of the HAP complex due to the higher cellular heme level in cells grown on galactose (Figs. 2*B* and 5, *A* and *B*).

Mitochondrial respiration correlates with cellular heme level

To confirm that the heme synthesis correlates with the concentration of ALA in the medium, we determined the cellular heme levels in *hem1*Δ, *hem1*Δ*hap1*Δ, *hem1*Δ*hap2*Δ, and *hem1*Δ*hap1*Δ*hap2*Δ cells grown in media containing glucose or galactose and different concentrations of ALA (Fig. 5, *A* and *B*). In both glucose- and galactose-grown cells, the cellular heme levels correlated with the ALA concentration in the medium. However, even at the highest concentration of ALA (300 μg/ml), the heme levels in *hem1*Δ cells grown on glucose or galactose did not reach the levels in wild-type cells grown on the corresponding carbon source (compare Figs. 2*B* and 5, *A* and *B*). In cells grown in glucose with the highest concentration of ALA, the heme levels in *hem1*Δ*hap1*Δ, *hem1*Δ*hap2*Δ, and *hem1*Δ*hap1*Δ*hap2*Δ cells did not significantly differ from *hem1*Δ cells. However, the heme levels in galactose-grown *hem1*Δ*hap1*Δ cells were elevated about 2-fold in comparison with the *hem1*Δ cells, whereas the heme levels in *hem1*Δ*hap2*Δ and *hem1*Δ*hap1*Δ*hap2*Δ cells were significantly decreased (Fig. 5*B*).

HAP4 transcription in *hem1*Δ cells grown on either glucose or galactose media supplemented with high concentrations of ALA was significantly increased in comparison with *HAP4* transcription in wild-type cells grown on the corresponding carbon source (compare Figs. 2*A* and 4, *A* and *B*) despite the fact that the cellular heme levels in *hem1*Δ cells were decreased (compare Figs. 2*B* and 5, *A* and *B*). This result suggests that one or several intermediates of the heme biosynthetic pathway, perhaps ALA itself, accumulates in *hem1*Δ cells supplemented with high concentrations of ALA and activates Hap1p and the HAP complex, resulting in increased *HAP4* transcription.

To determine whether respiration is affected by cellular heme levels, we measured oxygen consumption in *hem1*Δ, *hem1*Δ*hap1*Δ, *hem1*Δ*hap2*Δ, and *hem1*Δ*hap1*Δ*hap2*Δ cells grown in media containing glucose or galactose and different concentrations of ALA (Fig. 5, *C* and *D*). In glucose-grown cells, the oxygen consumption correlated with the ALA concentration in the medium. However, even at the highest concentration of ALA (300 μg/ml), the oxygen consumption in *hem1*Δ cells did not reach the oxygen consumption of wild-type cells. As expected, the highest oxygen consumption in *hem1*Δ*hap1*Δ, *hem1*Δ*hap2*Δ, and *hem1*Δ*hap1*Δ*hap2*Δ cells was reached in medium containing the highest concentration of ALA. Still, the values were reduced in comparison with *hem1*Δ cells (Fig. 5, *C* and *D*). We observed similar trends in *hem1*Δ, *hem1*Δ*hap1*Δ, *hem1*Δ*hap2*Δ, and *hem1*Δ*hap1*Δ*hap2*Δ cells grown on galactose, but the oxygen consumption reached at the highest concentrations of ALA were higher than in glucose-containing medium. This was expected because we reported previously that oxygen consumption in wild-type cells grown on galactose was about 2.5-fold higher than in wild-type cells grown on glucose (36).

Inactivation of ROX1 induces heme synthesis and mitochondrial respiration

The addition of excess ALA to *hem1*Δ cells did not result in cellular heme levels above the levels found in the wild-type cells grown on the corresponding carbon source (compare Figs. 2*B* and 5, *A* and *B*). The reason for this is currently not clear. It is probable that the exogenous ALA is not completely taken up by the cells or efficiently transported to the appropriate cellular compartment. It is also possible that the excess of exogenous ALA results in accumulation of intermediates of the heme biosynthetic pathway. These intermediates might not be effectively converted to heme and might affect activity of enzymes in the heme biosynthetic pathway (50).

In an effort to find a model with which we could test the effect of elevated cellular heme level on transcription of *HAP4* and mitochondrial respiration, we characterized the *rox1*Δ mutant. Rox1p is a repressor of anaerobic genes, such as *ANB1*. The heme-dependent repression by Rox1p is due to Hap1p-mediated activation of *ROX1* transcription. When the cellular level of heme increases, Hap1p is activated and stimulates *ROX1* transcription. Under hypoxic conditions when heme is absent, *ROX1* is not transcribed, and hypoxic genes are activated (32, 49, 51, 52). In addition, Rox1p represses transcription of its own gene (53, 54).

One of the genes regulated by Rox1p is *HEM13*, which encodes coproporphyrinogen III oxidase, one of the enzymes of the heme biosynthetic pathway (48). We speculated that derepression of *HEM13* upon inactivation of *ROX1* would result in an elevated cellular heme level. This hypothesis was correct; the heme level in glucose-grown cells was about 2.6-fold higher in *rox1*Δ cells than in the wild-type cells (Fig. 6*A*). In addition, the increased cellular level of heme correlated with increased oxygen consumption, which increased about 2.8-times in *rox1*Δ cells in comparison with wild-type cells (Fig. 6*B*). These results show that the oxygen consumption in *rox1*Δ cells grown on glucose is almost identical to the oxygen consumption of wild-type cells grown on galactose (36), suggesting that respiration is not repressed by glucose in *rox1*Δ cells. The increased cellular heme level and oxygen consumption in *rox1*Δ cells are associated with elevated ATP level and increased transcription of *HAP4*, *CIT1*, *SDH1*, and *SDH2* (Fig. 6, *C* and *D*). The increased heme and ATP levels, oxygen consumption, and transcription of the TCA cycle genes in *rox1*Δ cells require Hap1p and the HAP complex (Fig. 6). This finding is consistent with the notion that Hap1p and the HAP complex function as heme receptors and mediate the effect of heme on respiration.

Interestingly, the cellular ATP level was also significantly upregulated in *hap4*Δ cells (Fig. 6*C*). Because *HAP4* inactivation decreases oxygen consumption (Fig. 6*B*) and expression of TCA cycle genes (Fig. 6*D*), the increased ATP level in *hap4*Δ cells is likely not caused by increased activity of the TCA cycle, ETC, and OXPHOS. It seems more likely that activation of glycolysis is responsible for the increased ATP level, but the mechanism of this activation is not known.

Overexpression of HEM3 or HEM12 increases cellular heme level and induces mitochondrial respiration

Because Rox1p is a transcriptional repressor, it may directly or indirectly affect transcription of *HAP4* and genes required for the TCA cycle and OXPHOS, independently of its role in regulating the cellular heme level. To eliminate this possibility, we considered other approaches to increase cellular heme level. Heme synthesis can be increased by overexpressing *HEM3*, which encodes porphobilinogen deaminase, or *HEM12*, which encodes uroporphyrinogen decarboxylase (50, 55). To conclusively prove that it is the heme level that is responsible for the induction of mitochondrial respiration, we individually overexpressed *HEM3* and *HEM12* in wild-type cells.

Overexpression of *HEM3* or *HEM12* in wild-type cells increased the cellular heme level 2.2- and 2-fold, respectively (Fig. 7*A*). Importantly, overexpression of *HEM3* or *HEM12* increased oxygen consumption more than 3- and 4-fold, respectively (Fig. 7*B*). The oxygen consumption in glucose-grown cells overexpressing *HEM3* or *HEM12* is even higher than the oxygen consumption of wild-type cells grown on galactose or non-fermentable carbon sources (36). As expected, overexpression of *HEM3* or *HEM12* also increased the cellular ATP levels and transcription of *HAP4*, *CIT1*, *SDH1*, and *SDH2* (Fig. 7, *C* and *D*). Because Hem3p and Hem12p do not play any role in transcriptional regulation, the effect of overexpressing the corresponding genes on the induction of respiration and transcription of *HAP4* and genes encoding enzymes of the TCA

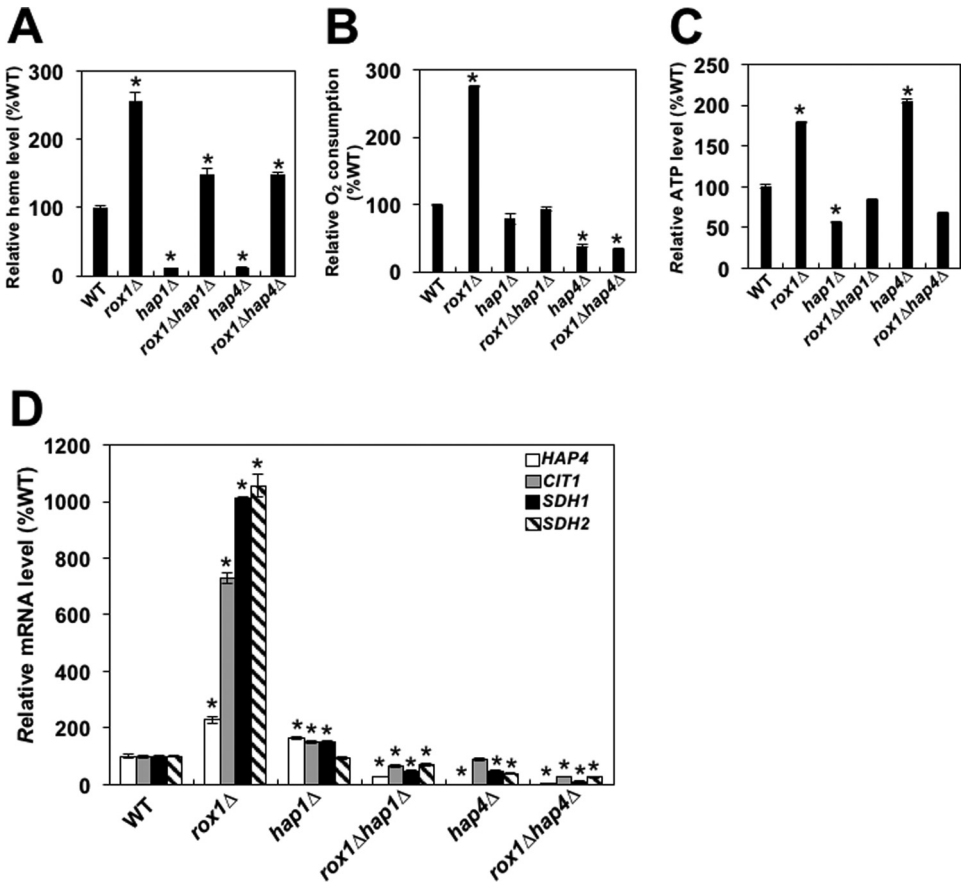

Figure 6. Inactivation of *ROX1* induces heme synthesis and mitochondrial respiration. Cellular heme levels (*A*), oxygen consumption (*B*), cellular ATP levels (*C*), and relative mRNA levels of the indicated genes (*D*) were determined in WT (W303-1a), *rox1*Δ (TZ917), *hap1*Δ (TZ766), *rox1*Δ*hap1*Δ (TZ992), *hap4*Δ (LG579), and *rox1*Δ*hap4*Δ (TZ974) strains. The ATP level in WT cells grown on glucose (YPD medium) was 0.58 μmol/10^{10} cells and was set as 100%. The cells were grown in YEP medium containing 2% glucose. The experiments were repeated three times, and the results are shown as means ± S.D. (*error bars*). Values that are statistically different ($p < 0.05$) from the WT cells are indicated by an *asterisk*.

cycle can be solely attributed to the increased cellular level of heme.

Discussion

The key finding of this study is that heme induces metabolic transition from fermentation to mitochondrial respiration even under glucose-repressing conditions (Fig. 8). Glucose represses many genes involved in the TCA cycle, ETC, and OXPHOS by a mechanism that is independent of PKA and Mig1p/Snf1p pathways. A number of these genes are regulated by the HAP complex (4, 11). Our results indicate that the mechanism of glucose repression of these genes involves low cellular heme level due to the low metabolic flux into the TCA cycle and insufficient availability of succinyl-CoA for ALA and heme synthesis. Low cellular heme level in glucose-grown cells results in low activity of the HAP complex and low transcription of HAP complex-regulated genes. Increasing heme synthesis activates the HAP complex and induces transcription of HAP complex-regulated genes and transition from fermentation to respiration.

In aerobic organisms, heme serves as a prosthetic group in proteins and enzymes that sense, transport, or use oxygen, such as hemoglobin, myoglobin, cytochrome complexes, catalases, and cyclooxygenases (31). Heme is also required for ATP production by the mitochondrial ETC and OXPHOS. To accomplish these diverse roles, heme controls activities of a number of proteins involved in signal transduction and transcription (31). In yeast, the major players involved in the heme transcriptional regulatory network are Hap1p and the HAP complex as well as repressors Rox1p and Mot3p.

Heme synthesis in yeast is regulated by the availability of oxygen, and heme serves as an intermediate in the signaling mechanism for sensing oxygen levels (32, 33). Our results suggest that the cellular heme level is also regulated by a carbon source; compared with glucose-grown cells, the heme levels in cells grown on galactose are 2.7 times higher (Fig. 2*B*). The increased heme level caused by addition of ALA in cells grown on glucose medium (Fig. 2*B*) indicates that the limiting step of heme synthesis in glucose-grown cells is the synthesis of ALA. ALA is produced from succinyl-CoA and glycine by 5-aminolevulinate synthase, encoded by the *HEM1* gene. Because the metabolic flux into the TCA cycle is low in glucose-grown cells (7, 8), it is likely that the synthesis of succinyl-CoA, an intermediate of the TCA cycle, is limiting for the production of ALA and heme (Figs. 1 and 8). Derepression of *HEM13* in *rox1*Δ cells or overexpression of *HEM3* or *HEM12* leads to increased conversion of ALA to heme (Figs. 6 and 7), which in turn induces transcription of *HAP4* and activates Hap1p and the HAP complex. Activation of Hap1p and the HAP complex leads to

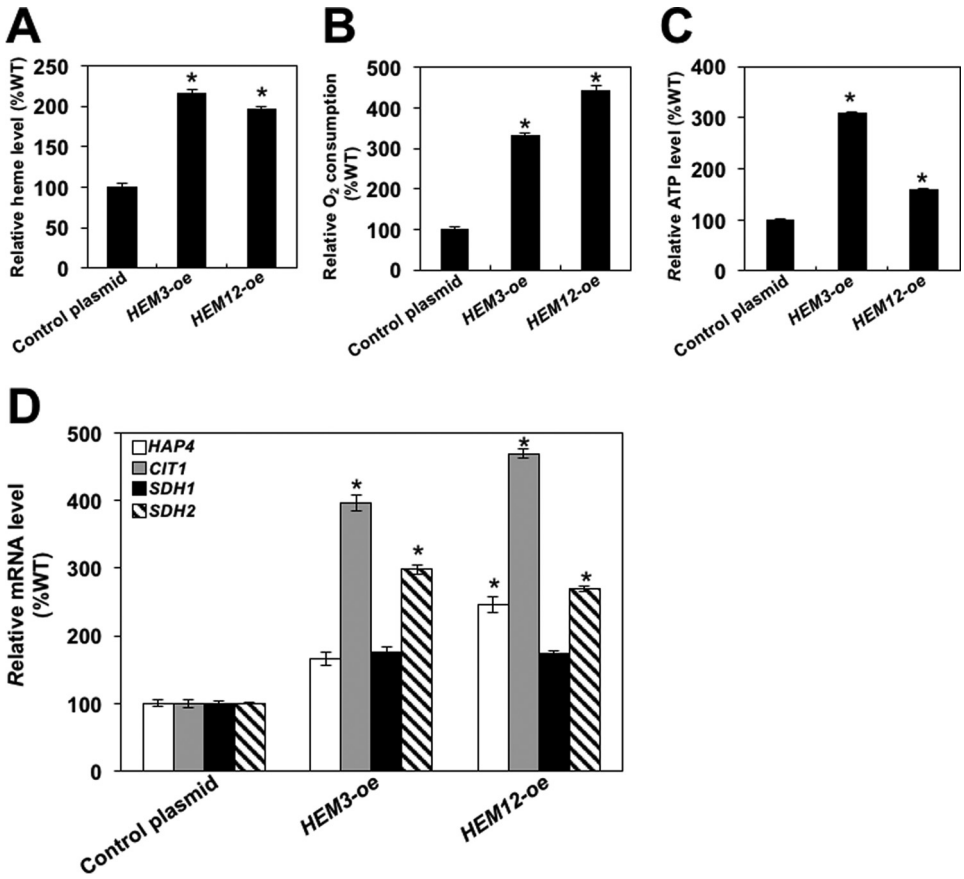

Figure 7. Overexpression of *HEM3* or *HEM12* increases cellular heme level and induces mitochondrial respiration. Cellular heme levels (*A*), oxygen consumption (*B*), cellular ATP levels (*C*), and relative mRNA levels of the indicated genes (*D*) were determined in wild-type cells containing either the control plasmid (TZ1099) or high-copy-number plasmid expressing *HEM3* (*HEM3-oe*; strain TZ1100) or *HEM12* (*HEM12-oe*; strain TZ1101). The cells were pregrown under selection in synthetic complete medium and inoculated to an $A_{600\ nm}$ of 0.2 into YEP medium containing 2% glucose and grown for two generations. The experiments were repeated three times, and the results are shown as means ± S.D. (*error bars*). Values that are statistically different ($p < 0.05$) from the wild-type cells containing the control plasmid are indicated by an *asterisk*.

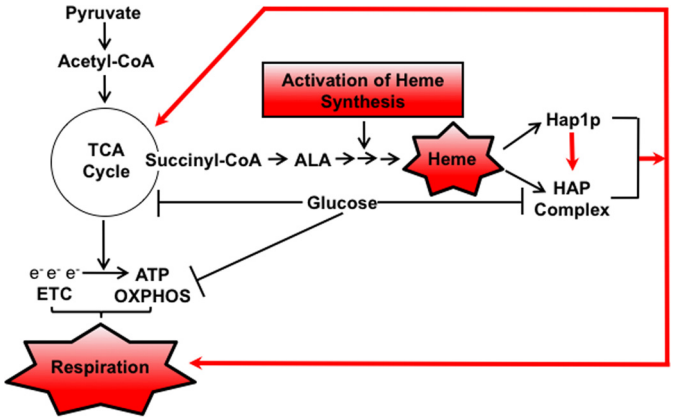

Figure 8. Increased synthesis of heme overcomes glucose repression of mitochondrial respiration. Glucose represses TCA cycle genes and synthesis of succinyl-CoA, resulting in a low level of ALA and heme. Activation of heme synthesis by overexpressing *HEM3* or *HEM12* or by derepressing *HEM13* (in *rox1*Δ cells) results in elevated levels of heme and activation of Hap1p and the HAP complex, leading to activation of genes encoding enzymes of the TCA cycle, ETC, and OXPHOS.

increased expression of genes encoding the TCA cycle enzymes and increased synthesis of succinyl-CoA, ALA, and heme. The negative regulator in this mechanism is Rox1p. Transcription of *ROX1* is activated by heme in a Hap1p-dependent manner, and Rox1p controls heme synthesis by repressing *HEM13* and *HEM14* (48, 49). This model is also consistent with the fact that the addition of ALA to wild-type cells results in about a 4-fold increase of cellular heme level (Fig. 2*B*) and that the same addition of ALA to *hem1*Δ cells results in cellular heme levels that correspond to only about 70% of the wild-type level (Fig. 5*A*). We interpret this result to mean that the addition of ALA to wild-type cells increases heme synthesis, resulting in increased activation of Hap1p and the HAP complex, increased transcription of *HAP4* and genes encoding TCA cycle enzymes, and increased synthesis of succinyl-CoA and, therefore, ALA. Introducing the *hem1*Δ mutation prevents *de novo* synthesis of ALA and does not allow the cellular heme to reach the wild-type levels.

In *hem1*Δ cells grown on glucose, high concentrations of ALA do not increase heme levels (Fig. 5*A*), but they do lead to about a 4-fold increase in *HAP4* transcription (Fig. 4*A*). However, this increased *HAP4* transcription is not accompanied by induction of respiration (Fig. 5*C*). Thus, increased transcription of *HAP4* in the absence of elevated heme level is not able to induce respiration. In contrast, increased heme synthesis in *rox1*Δ cells or in cells overexpressing *HEM3* or *HEM12* results in the induction of respiration despite the fact that it induces only a relatively modest increase in *HAP4* transcription (Figs. 6 and 7). These findings indicate that the cellular level of heme is

Table 1
Yeast strains used in this study

Strain	Genotype	Source/Ref.
W303-1a	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100*	R. Rothstein
W303-1α	*MATα ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100*	R. Rothstein
W303	*MATa/MATα ade2–1/ade2–1 his3–11,15/his3–11,15 leu2–3,112/leu2–3,112 trp1–1/ trp1–1ura3–1/ ura3–1 can1–100/ can1–100*	R. Rothstein
YPL101W	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 hap1::KAN*	Open Biosystems
TZ766	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hap1::KAN*	This study
YGL237C	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 hap2::KAN*	Open Biosystems
TZ742	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hap2::KAN*	This study
FY2867	*MATa ura3Δ0 his3Δ200 leu2Δ0 lys2–128 hap2Δ0::LEU2*	59
TZ742	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hap2::LEU*	This study
TZ354	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 pda1::KAN*	36
TZ341	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 mpc1::KAN*	36
FY2612	*MATa ura3Δ0 his3Δ0 his3Δ0 lsy2–128 myc-HAP1*	59
HAP4-Myc9	*MATa ura3Δ0 his3Δ200 leu2Δ0 lys2–128 myc-HAP4::HPH*	60
TZ909	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 myc-HAP4::HPH*	This study
YDR232W	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 hem::KAN*	Open Biosystems
TZ779	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hem1::KAN*	This study
TZ864	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hem1:: KAN hap1::KAN*	This study
TZ881	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hem1:: KAN hap2::LEU*	This study
TZ884	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hem1:: KAN hap1:: KAN hap2::LEU*	This study
YPR065W	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 rox1::KAN*	Open Biosystems
TZ917	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 rox1::KAN*	This study
TZ992	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 rox1:: KAN hap1::KAN*	This study
YKL109W	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 hap4::KAN*	Open Biosystems
LG579	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 hap4::KAN*	36
TZ974	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100 rox1:: KAN hap4::KAN*	This study
TZ1099	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100* pRS426 [*URA3*, 2μ]	This study
TZ1100	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100* pRS426-*HEM3* [*URA3*, 2μ, *HEM3*]	This study
TZ1101	*MATa ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 ssd1-d2 can1–100* pRS426-*HEM12* [*URA3*, 2μ, *HEM12*]	This study

the primary signal that triggers the metabolic transition from fermentation to respiration (Fig. 8). The heme-induced activation of *HAP4* transcription is part of the cellular response to increased heme level, but when *HAP4* transcription is dissociated from heme synthesis, it does not induce respiration (compare Figs. 4 and 6). This conclusion underscores the importance of Hap1p and HAP complex activation by heme.

How does heme orchestrate the transition of the metabolic mode from fermentation to respiration? Our results show that the transition requires both Hap1p and the HAP complex. The increased respiration due to the increased synthesis of heme in *rox1*Δ cells is attenuated by introducing *hap1*Δ or *hap4*Δ mutation (Fig. 6). The requirement for *HAP4* is not surprising because *HAP4* and the integrity of the HAP complex are required for induction of respiration and viability when cells are grown on non-fermentable carbon sources (28). In addition, overexpression of *HAP4* results in increased respiration even in cells grown on glucose (24–26). The requirement of Hap1p for heme-induced respiration is consistent with the role of Hap1p in the regulation of *HAP4* transcription and suggests that activation of the HAP complex by heme is alone not sufficient for induction of respiration. Our results thus indicate that heme has at least two targets required for activation of respiration: Hap1p and the HAP complex. However, we cannot exclude the possibility that heme directly regulates other proteins important for respiration. For example, Mss51p, a *COX1* mRNA-specific translational activator and Cox1p chaperone, is a heme-binding protein whose function in cytochrome *c* oxidase biogenesis is also regulated by heme (56).

The transcriptional regulation of *HAP4* is not completely understood. The *HAP4* promoter contains Cat8p-binding sites; however, *cat8*Δ mutation has no effect on *HAP4* transcription (57). The *HAP4* promoter also contains a canonical Mig1p-binding site; however, Mig1p binding to the *HAP4* promoter has not been demonstrated, and the *mig1*Δ mutation does not result in activation of respiration (7, 42, 58). Our results show that both Hap1p and Hap4p are recruited to the *HAP4* promoter and that *HAP4* transcription is stimulated by heme (Figs. 2 and 4). Thus, heme regulates both the activity of HAP complex (27) and transcription of *HAP4* (Figs. 2–4).

Altogether, this study suggests that the glucose-mediated repression of respiration in budding yeast is at least partly due to the low cellular heme level. The metabolic flux into the TCA cycle is low in glucose-grown cells (7, 8) partly due to the high activity of pyruvate decarboxylase Pdc1p (5, 6), which limits the amount of pyruvate available for conversion to acetyl-CoA in mitochondria. This results in low levels of succinyl-CoA, a precursor for ALA and heme synthesis. Increasing conversion of ALA into heme activates Hap1p and the HAP complex, which results in induction of TCA cycle genes, increased metabolic flux into the TCA cycle, and increased synthesis of succinyl-CoA. This mechanism also explains the observed activation of the HAP complex during the diauxic shift (23). As glucose is depleted and cells begin to utilize ethanol, the metabolic flux into the TCA cycle increases together with the synthesis of succinyl-CoA, ALA, and heme, leading to the activation of Hap1p and the HAP complex and induction of TCA cycle, ETC, and OXPHOS genes.

Experimental procedures

Yeast strains, plasmids, and media

All yeast strains used in this study are listed in Table 1. Standard genetic techniques were used to manipulate yeast strains and to introduce mutations from non-W303 strains into the W303 background (61). To construct *HEM3* and *HEM12* overexpression plasmids, *HEM3* and *HEM12* genomic sequences, spanning 1.0 kb upstream and 400 bp downstream of the coding

region, were cloned by PCR using Phusion high-fidelity DNA polymerase (M0530S, New England Biolabs) with primers specific to *HEM3* (5′-AGGGTTAGACTAGTATATCAATCCCG-3′ and 5′-TAATAACTCGAGATTTCATTTTTTATATACAAGCTA-3′) and *HEM12* (5′-TGCTTCTGTCTAGAAAAACGAGC-3′ and 5′-CATTTTACTCGAGTTTTGTGAATTCATGC-3′). The *HEM3* forward and reverse primers contain SpeI and XhoI sites, respectively, and the *HEM12* forward and reverse primers contain NotI and XhoI sites, respectively. Following double digestion with the appropriate restriction enzymes, *HEM3* and *HEM12* genomic DNA fragments were ligated into the high-copy-number plasmid pRS426. Cells were grown at 28 °C in YEP medium containing 2% glucose, 2% galactose, or 2% acetate or under selection in synthetic complete medium containing 2% glucose and, when appropriate, lacking specific nutrients to select for a particular genotype.

Oxygen consumption

Cells were grown to an $A_{600\,nm}$ of 0.6 in YEP medium containing either 2% glucose or galactose, and 9×10^6 cells were harvested by centrifugation. Cells were resuspended in a buffer containing 10 mM HEPES, 25 mM K_2HPO_4, pH 7.0, and incubated at 30 °C in an oxygen consumption chamber (Instech Laboratories, Inc.) connected to a NeoFOX fluorescence-sensing detector using NeoFOX software (Ocean Optics, Inc.). Results were calculated as pmol of $O_2/10^6$ cells/s and expressed as percentages of the wild-type value. The oxygen consumption rate in wild-type (WT) cells grown on glucose (YPD medium) was 5.08 pmol/10^6 cells/s and was set as 100%.

ATP and heme assays

For the ATP assay, cells were grown to an $A_{600\,nm}$ of 0.6 in YEP medium containing 2% glucose, and 9×10^7 cells were harvested by centrifugation and lysed in 5% trichloroacetic acid with prechilled glass beads. Cell lysate was neutralized to pH 7.5 with 10 M KOH and 2 M Tris-HCl, pH 7.5. ATP levels were measured using the ENLITEN ATP assay (FF2000, Promega) according to the manufacturer's instructions and normalized by the number of cells. The ATP level in WT cells grown on glucose (YPD medium) was 0.58 μmol/10^{10} cells and was set as 100%. For the heme assay, cells were grown to an $A_{600\,nm}$ of 0.8 in YEP medium containing either 2% glucose or galactose, and 3×10^8 cells were harvested by centrifugation and lysed in 200 μl of 1% NaOH and 5% Triton with prechilled glass beads. Cell lysate was neutralized to pH 7.5 with 1 M HCl and 2 M Tris-HCl, pH 7.5. Heme was assayed using a heme colorimetric assay kit (K672-100, BioVision). The heme level in WT cells grown on glucose (YPD medium) was 17.3 pmol/10^{10} cells and was set as 100%.

Real-time RT-PCR

Real-time RT-PCR was performed as described (62) using the following primers: *ACT1*, 5′-TATGTGTAAAGCCGGTTTTGC-3′ and 5′-GACAATACCGTGTTCAATTGGG-3′; *HAP4*, 5′-CCGCAAAGACTTTTCTACACAGG-3′ and 5′-TGTTATGATGGTTGGTATTTGGG-3′; *CIT1*, 5′-CAGCGATATTATCAACAACTAGCA-3′ and 5′-TAGTGGCGAGCATTCAATAGTG-3′; *SDH1*, 5′-CTCCAAGTTGACTTTGCTCAGAA-3′ and 5′-ACGCGGAACCGTTTACAGA-3′; and *SDH2*, 5′-ATTGAGAAGGAAGGCCTTTTGT-3′ and 5′-AGTTTTCAATCTGGGGGTATGC-3′.

ChIP assays

In vivo chromatin cross-linking and immunoprecipitation were performed essentially as described (63, 64). Immunoprecipitation was performed with the anti-myc antibody (2276, Cell Signaling Technology). The primers used for real-time PCR are as follows: *POL1*, 5′-TCCTGACAAAGAAGGCAATAGAAG-3′ and 5′-TAAAACACCCTGATCCACCTCTG-3′; *HAP2*, 5′-AGACGAAACGGATGCGAAAT-3′ and 5′-AAGCTGCTGCCGTTGATGT-3′; *HAP3*, 5′-ATGAATACCAACGAGTCCGAACA-3′ and 5′-GAAATTTGCTGCAAACTGCC-3′; *HAP4-1*, 5′-GAAATAGATGCATTTTATGTGCGA-3′ and 5′-GGTTTGCATTCTATTCGTTACCC-3′; *HAP4-2*, 5′-CTATGAGATCACTATTTTGCCGGA-3′ and 5′-GAAATTCTCAGCAGAGGTTATCCC-3′; *HAP4-3*, 5′-CTTATGTTACGGATCTTGCACG-3′ and 5′-TCTGCAAAATCGATACATGACAC-3′; *HAP4-4*, 5′-TGGTTGTTTAGGTCCATCTCCTT-3′ and 5′-AAAACTGGGGTTTAGAGAGGTGA-3′; *HAP4-5*, 5′-CGCATAGGAAGAGAAAAAACACA-3′ and 5′-CAGCAACCCATTAAAATGCTC-3′; *HAP4-6*, 5′-CCGCAAAGACTTTTCTACACAGG-3′ and 5′-TGTTATGATGGTTGGTATTTGGG-3′; *HAP5*, 5′-GACTGATAGGAATTTCTCACCACA-3′ and 5′-CTCTCTGAATCATCGTACTGGGTT-3′; *ROX1*, 5′-CACCTAAGATTCCAAGACCCAA-3′ and 5′-GCTGTCTGAACAGAATAAATGCG-3′; *CYC1*, 5′-TTCTGCTAAGAAAGGTGCTACACT-3′ and 5′-ACCATGCAAGTTTGGACCAA-3′; *CIT1*, 5′-CAGCGATATTATCAACAACTAGCA-3′ and 5′-TAGTGGCGAGCATTCAATAGTG-3′; *QCR7*, 5′-ACGTCTATTGCGAGAATTGGTG-3′ and 5′-AGCCCTAACTTCTTGTAACCTGC-3′; and *KGD2*, 5′-TAAGGTTCGTGTCTTCGCAAAC-3′ and 5′-CCGACAATTTTGACAGTAGATGC-3′.

Replicative life span

Yeast replicative life span was determined as described (65, 66). Briefly, virgin daughter cells were allowed to grow into mother cells and produce daughter cells on YPD medium with and without ALA (300 μg/ml). The daughter cells were microdissected from mother cells and counted until the mother cells could no longer divide. Forty cells were analyzed in each experiment.

Statistical analysis

The results represent at least three independent experiments. Numerical results are presented as means ± S.D. Data were analyzed using an InStat software package (GraphPad, San Diego, CA). Statistical significance was evaluated by one-way analysis of variance, and $p < 0.05$ was considered significant.

Author contributions—T. Z. conceived the project, conducted most of the experiments, analyzed the results, prepared the figures, and wrote the paper. P. B. designed and conducted some of the experiments. J. Z. conducted some of the experiments. A. V. conceived the project, analyzed the data, wrote the paper, and coordinated the study.

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