Received: 8 June 2022 | Accepted: 1 August 2022

DOI: 10.1111/1462-2920.16157

RESEARCH ARTICLE

environmental microbiology Applied Microbiology International

Transcriptional regulation of ergosterol biosynthesis genes in response to iron deficiency

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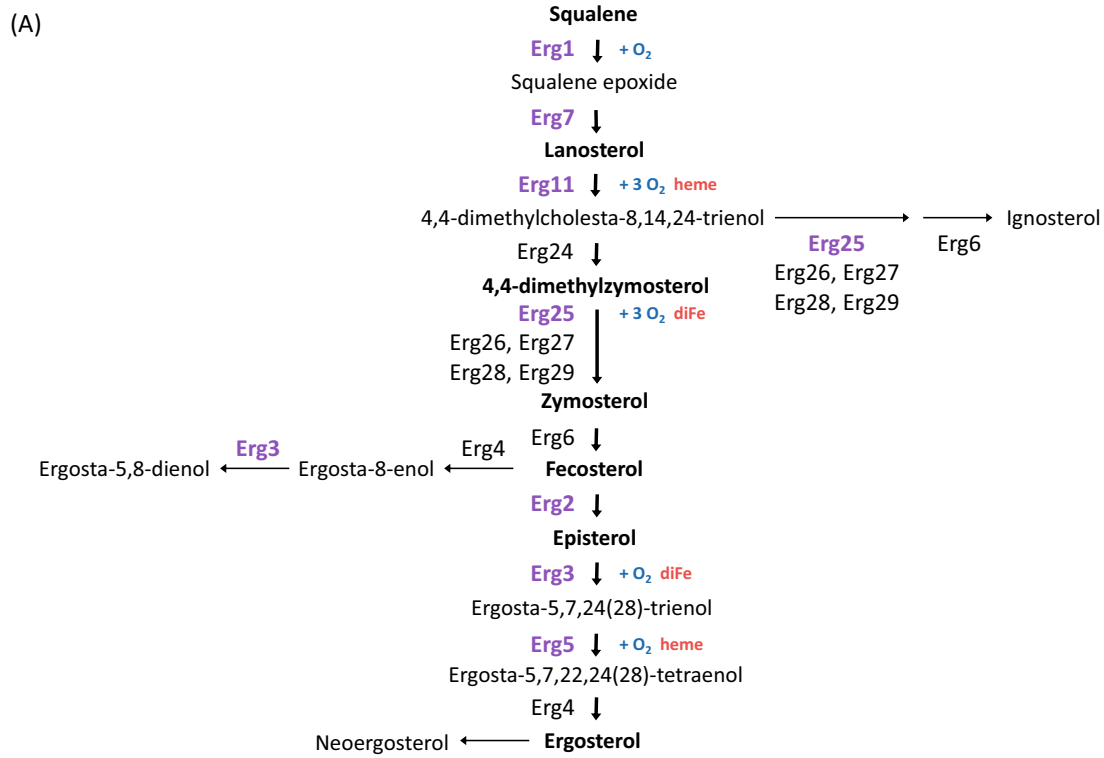
Funding information
Consejo Superior de Investigaciones Científicas Open Access Publication Support Initiative; Generalitat Valenciana, Grant/Award Numbers: ACIF/2019/214, AICO/2020/086; MCIN/AEI/10.13039/501100011033, Grant/Award Numbers: FPU2017/03542, PID2020-116940RB-I00, PID2020-120066RB-I00

Abstract

Iron participates as an essential cofactor in the biosynthesis of critical cellular components, including DNA, proteins and lipids. The ergosterol biosynthetic pathway, which is an important target of antifungal treatments, depends on iron in four enzymatic steps. Our results in the model yeast *Saccharomyces cerevisiae* show that the expression of ergosterol biosynthesis (*ERG*) genes is tightly modulated by iron availability probably through the iron-dependent variation of sterol and heme levels. Whereas the transcription factors Upc2 and Ecm22 are responsible for the activation of *ERG* genes upon iron deficiency, the heme-dependent factor Hap1 triggers their Tup1-mediated transcriptional repression. The combined regulation by both activating and repressing regulatory factors allows for the fine-tuning of *ERG* transcript levels along the progress of iron deficiency, avoiding the accumulation of toxic sterol intermediates and enabling efficient adaptation to rapidly changing conditions. The lack of these regulatory factors leads to changes in the yeast sterol profile upon iron-deficient conditions. Both environmental iron availability and specific regulatory factors should be considered in ergosterol antifungal treatments.

INTRODUCTION

Sterols, mainly cholesterol in animals and ergosterol in fungi, are key components of eukaryotic cell membranes that maintain the fluidity, permeability and activity of membrane-associated proteins. Dynamic changes in the sterol composition allow yeast cells to adapt to metabolic remodelling and environmental stresses (Ernst et al., 2018; Johnston et al., 2020). However, the rate of ergosterol biosynthesis is limited by iron bioavailability because the late biosynthetic pathway includes enzymes that utilize iron as an essential redox cofactor, such as the hemoproteins Erg11 and Erg5, and the oxo-diiron enzymes Erg25 and Erg3 (Figure 1A) (Jorda & Puig, 2020). Iron is also indispensable for other critical biological processes, such as mitochondrial respiration or the synthesis of DNA, proteins, sphingolipids, and unsaturated fatty acids. Despite the high abundance of iron, its bioavailability is extremely low. Consequently, cells have developed refined



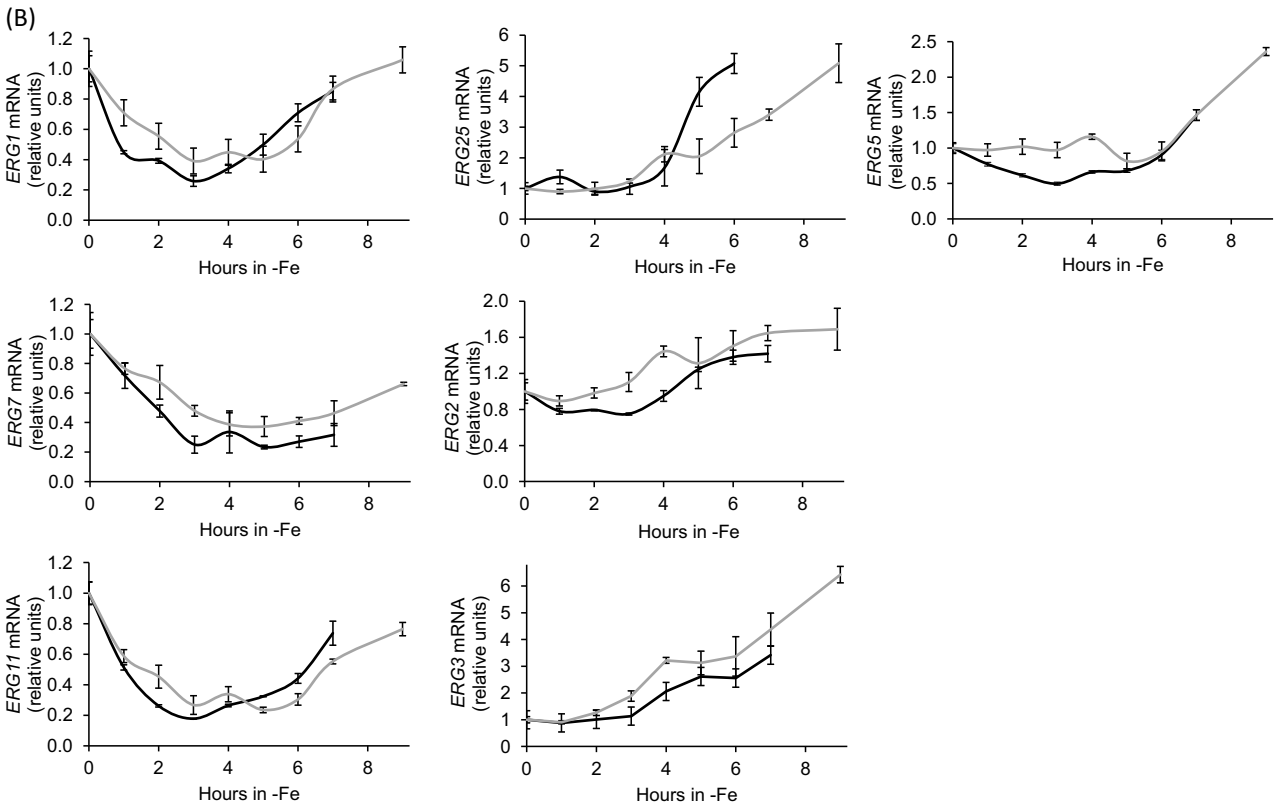


FIGURE 1 Iron deficiency alters transcript levels of ergosterol biosynthesis (*ERG*) genes. (A) Late ergosterol biosynthesis pathway in *S. cerevisiae*. Enzymes, intermediates and requirements of oxygen and iron are indicated. Enzymes whose gene expression is investigated in this work are in purple. (B) Kinetics of *ERG* mRNA levels under iron deficiency. Wild-type FY2609 (black line) and BY4741 (grey line) strains were cultivated overnight in SC medium at 30°C for 15 h to exponential phase. Then, an aliquot was extracted (0 h) and 100 μM BPS were added to the medium (–Fe). Cells were cultivated for 7 h (FY2609 strain) or 9 h (BY4741 strain), and aliquots were removed every hour for total RNA extraction. The mRNA levels of the indicated *ERG* genes were determined by RT-qPCR and were normalized with *ACT1*. Data represent the average and standard deviation (SD) of three biologically independent experiments.

regulatory strategies to properly respond to iron depletion and its secondary effects such as ergosterol and heme depletion. Transcriptomic and proteomic studies suggest that the expression of ergosterol biosynthesis (*ERG*) genes is heterogeneously altered by iron deficiency (Hausmann et al., 2008; Navarrete-Perea et al., 2021; Puig et al., 2005; Romero et al., 2019). Nevertheless, the molecular mechanisms that contribute to *ERG* regulation under iron depletion have not yet been investigated.

In *S. cerevisiae*, the expression of many *ERG* genes is regulated at the transcriptional level by sterol abundance through two paralog transcription activators, Upc2 and Ecm22 (Jorda & Puig, 2020). Both transcription factors contain an amino-terminal Zn_2-Cys_6 DNA-binding domain that enables their binding to TATACGA DNA motifs, known as sterol regulatory elements (SREs), which are identical to the anaerobic response elements AR1c, and are located in the promoter of *ERG* genes (Gallo-Ebert et al., 2013; Vik & Rine, 2001). Upc2 also binds to TAAACGA anaerobic response (AR1b) elements within some *ERG* promoters (Gallo-Ebert et al., 2013). Under normal growth conditions, Ecm22 is more abundant at *ERG* promoters promoting their expression; however, when sterols are depleted, Ecm22 levels drop, while Upc2 increases its abundance and replaces Ecm22 at the promoters of *ERG* genes to strongly activate expression (Davies et al., 2005; Davies & Rine, 2006). Structural studies with Upc2 have demonstrated that a conserved carboxy-terminal α-helical fold with a deep hydrophobic pocket serves as both an ergosterol-binding and sensing domain (Yang et al., 2015). Thus, under normal conditions, ergosterol binding traps Upc2 in the cytosol, whereas ergosterol depletion leads to its nuclear accumulation and transcriptional activation (Yang et al., 2015).

In addition to Ecm22, the heme-binding protein Hap1 also contributes to the basal transcription of some *ERG* genes (Becerra et al., 2002; Davies & Rine, 2006; Tamura et al., 2004; Turi & Loper, 1992). Hap1 cooperatively binds as a dimer through conserved C6 zinc clusters to asymmetric DNA sites containing a direct repeat of two CGG/CGC triplets (Bergenholm et al., 2018; Gordan et al., 2011; Harbison et al., 2004; MacIsaac et al., 2006; Zhang & Guarente, 1994). The activity of Hap1 is regulated by heme levels through multiple elements, including its association with several chaperones and co-chaperones. Hap1 dimers are in continuous association with the chaperone Hsp70 and the co-chaperones Ydj1 and Sro9, and only the presence of heme enhances the association of this complex with the chaperone Hsp90, which leads to Hap1 activation (Hon et al., 2001; Lan et al., 2004; Zhang & Hach, 1999). Thereby, Hap1 is continuously repressed while heme allows its transient activation by, at least in part, Hsp90 interaction (Lan et al., 2004). In addition, under hypoxic conditions, heme-free Hap1 can recruit general co-repressors, like Tup1/Ssn6 and Set4, and directly repress the transcription of many genes, including some *ERG* genes (Hickman & Winston, 2007; Serratore et al., 2018). Therefore, Hap1 can act as a heme-bound activator and also as a heme-free Tup1-mediated repressor, as occurs in its regulation of the translation elongation factor eIF5A encoding gene *TIF51A* in response to changes in the mitochondrial functional status (Barba-Aliaga et al., 2020; Barba-Aliaga & Alepuz, 2022). Since ergosterol biosynthesis also depends on oxygen at critical enzymatic steps of the pathway (Figure 1A), oxygen depletion limits ergosterol production leading to the activation of Upc2 and Ecm22 (Davies & Rine, 2006; Jorda & Puig, 2020; Kwast et al., 2002). The crosstalk between opposite regulatory factors under hypoxic conditions leads to different expression patterns among *ERG* genes, which in turn depend on the severity of oxygen restriction (Jorda & Puig, 2020).

In this study, we investigated the role of the transcriptional factors Upc2, Ecm22 and Hap1 on *ERG* genes expression under iron deficiency conditions and their contribution to yeast sterol profile.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

The description of *S. cerevisiae* strains used in this report is shown in Table S1. Prior to experiments, yeast cells were cultivated overnight at 30°C in liquid synthetic complete (SC) medium (0.17% yeast nitrogen base without amino acids and without ammonium sulfate [Pronadisa], 0.5% ammonium sulfate [Panreac], 2% glucose [Panreac], and 2 g/L Kaiser drop-out [Formedium]) lacking specific requirements when necessary. For experiments shown in Figures 1 and 2, cells were re-inoculated in 50 ml of SC and incubated for 15 h at 190 rpm to early exponential phase. At that moment, 100 μM of the Fe^{2+}-specific chelator bathophenanthroline disulfonic acid disodium (BPS) (Sigma) was added to create iron-deficient media. For experiments in Figures 3 to 6, cells were re-inoculated at an OD_{600} of 0.2 in SC medium without (+Fe) or with 100 μM BPS (−Fe) and incubated for 6–9 h.

RNA analyses

Total RNA extraction and determination of mRNA levels by RT-qPCR were performed as previously

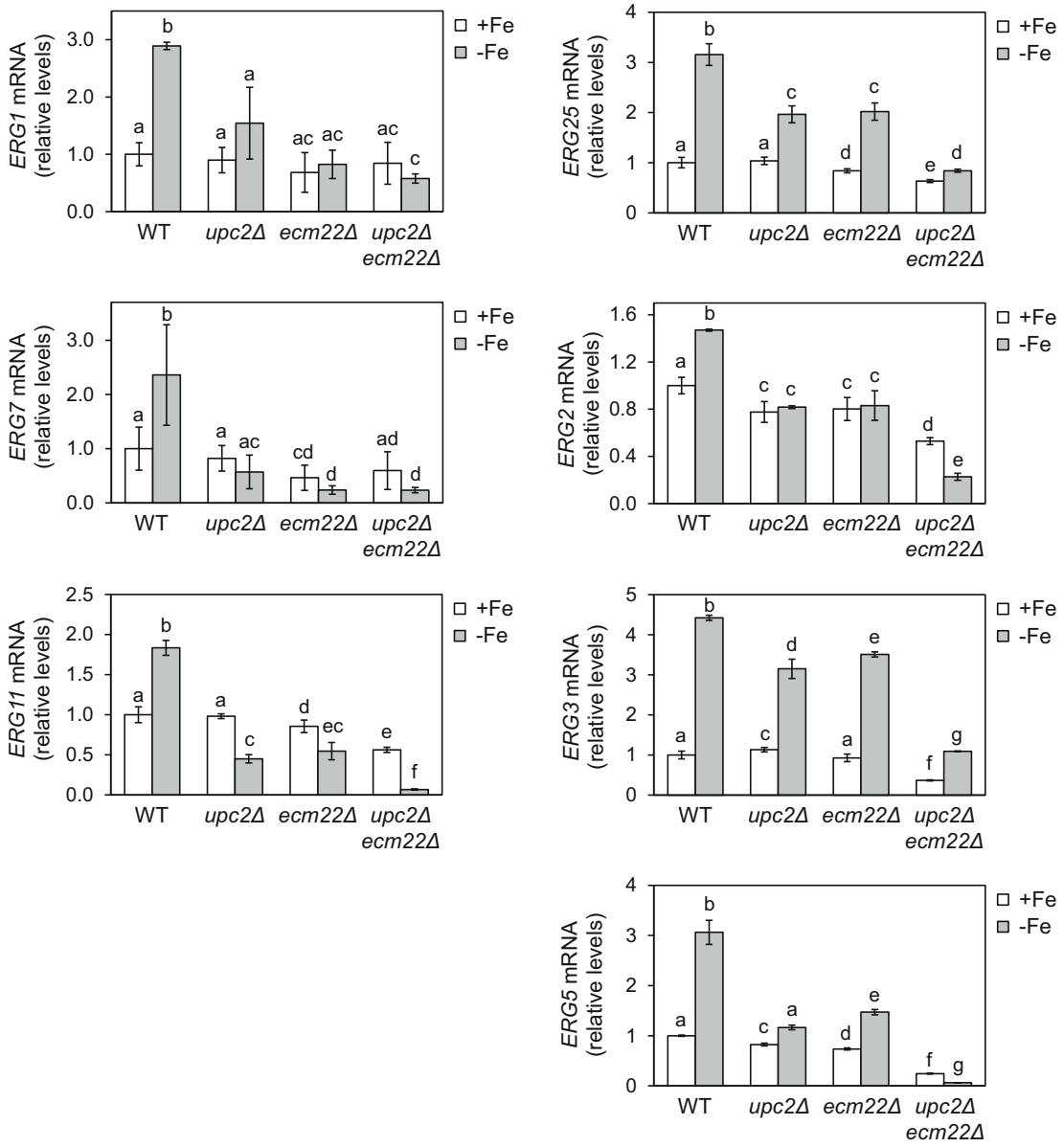


FIGURE 2 Deletion of *UPC2* and *ECM22* limits the upregulation of *ERG* genes under iron deficiency. WT (W303), *upc2*Δ, *ecm22*Δ, and *upc2*Δ*ecm22*Δ cells were grown for 15 h to exponential phase in SC medium (+Fe) and then 100 μM BPS were added, and cells were cultivated for 9 h (−Fe). *ERG* mRNA levels, normalized to *ACT1*, were determined by RT-qPCR. Data display the average and SD of three biological samples relative to WT cells in +Fe conditions. Different letters above bars indicate statistically significant differences (p value < 0.05). See Experimental Procedures for more details

described (Sanvisens et al., 2014). Primers used for RT-qPCR are listed in Table S2.

Western blot analyses

Total protein extraction, resolution by SDS-PAGE and detection on nitrocellulose membranes were performed as previously described (Romero et al., 2018). The primary antibodies used were anti-c-myc (9E10, Roche) and anti-Pgk1 (22C5D8, Invitrogen). Images were obtained with an ImageQuant LAS 4000 mini Biomolecular Imager (GE Healthcare Life Sciences).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Barba-Aliaga & Alepuz, 2022) in strains expressing *myc-HAP1* or *TUP1-myc* allele and also in strains expressing untagged *HAP1* or *TUP1* as control for background binding. For immunoprecipitation, an anti-c-myc

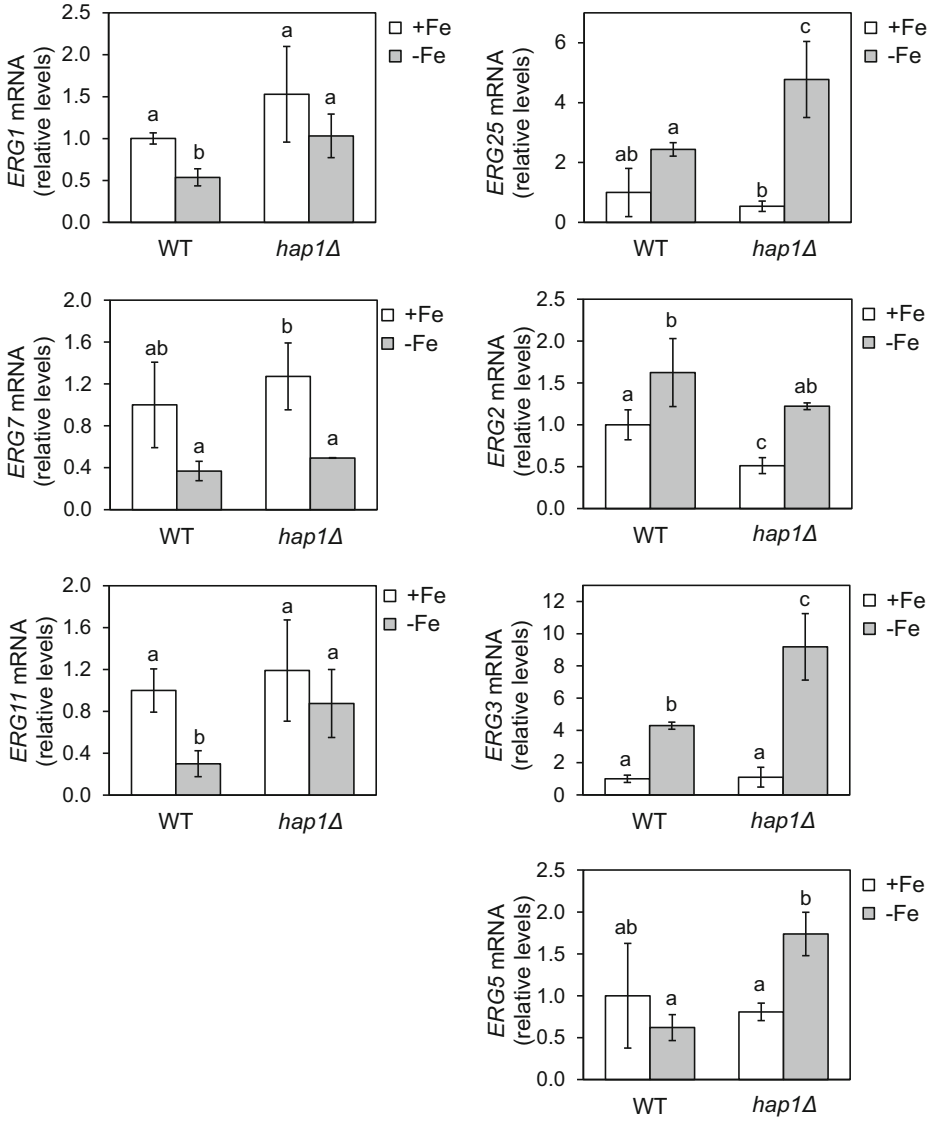


FIGURE 3 Deletion of *HAP1* up-regulates *ERG* gene expression under iron deficiency. WT (BY4741) and *hap1*Δ cells were grown to exponential phase in SC medium without (+Fe) or with 100 μM BPS (−Fe) for 7 h. *ERG* mRNA levels, normalized to *ACT1*, were determined by RT-qPCR. Data show the average and SD of three biological replicates relative to WT cells in +Fe conditions. Different letters above bars indicate statistically significant differences (p value <0.05).

antibody (Invitrogen) was used. We used primers listed in Table S2 to quantify by qPCR the binding of Hap1 and Tup1 to the following promoter sequences, which contain putative Hap1-binding sites: *ERG1* (−455 to −329), *ERG7* (−624 to −501), *ERG11* (−697 to −590), *ERG25* (−669 to −547), *ERG2* (−491 to −370), *ERG3* (−380 to −272), and *ERG5* (−482 to −359). Binding to *ACT1* promoter was used to normalize.

Sterol profiling

Analysis of sterol cell composition was performed as previously described (Jorda et al., 2021). After normalization with the α-cholestane area, chromatographic peak areas were used to calculate relative sterol abundance.

Statistical analyses

The statistical analysis of the relative gene expression was performed by the pair wise fixed reallocation randomization test (Pfaffl et al., 2002) using the InfoStat software. For the remaining parameters, statistical significance was evaluated with tailed t-student test. When bars share at least one letter, no significant differences exist between values, whereas when they do not share any common letter, values are statistically different (p value < 0.05).

RESULTS AND DISCUSSION

Expression pattern of *ERG* genes in response to iron deficiency

The expression of ergosterol biosynthesis (*ERG*) genes is finely regulated as a function of environmental signals that include oxygen and sterol availability (Jorda & Puig, 2020). Despite iron is essential for sterol synthesis, little is known about the effect of iron bioavailability on the expression pattern of *ERG* genes. Here, we determined by RT-qPCR the mRNA expression pattern of several *ERG* genes during the progress of iron depletion achieved by the addition of the Fe^{2+}-specific chelator BPS (Figure 1B). We observed that the abundance of *ERG1*, and *ERG11* transcripts decreased sharply during the initial hours of iron starvation (up to 3–5 h) and then it gradually increased (Figure 1B). In the case of *ERG7*, a similar initial downregulation was reported, but only a mild or no later up-regulation was obtained (Figure 1B). In contrast, *ERG25*, *ERG2*, *ERG3* and *ERG5* mRNAs downregulation at the initial stages of iron starvation was very subtle (*ERG2* and *ERG5*) or absent (*ERG25* and *ERG3*) and a marked upregulation occurred after a few hours of iron limitation (Figure 1B). These data indicate that two general *ERG* mRNA expression patterns are observed in response to iron starvation, whereas the three initial genes of the ERG pathway (*ERG1*, *ERG7* and *ERG11*) display both an initial drop in transcript levels followed by a strong induction, downstream *ERG* genes (*ERG25*, *ERG2*, *ERG3* and *ERG5*) only respond by up-regulating their mRNA levels, when iron is scarce.

Previous studies have determined Erg protein abundance during the progress of iron deficiency (Navarrete-Perea et al., 2021; Shakoury-Elizeh et al., 2010). Western blot analyses with yeast cells expressing carboxy-terminal tagged Erg proteins reported a drop of Erg1, Erg11 and Erg5 protein abundance and an upregulation of Erg25, Erg6 and Erg3 upon iron limitation (Shakoury-Elizeh et al., 2010), whereas a more recent proteomics analysis has shown an upregulation of most Erg protein levels when iron is scarce, with the exception of Erg1, Erg7, Erg11 and Erg5, which display a significant decrease (Navarrete-Perea et al., 2021). Although different strains and iron-deficient growth conditions have been used in all these studies, we can conclude that both mRNA and protein levels of the initial enzymes of the ERG pathway are, at least transiently, down-regulated when iron is scarce, whereas steps later in the pathway are up-regulated. Nevertheless, there is a discrepancy between the mRNA and protein levels of *ERG5*, which encodes for the last enzyme of the pathway, suggesting that an additional regulatory mechanism may exist.

Previous genome-wide studies performed during the progress of iron deficiency with the Genomic Run-On experimental approach, which determines gene-specific transcriptional rates (Garcia-Martinez et al., 2004), suggest that the changes that *ERG* transcripts experience during the progress of iron depletion (both initial downregulation and later upregulation) may be, to a large extent, a consequence of changes in their transcription rate (Romero et al., 2019) (Figure S1). Therefore, we decided to explore the contribution of the transcription factors Upc2, Ecm22 and Hap1, which have been previously reported to control *ERG* gene expression in response to changes in oxygen or ergosterol levels (Jorda & Puig, 2020), to the regulation of *ERG* genes in response to iron starvation.

Upc2 and Ecm22 independently contribute to the iron deficiency-induced expression of *ERG* genes

Upc2 and Ecm22 are the main transcription factors responsible for the basal and induced expression of *ERG* genes by oxygen and sterol levels (Jorda & Puig, 2020). Interestingly, we have recently reported that both transcription factors carry out a central role in defining the sterol pattern of iron-deficient yeasts (Jorda et al., 2021) (Figure S2), suggesting that they could play a relevant function in *ERG* gene regulation when iron availability is challenged. To explore this possibility, we determined *ERG* mRNA levels by RT-qPCR in wild-type (WT), *upc2*Δ, *ecm22*Δ and *upc2*Δ*ecm22*Δ strains in iron-sufficient (+Fe) and severe iron-deficient (–Fe, 9 h) conditions. Statistical analysis indicates that the basal mRNA levels of *ERG1*, *ERG7*, *ERG11* and *ERG25* do not change in *upc2*Δ cells as compared to WT cells, whereas *ERG2*, *ERG3* and *ERG5* transcript levels are slightly altered (Figure 2). With the exception of *ERG1* and *ERG3*, all *ERG* genes significantly diminished their basal expression in *ecm22*Δ cells and, in a more pronounced manner, in *upc2*Δ*ecm22*Δ double mutant (Figure 2). In accordance with previous publications (Davies et al., 2005; Davies & Rine, 2006; Vik & Rine, 2001), these results indicate that both transcription factors contribute to the basal expression of *ERG* genes, with a predominant Ecm22 contribution. As shown above (Figure 1B), the abundance of *ERG* transcripts in WT cells increased after multiple hours of cultivation in iron-deficient conditions (Figure 2). However, we observed that both *upc2*Δ and *ecm22*Δ single mutants displayed important defects in *ERG* gene upregulation by iron depletion, which were exacerbated in the *upc2*Δ*ecm22*Δ double mutant (Figure 2). These results strongly suggest that both Upc2 and Ecm22 independently contribute to the activation of *ERG* genes in response to iron deficiency. A similar pattern has been reported in response to ergosterol depletion. Although Upc2 has been suggested to be the main

transcriptional regulator of *ERG* genes under sterol deficiency, Ecm22 also contributes to *ERG* expression in these conditions, and only the lack of both *UPC2* and *ECM22* prevents *ERG* induction by ergosterol depletion (Davies et al., 2005; Davies & Rine, 2006; Vik & Rine, 2001; Yang et al., 2015).

We have recently observed that iron replete *upc2*Δ*ecm22*Δ cells contain significant lower relative levels of ergosterol than the rest of the strains (Jorda et al., 2021) (Figure S2), which according to its *ERG* genes expression pattern is probably due the lower transcript levels of most *ERG* genes (Figure 2). Remarkably, *upc2*Δ*ecm22*Δ cells accumulate considerable amounts of an intermediate sterol, episterol, which is the substrate of Erg3 and Erg5 enzymes (Figure 1A). This is probably due to the strong downregulation observed for these transcripts in *upc2*Δ*ecm22*Δ cells under normal growth conditions compared to wild-type cells (Figure 2). A similar trend (high episterol and low ergosterol) was also observed when *upc2*Δ*ecm22*Δ cells were cultivated in iron-limited conditions (Jorda et al., 2021) (Figure S2). Surprisingly, the partial defect in *ERG* genes upregulation by iron depletion that the *upc2*Δ and *ecm22*Δ single mutants exhibit did not lead to any decrease in ergosterol relative levels or accumulation of the initial sterols of the pathway (squalene, lanosterol) (Jorda et al., 2021) (Figure S2). Moreover, whereas deletion of *UPC2* or *ECM22* has no effect on yeast growth under iron-deficient conditions, their double deletion leads to a severe iron depletion sensitivity (Jorda et al., 2021). These data suggest that the transcriptional activation of *ERG* genes by the combined effect of Upc2 and Ecm22 is crucial for adequate ergosterol biosynthesis and growth during adaptation to iron depletion.

Hap1, in association with Tup1, promotes the repression of *ERG* genes during early iron deficiency

Transcription rate and mRNA kinetics suggest that *ERG* genes become transcriptionally repressed during the initial stages of iron depletion (Figures 1 and S1). Previous studies have reported that, when heme synthesis decreases due to oxygen depletion, the heme-dependent transcription factor Hap1 switches from activator to repressor of some *ERG* genes, including *ERG11*, *ERG2*, *ERG3* and *ERG5* (Hickman & Winston, 2007; Serratore et al., 2018). In agreement with the requirement of iron for heme biosynthesis, previous studies have also reported that iron-deficient yeast cells suffer a marked fall in intracellular heme levels (Shakoury-Elizeh et al., 2010). Therefore, we hypothesized that Hap1 could function as a repressor of *ERG* genes transcription under iron deficiency conditions. To test this hypothesis, we compared *ERG* mRNA levels in WT and *hap1*Δ strains at a time when iron deficiency mainly led to downregulation of *ERG1*, *ERG7* and *ERG11* genes (Figure 3). As previously reported (Davies & Rine, 2006), Hap1 was necessary for basal expression of *ERG2* gene, as *ERG2* mRNA levels decreased in *hap1*Δ mutant in normal growth conditions (Figure 3). However, no Hap1-dependent effect was observed for the basal expression levels of the rest of *ERG* genes assayed here (Figure 3). Importantly, with the exception of *ERG7* and *ERG2*, all *ERG* genes assayed displayed an approximately 2-fold induction upon iron depletion when *HAP1* gene was deleted (Figure 3), which is consistent with Hap1 contributing to the downregulation of *ERG* genes in response to iron deficiency.

Genome-wide analyses suggested that Hap1 binds to particular *ERG* promoters (Bergenholm et al., 2018; Harbison et al., 2004), whereas several ChIP studies have demonstrated direct binding of Hap1 transcription factor to *ERG11*, *ERG2*, *ERG3* and *ERG5* genes (Davies & Rine, 2006; Hickman & Winston, 2007; Serratore et al., 2018). Additionally, a recent study determined that an *erg3*Δ mutant, with altered sterol content, showed an increase in SAGA-complex occupation on many of the thus induced ERG promoters, and, interestingly, the purified SAGA complexes were enriched in Hap1, as well as in Upc2 (Dewhurst-Maridor et al., 2017), suggesting a role for this complex in the recruitment of these factors. To test whether Hap1 directly represses the transcription of *ERG* genes in response to iron deficiency, we studied its association to *ERG* gene promoters in +Fe and −Fe conditions by ChIP with a functional myc-tagged version of Hap1. ChIP probes were designed to include potential Hap1-binding sites within each *ERG* gene promoter (see Experimental Procedures section). As previously reported (Hickman & Winston, 2007), we observed that Hap1 protein was enriched at *ERG* gene promoters in iron-sufficient conditions when compared to Hap1-untagged cells (Figure 4). Significantly, Hap1 was also present in *ERG* gene promoters in iron-starved conditions, although Hap1 protein recruited was significantly diminished compared to iron replete conditions (Figure 4). These results are consistent with previous studies showing that Hap1 affinity for DNA decreases in heme-deficient cells (Hon et al., 1999; Hon et al., 2001; Lan et al., 2004; Zhang & Hach, 1999). However, no decrease in Hap1 association to *ERG* promoters has been observed upon hypoxia (Hickman & Winston, 2007; Serratore et al., 2018). As previously shown in proteomic assays (Navarrete-Perea et al., 2021), we observed that Hap1 protein levels increase upon iron starvation (Figure S3), which may partially compensate its diminished DNA-binding capacity.

To further assess the mechanism for Hap1-dependent repression of *ERG* genes in low iron con

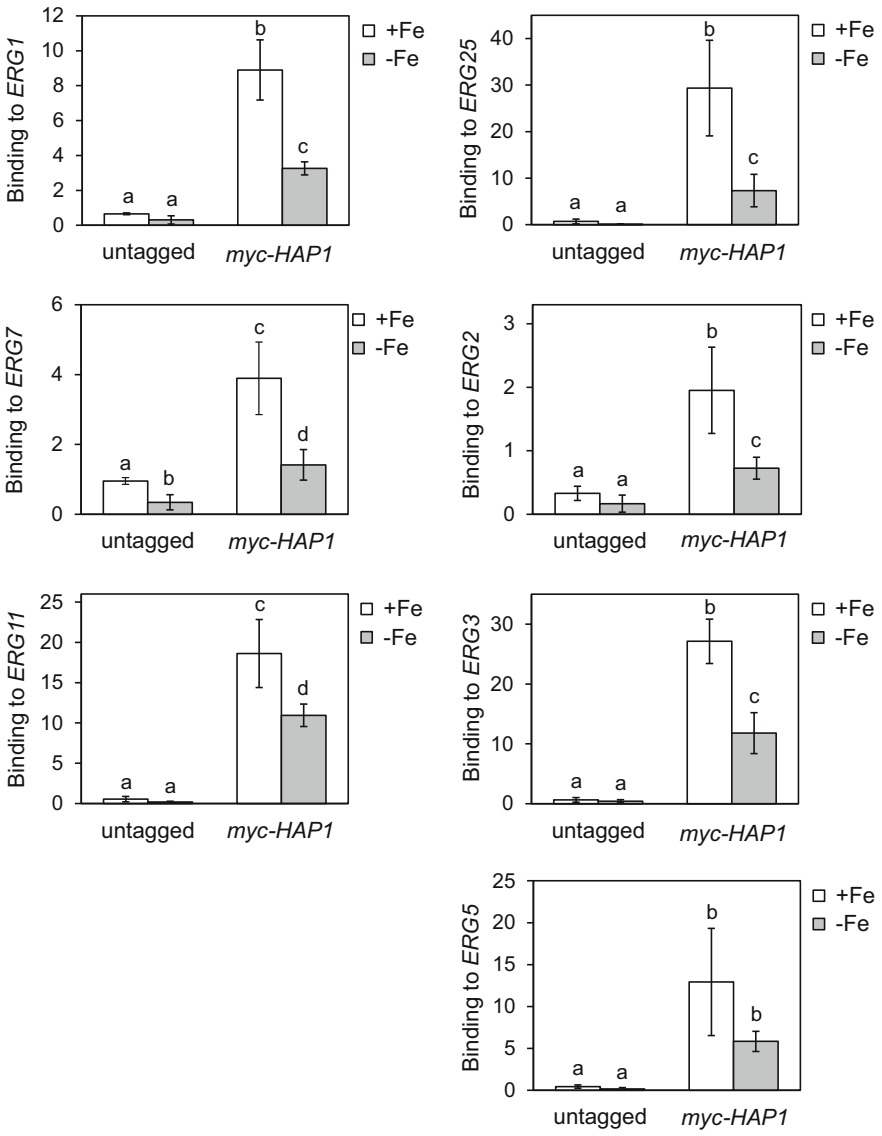


FIGURE 4 Hap1 is recruited to the promoters of *ERG* genes under both iron-sufficient and iron-deficient conditions. WT (FY2609) cells expressing untagged *HAP1* or *HAP1* fused to a myc epitope tag were grown as described in Figure 3. Proteins were extracted and immunoprecipitated with anti-myc antibody, and binding to *ERG* promoter regions was determined by RT-qPCR. Data show the average and SD of three independent experiments normalized to *ACT1*. Different letters above bars indicate statistically significant differences (p value < 0.05).

ditions, we analysed the recruitment of Tup1 to *ERG* promoters and its Hap1 dependence by performing ChIP experiments with Tup1-myc in both WT and *hap1*Δ cells grown under +Fe and −Fe conditions (Figure 5). We observed that Tup1 was only physically associated with *ERG* promoters under iron deficiency and that the deletion of *HAP1* abolished Tup1 recruitment (Figure 5). Moreover, we did not detect enrichment of Tup1 to promoters of *ERG7* and *ERG2* genes (Figure 5), whose mRNA levels were the only ones not being increased by *HAP1* deletion under iron-depleted conditions (Figure 3). With the exception of *ERG7* and *ERG2*, these results suggest that Hap1 directly represses *ERG* gene expression during the initial stages of iron deficiency by recruiting the corepressor Tup1. Recently, it has been shown that iron depletion also induces the Hap1-Tup1-mediated repression of the eIF5A encoding gene *TIF51A* (Barba-Aliaga & Alepuz, 2022). Similar to iron depletion, previous studies have shown that the recruitment to *ERG* gene promoters of corepressor factors, such as Tup1/Ssn6 and also Set4, increases upon hypoxic conditions in a Hap1-dependent manner (Hickman & Winston, 2007; Serratore et al., 2018). These results indicate that Hap1 functions as a repressor of *ERG* genes in response to both iron deficiency and hypoxia.

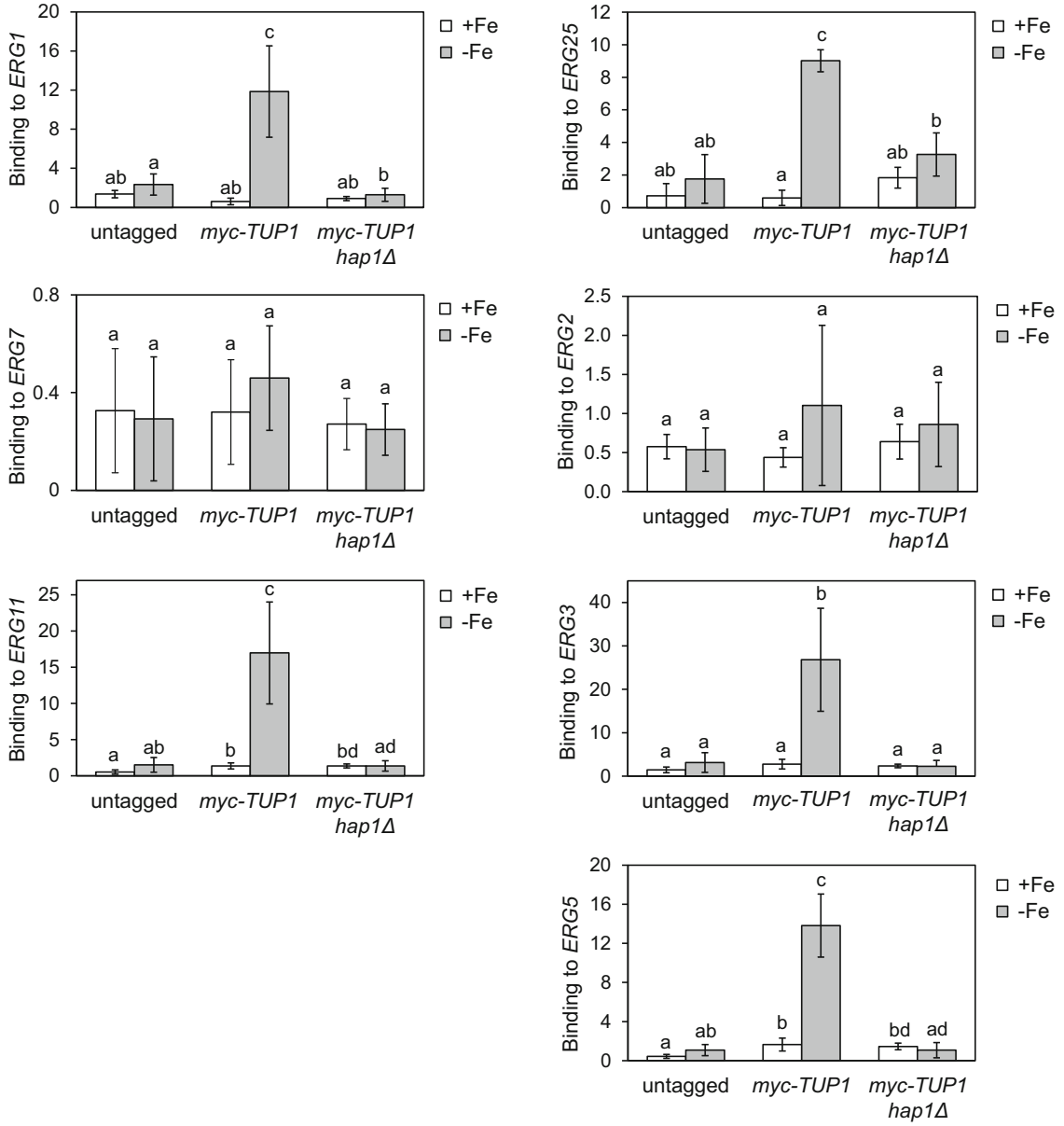


FIGURE 5 Tup1 is recruited to *ERG* gene promoters by Hap1 in iron-deficient conditions. WT (BY4741) cells expressing untagged *TUP1*, and WT and *hap1*Δ cells expressing *TUP1* fused to a myc epitope tag, were grown as described in Figure 3. The ChIP experiments were performed as in Figure 4. Data show the average and SD of three independent experiments normalized to *ACT1*. Different letters above bars indicate statistically significant differences (p value < 0.05).

Contribution of Hap1 to the sterols profile of yeast cells

To study the effect of Hap1 on yeast sterol content, we used gas chromatography mass spectrometry to determine the sterol profile of WT and *hap1*Δ cells cultivated under +Fe and −Fe conditions. In accordance with previous studies with WT cells (Jorda et al., 2021; Shakoury-Elizeh et al., 2010), iron deficiency caused a reduction in ergosterol and zymosterol content and an increase in the relative levels of squalene, lanosterol and 4,4-dimethylzymosterol (4,4-DMZ) in both strains (Figures 6 and S4). The relative distribution of sterols was only slightly altered in the *hap1*Δ mutant as compared to WT cells (Figures 6 and S4). These changes did not alter the fitness of *hap1*Δ mutant under either +Fe or −Fe conditions (Figure S5). We observed that *hap1*Δ cells displayed a slight increase in ergosterol and ergosta-5,8-dienol relative levels, and undetectable levels of episterol in both growth conditions (Figures 6 and S4). The lack of episterol, which is the product of Erg2 catalysis, in *hap1*Δ strain is consistent with the *ERG2* downregulation that occurs upon *HAP1* deletion (Figure 3), although no accumulation of its substrate, fecosterol, was observed (Figures 6 and S4). The last enzymes of the ERG pathway (Erg2-6) display a low

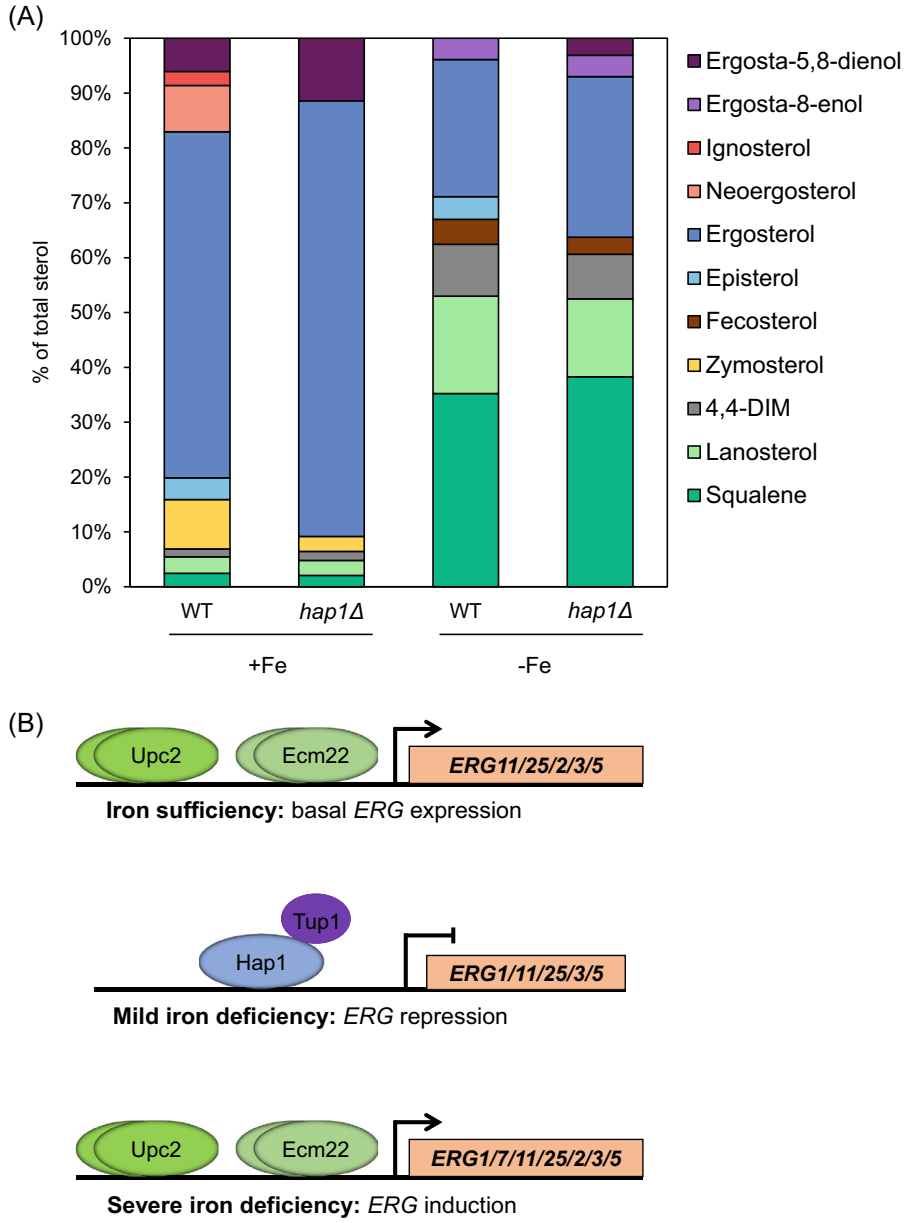


FIGURE 6 Sterols profile of *hap1*Δ cells and regulatory model. (A) Yeast WT (FY2609) and *hap1*Δ cells were grown to exponential phase in SC without (+Fe) or with 100 μM BPS (−Fe) for 9 h. Sterol species were quantified by gas chromatography mass spectrometry and their relative abundance was represented in a stacked bar chart. Data show the average of four biologically independent experiments. (B) Proposed transcriptional regulatory mechanism for yeast *ERG* genes in response to iron deficiency. Under basal conditions, Upc2 and Ecm22 are responsible for the expression of most *ERG* genes analysed. Upon mild iron deficiency, a Hap1-mediated repression via Tup1 recruitment is observed for particular *ERG* genes, probably due to a decrease in heme levels. Upon severe iron depletion, Upc2 and Ecm22 activate the transcription of ERG genes, probably due to a decrease in ergosterol levels.

specificity for their substrates and accept a broad range of similar sterol compounds. In fact, deletion of any of these genes is not lethal in yeast and leads to the accumulation of new sterol species instead of simply their substrate in the pathway (Johnston et al., 2020). It is tempting to speculate that the lack of fecosterol in *hap1*Δ cells could be the result of its conversion into ergosta-5,8-dienol, which accumulates in *hap1*Δ mutant, due to Erg3 and Erg4 enzymatic catalysis. In this sense, we observed that *ERG3* was up-regulated by *HAP1* deletion (Figure 3). In the same sense, the function of the iron-dependent enzyme Erg3 may be diminished in iron deficiency, which could explain the detection of ergosta-8-enol in iron-deficient cells, due to the unique action of Erg4 on fecosterol. There are also some growth-specific differences between the sterol pattern of WT and *hap1*Δ cells. Under normal conditions, WT cells seem to convert a small portion of ergosterol into neoergosterol, a potential ergosterol-derived metabolite (Barrero2002), leading to slightly

lower ergosterol relative levels than *hap1*Δ mutants (Figure 6 and S4). Moreover, ignosterol, which is formed upon defects in Erg24 function (Lai et al., 1994), was detected in iron-sufficient WT cells, but not *hap1*Δ mutant (Figures 6 and S4). Finally, under iron replete conditions, zymosterol relative levels are lower in *hap1*Δ than in WT cells, whereas upon iron-deficiency, *hap1*Δ cells exhibit slightly reduced levels of lanosterol as comparted to WT cells (Figures 6 and S4). Studies beyond *ERG* gene expression data would be necessary to propose potential mechanisms for such sterol pattern changes.

The combined action of multiple factors modulates ergosterol biosynthesis during yeast adaptation to iron deficiency

Iron participates as an enzymatic cofactor in most cellular metabolic pathways. Consequently, iron deficiency leads to the drop in the biosynthesis of iron cofactors (heme, Fe-S clusters, oxo-iron) and to the decrease in the activity of many iron-dependent enzymes. Thus, upon iron deficiency, yeast cells display a decrease in the synthesis rate of critical components of DNA (dNTPs), proteins (amino acids) and lipids (including unsaturated fatty acids, sphingolipids and ergosterol). Moreover, the central role of iron in the mitochondrial electron transport chain also leads to a decrease in oxygen consumption and energy generation via respiration. All these secondary effects act as stimuli to regulate particular signalling pathways that alter yeast gene expression. Similar to previous observation in response to hypoxia, the regulation of *ERG* genes expression upon iron limitation described here could be due in part to heme and ergosterol depletion, which would signal Hap1 and Upc2/Ecm22 and trigger the transcriptional repression or activation of *ERG* genes, respectively (Figure 6B). The combined regulation by multiple activating and repressing regulatory factors may allow for the fine-tuning of *ERG* transcript levels along the progress of iron deficiency, enabling efficient adaptation to rapidly changing conditions.

In recent years, ergosterol biosynthesis has been greatly studied in *S. cerevisiae*, leading to the identification of many conserved but also fungal-specific steps (Demuyser & Van Dijck, 2019). The ergosterol biosynthesis is one of the most important targets for the development of antifungal agents in health and agriculture. An extensive knowledge of the molecular mechanisms that contribute to the regulation of sterols biosynthesis is crucial for the design of new antifungal strategies that overcome the growing appearance of fungal pathogen resistance. In addition, *S. cerevisiae* can be used as a biofactory to produce heterogeneous sterols and steroids valuables for food and pharmaceutical industries (Gu et al., 2021). In this regard, several strains here analysed show, under iron deficiency, an increase in the relative amount of sterols such as squalene or lanosterol, of great biotechnological value, which deserves a more profound analysis as a possible way to produce these metabolites. Therefore, a better understanding of *ERG* regulation seems strategic given that the native regulation network of yeast cells limits the efficiency of sterols biosynthesis and fungal susceptibility.

ACKNOWLEDGEMENTS

The authors are grateful to the members of the Iron Homeostasis laboratory for technical and scientific assistance. The authors thank Dr. Fred Winston and Dr. Jasper Rine for yeast strains used in this work. This research was supported by grant PID2020-116940RB-I00 funded by MCIN/AEI/10.13039/501100011033 to Sergi Puig, grants PID2020-120066RB-I00 funded by MCIN/AEI/10.13039/501100011033 and AICO/2020/086 by 'Generalitat Valenciana' to Paula Alepuz, and grant RED2018-102467-T funded by MCIN/AEI/10.13039/501100011033 to Sergi Puig and Paula Alepuz. Tania Jordá was a recipient of a predoctoral fellowship ACIF/2019/214 funded by 'Generalitat Valenciana', and Marina Barba-Aliaga was a recipient of a predoctoral fellowship (FPU2017/03542) funded by MCIN/AEI/10.13039/501100011033 and by ESF Investing in your future. The authors also acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are openly available in Digital CSIC (https://digital.csic.es).

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How to cite this article: Jordá, T., Barba-Aliaga, M., Rozès, N., Alepuz, P., Martínez-Pastor, M.T. & Puig, S. (2022) Transcriptional regulation of ergosterol biosynthesis genes in response to iron deficiency. *Environmental Microbiology*, 24(11), 5248–5260. Available from: https://doi.org/10.1111/1462-2920.16157