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The yeast mRNA-binding protein Cth2 post-transcriptionally modulates ergosterol biosynthesis in response to iron deficiency

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ABSTRACT

Sterol synthesis is an iron-dependent metabolic pathway in eukaryotes. Consequently, fungal ergosterol biosynthesis (ERG) is down-regulated in response to iron deficiency. In this report, we show that, upon iron limitation or overexpression of the iron-regulated mRNA-binding protein Cth2, the yeast *Saccharomyces cerevisiae* down-regulates the three initial enzymatic steps of ergosterol synthesis (*ERG1*, *ERG7* and *ERG11*). Mechanistically, we show that Cth2 protein limits the translation and promotes the decrease in the mRNA levels of these specific *ERG* genes, which contain consensus Cth2-binding sites defined as AU-rich elements (AREs). Thus, expression of *CTH2* leads to the accumulation of initial sterol intermediates, such as squalene, and to the drop of ergosterol levels. Changes in *CTH2* expression levels disturb the response of yeast cells to stresses related to membrane integrity such as high ethanol and sorbitol concentrations. Therefore, *CTH2* should be considered as a critical regulatory factor of ergosterol biosynthesis during iron deficiency.

1. Introduction

In order to adapt to metabolic changes and environmental stresses, yeast cells tightly adjust the lipid composition of their membranes, thus modulating fluidity, permeability and the activity of membrane-associated proteins [1,2]. One of the essential membrane lipids is sterols, within which ergosterol is the most abundant in fungal cells, mainly residing in the plasma membrane. Understanding the regulation of ergosterol biosynthesis is important due to the fact that it is one of the major targets of antifungal therapies. Ergosterol biosynthesis rate is limited by iron bioavailability because iron is a vital redox cofactor for multiple enzymes of the late biosynthetic pathway, such as the hemoproteins Erg11 and Erg5, and the oxo-diiron enzymes Erg25 and Erg3 (Fig. 1) [3]. Being iron bioavailability extremely low, cells have evolved refined regulatory strategies to properly respond to iron deficiency and its side effects, including ergosterol and heme depletion. Previous studies have shown that the expression of ergosterol biosynthesis (*ERG*) genes in *S. cerevisiae* is significantly altered during the progress of iron deficiency [4–9]. We have recently characterized that the action of both transcriptional activators (Upc2/Ecm22) and repressors (Hap1-Tup1) strongly contributes to *ERG* genes expression pattern in low iron conditions [9]. However, little is known about the contribution of post-transcriptional mechanisms to the regulation to *ERG* genes expression.

In response to iron deficiency, yeast cells activate the expression of the RNA-binding protein Cth2 that, in coordination with its partially redundant paralog Cth1, promotes a metabolic remodeling of cellular processes to optimize iron utilization [4,10,11]. Cth1 and Cth2 proteins contain two $Cx_8Cx_5Cx_3H$ tandem zinc fingers (TZFs) that specifically bind to AU-rich elements (ARE) within the 3′ untranslated region (3′UTR) of many target mRNAs, promoting their degradation and inhibiting their translation [4,10,12–15]. Genome-wide studies in iron-deficient cells suggest that some *ERG* genes containing putative AREs are under Cth1 and Cth2 regulation [4,10]. Consistent with this, we have recently reported a negative correlation between *CTH2* and *ERG1* expression [16].

In this study, we investigated the role of the post-transcriptional factor Cth2 on *ERG* genes expression under iron deficiency conditions, and its contribution to yeast sterol profile and stress adaptation.

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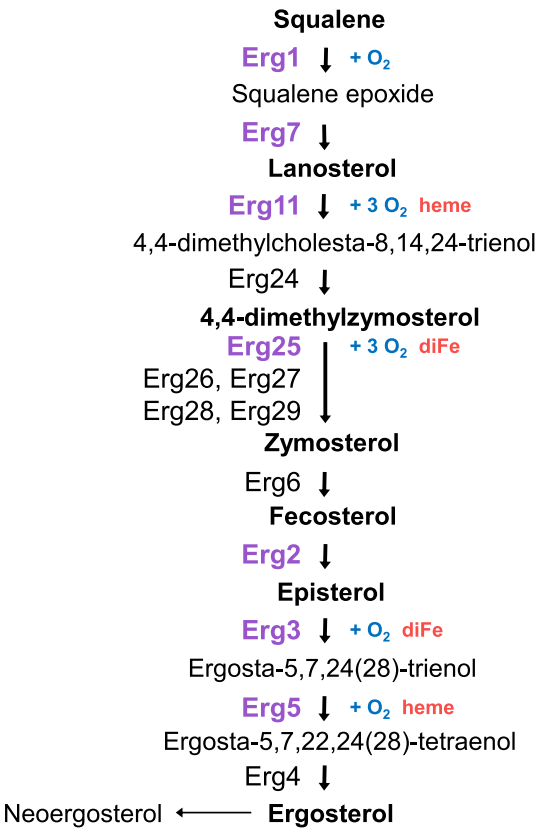

Fig. 1. Late ergosterol biosynthesis pathway in the budding yeast *Saccharomyces cerevisiae*. Enzymic steps between sterol intermediates are indicated with arrows, with their corresponding catalyzing enzyme on the left. Oxygen and iron requirements have been indicated. Enzymes: Erg1, squalene epoxidase, oxygen-dependent; Erg7, lanosterol synthase; Erg11 (Cyp51), lanosterol C-14 demethylase, heme- and oxygen-dependent; Erg24, sterol C-14 reductase; Erg25, sterol C-4 methyloxydase, iron- and oxygen-dependent; Erg26, sterol C-3 dehydrogenase (C4-decarboxylase); Erg27, sterol C-3 ketoreductase; Erg6, sterol C-24 methyltransferase; Erg2, sterol C-8 isomerase; Erg3, sterol C-5 desaturase, iron- and oxygen-dependent; Erg5, sterol C-22 desaturase, heme- and oxygen-dependent; Erg4, sterol C-24 reductase. Enzymes and intermediates analyzed in this study are shown in purple and bold, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Strains, plasmids and growth conditions

In this work, we used the *S. cerevisiae* yeast strains W303 (HTLU-2832-1B, *MATa*, *HIS3*, *TRP1*, *LEU2*, *URA3*, *ADE2*, *can1*), a prototroph strain from Dr. Fred Cross; W303 *cth1Δcth2Δ* (*cth1::hphB cth2::KanMX4*); BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*), BY4741 *cth1Δcth2Δ* (*cth1::KanMX4, cth2::HisMX6*), and BY4741 *cth1Δcth2Δsdh4Δ* (*cth1::KanMX4, cth2::HisMX6, sdh4::hphB*). When indicated, yeasts were transformed with the centromeric low-copy plasmids pRS415, pRS416, pRS415-CTH2, pRS416-CTH2, p416TEF-CTH2, or pRS416-SDH4 [4,10,12]. Cells were cultivated in synthetic complete [17] media [0.17 % yeast nitrogen base without ammonium sulfate and without amino acids, 0.5 % ammonium sulfate, 2 % glucose, and 2 g/L Kaiser drop-out (Formedium)] lacking particular requirements at 30 °C and 190 rpm to exponential growth phase. To induce iron depletion, 100 μM of the Fe^{2+}-specific chelator bathophenanthroline disulfonic acid disodium (BPS) (Sigma) was added for the indicated times. For growth spot assays on solid media, yeast cells were cultivated to exponential phase and then spotted in 10-fold serial dilutions.

2.2. RNA analyses

RNA isolation and RT-qPCR for specific transcripts were performed as previously described [18]. Primers used for RT-qPCR experiments have been previously described [9].

2.3. Polyribosome profiles and sterol analyses

Both were performed as previously described, respectively [14,19].

2.4. Statistical analyses

We used the pair wise fixed reallocation randomization test InfoStat software for the statistical analysis of gene expression data [20]. Tailed t-student test was used for the remaining data. When values were statistically different (p-value <0.05), they did not share any letter in the graphical representation, whereas when no significant differences existed, data shared at least one common letter.

3. Results

3.1. Effect of Cth1 and Cth2 mRNA-binding proteins on ERG gene expression pattern during iron deficiency

Previous genome-wide studies suggested that, upon iron limitation, the iron-regulated mRNA-binding proteins Cth1 and Cth2 could modulate the expression of particular *ERG* genes, which contain putative AREs within their 3′UTR [4,10]. More recently, we have reported that cells lacking *CTH2* up-regulate *ERG1* expression upon iron-deficient conditions, whereas *CTH2* overexpression diminishes *ERG1* transcript abundance [16]. To explore how Cth1 and Cth2 proteins influence the expression profile of *ERG* genes during iron limitation, we first cultivated a prototroph W303 wild-type strain and its *cth1Δcth2Δ* derivative in iron-deficient conditions, achieved by the addition of the extracellular Fe^{2+}-specific chelator BPS to the growth medium. Then, we determined the transcript levels of several *ERG* genes by RT-qPCR using specific oligonucleotides [9]. As recently reported for BY4741 and FY2609 yeast strains [9], we observed that *ERG1*, *ERG7* and *ERG11* transcripts are also initially down-regulated in wild-type W303 strain (Fig. 2A–C). However, *ERG25* and especially *ERG3* transcript abundance increased during the progress of iron deficiency (Fig. 2D–E). Finally, the mRNA levels of *ERG2* and *ERG5* transcripts decreased, but recovered their initial values after 9 h of iron depletion (Fig. 2F–G). The comparison of the expression pattern of wild-type and *cth1Δcth2Δ* strains suggested that *CTH1* and *CTH2* contribute to the down-regulation of *ERG1*, *ERG7*, *ERG11*, *ERG25* and *ERG3* mRNAs during iron deficiency (Fig. 2). In the case of *ERG1* and *ERG11*, up-regulation was also observed at basal levels (time zero) in *cth1Δcth2Δ* cells (Fig. 2A and C). These results suggest that the mRNA-binding proteins Cth1 and Cth2 contribute to the expression of several yeast *ERG* genes when iron is scarce.

3.2. Cth2 down-regulates the expression of the initial genes of the late ergosterol biosynthesis pathway

We decided to focus on studying the effect of Cth2 on *ERG* genes since *CTH2* is highly expressed in response to iron depletion and triggers a predominant regulatory function as compared to Cth1 [10]. To exclude interferences from the partially redundant Cth1 protein [10], we performed the experiments in a *cth1Δcth2Δ* genetic background transformed with either a *CTH2*-expressing plasmid (*CTH2* cells), empty vector (*cth1Δcth2Δ* cells), or a plasmid constitutively expressing *CTH2* under the control of the *TEF2* promoter (P_{TEF}-*CTH2* cells) [10]. No growth differences were observed for *CTH2* and *cth1Δcth2Δ* cells under iron-sufficient conditions, whereas overexpression of *CTH2* under iron-sufficient conditions or iron depletion led to a slight growth defect (Fig. S1). Firstly, we confirmed that *CTH2* mRNA levels were up-

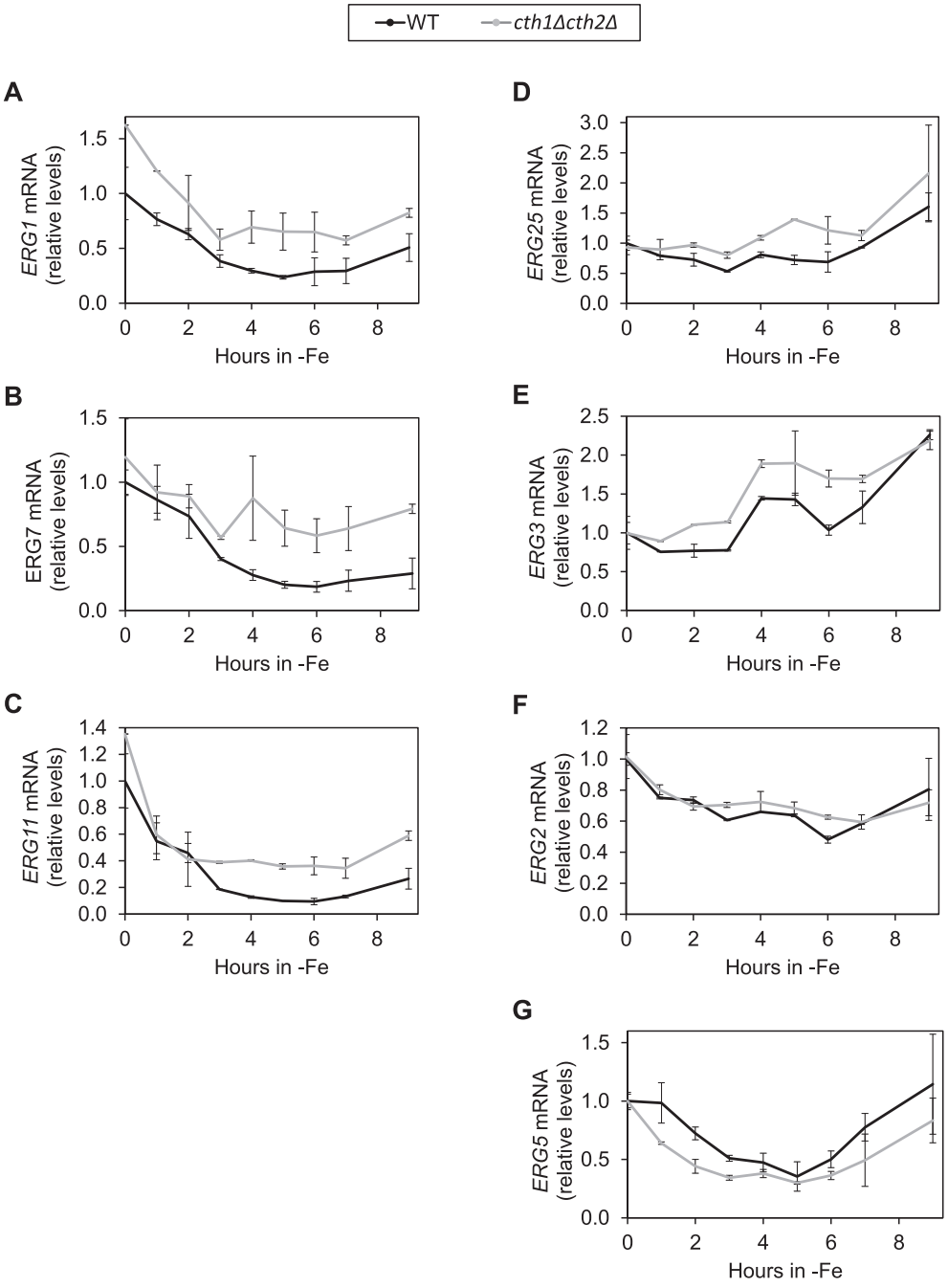

Fig. 2. *ERG* transcript levels in response to iron deficiency in wild-type and *cth1Δcth2Δ* cells. Wild-type W303 (black line) and W303 *cth1Δcth2Δ* (grey line) cells were cultivated overnight (~16 h) in SC at 30 °C to reach exponential phase. Then, 100 μM BPS was added for 9 h, and aliquots were obtained every hour for RNA extraction. *ERG* mRNA levels were determined by RT-qPCR and normalized to *ACT1*. Values are relative to wild-type W303 cells at time zero. The average and standard deviation (SD) of three biologically independent experiments are represented.

regulated in response to iron depletion, whereas *TEF2* promoter increased the expression of *CTH2* in iron replete conditions (Fig. 3A). Then, we checked whether changes in *CTH2* expression levels altered the abundance of *ERG* transcripts by iron (Fig. 3B–H). We observed that *ERG1*, *ERG7* and *ERG11* down-regulation by iron deficiency was fully abolished in *cth1Δcth2Δ* cells, and in turn *CTH2* overexpression limited their expression in iron-sufficient conditions (Fig. 3B–D), suggesting that they are down-regulated when Cth1 and Cth2 are expressed. *ERG25*, *ERG12* and *ERG13* also displayed a slight up-regulation in iron-deficient *cth1Δcth2Δ* cells as compared to wild-type (Figs. 3E and S2A). However, no down-regulation was observed for these nor other *ERG* genes when *CTH2* was overexpressed (Figs. 3E–H and S2A). As a control, we determined the mRNA levels of two well-known Cth2 target transcripts, *SDH4* and *HEM15*, which encode for a subunit of succinate dehydrogenase and ferrochelatase, respectively [4]. As expected, the expression of both *SDH4* and *HEM15* decreased in a Cth2-dependent manner (Fig. S2B). Altogether, these results suggest that, in response to iron deficiency, Cth2 protein promotes the down-regulation of *ERG* transcripts within the initial steps of the late ergosterol biosynthetic pathway.

Recent data have demonstrated that Cth2 is also able to limit the expression of its target mRNAs by inhibiting their translation [14,15]. To explore whether Cth2 specifically inhibits the translation of particular *ERG* genes in addition to promote their degradation, we performed polyribosome profiles under both iron replete and depleted conditions, and we determined the position of *ERG* mRNAs in the different polyribosomal fractions (Fig. 4). We observed that cells lacking *CTH2* (*cth1Δcth2Δ*) displayed a shift of *ERG1*, *ERG7* and *ERG11* transcripts from monosomal (M) to polysomal (P) fractions as compared to *CTH2*-expressing cells, leading to a higher P/M ratio, which is indicative of increased translation (Fig. 4A–D). However, no significant changes in

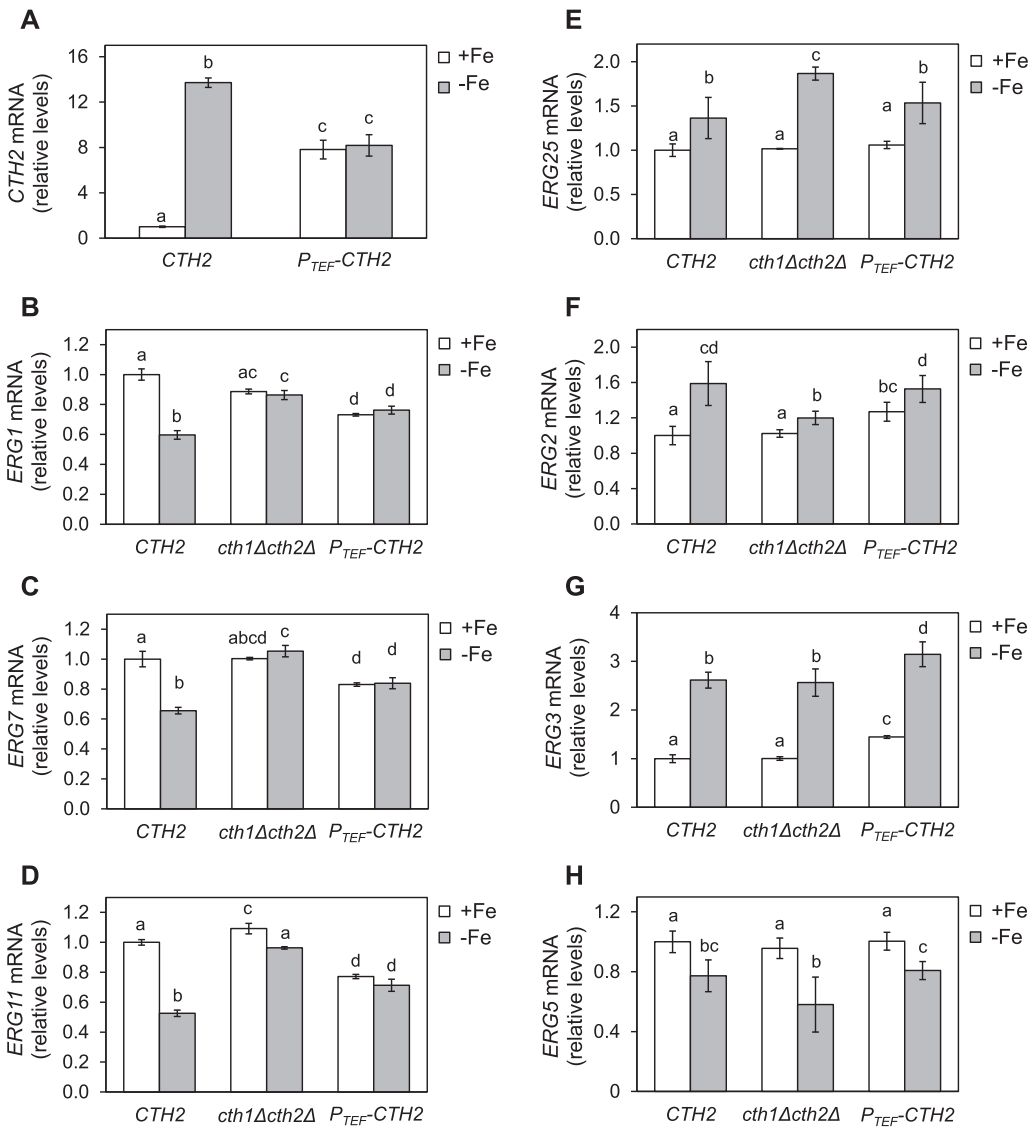

Fig. 3. *CTH2* expression down-regulates specific *ERG* genes. Yeast BY4741 *cth1Δcth2Δ* cells transformed with pRS416-CTH2 (*CTH2*), pRS416 empty vector (*cth1Δcth2Δ*) or p416TEF-CTH2 (P_{TEF}-*CTH2*) were grown for 6 h to exponential phase in SC-Ura (+Fe) or SC-Ura with 100 μM BPS (–Fe). Total RNA was extracted, and *CTH2* (A) and *ERG* (B–H) mRNA levels, normalized to *ACT1*, were determined by RT-qPCR. Data show the average and SD of three biological replicates relative to *CTH2*-expressing cells in +Fe conditions. Different letters above bars indicate statistically significant differences (p-value <0.05).

transcript association to polysomes were observed for the rest of *ERG* genes analyzed (Fig. 4A and E–H). These data are consistent with Cth2 specifically limiting the expression of the ARE-containing mRNAs *ERG1*, *ERG7* and *ERG11* by promoting their decay and inhibiting their translation.

3.3. *CTH2* expression modulates the yeast sterols profile

To ascertain the effect of Cth2 on sterol metabolism, we investigated how changes in *ERG* genes expression induced by Cth2 influenced yeast sterol composition. Expectably, upon iron depletion all yeast strains assayed (cells expressing normal *CTH2* levels, *cth1Δcth2Δ* mutant, and *CTH2*-overexpressing cells) accumulated squalene and 4,4-dimethylzymosterol (DMZ), and diminished their levels of ergosterol and zymosterol (Figs. 5A and S3). Since *CTH2* is specifically expressed upon iron depletion, we first examined the effect of deleting *CTH2* in the sterol pattern of iron-deficient yeasts (*CTH2* vs *cth1Δcth2Δ* cells in –Fe conditions). We observed that *cth1Δcth2Δ* cells displayed a decrease in squalene levels and a rise in 4,4-DMZ intermediate (Figs. 5A and S3), which is consistent with the up-regulation of the initial steps of ergosterol biosynthesis (*ERG1*, *ERG7*, and *ERG11*) observed in this mutant (Figs. 2A–C and 3B–D), since squalene and 4,4-DMZ are the substrate and product, respectively, of the initial part of the pathway (Fig. 1). Remarkably, ergosterol levels did not rise in *cth1Δcth2Δ* cells, probably due to the low enzymatic activity of Erg25, Erg3 and Erg5 under iron starvation conditions. Then, we analyzed the effect of overexpressing *CTH2* in iron replete conditions (*CTH2* vs P_{TEF}-*CTH2* cells in +Fe conditions). Overexpression of *CTH2* led to the relative accumulation of initial sterol intermediates (squalene and lanosterol) and the decrease of downstream sterol compounds of the pathway (zymosterol, episterol and ergosterol) (Figs. 5A and S3), which could be attributed to the drop in *ERG1*, *ERG7* and *ERG11* mRNA levels caused by the expression of *CTH2* in iron-sufficient conditions (Fig. 3B–D). Under iron deficiency, the expression of *CTH2* with the *TEF2* promoter, instead of its native promoter, also led to a slight decrease in *CTH2* mRNA levels (Fig. 3A), which caused a parallel increase in *ERG1*, *ERG7* and *ERG11* transcript abundance (Fig. 3B–D). This regulation was translated into a decrease in squalene and an increase in 4,4-DMZ and ergosterol levels (Figs. 5A and S3). Altogether, these results demonstrate that *CTH2* expression alters the expression of *ERG* genes and consequently the relative abundance of sterol intermediates and ergosterol.

The lipid composition of cellular membranes is crucial for the adaptation to multiple stress conditions, including high ethanol concentrations and osmotic stress [21,22]. To investigate the importance of the regulation of sterols formation by Cth2 on cellular adaptation to stress, we tested the growth of yeast cells expressing different levels of

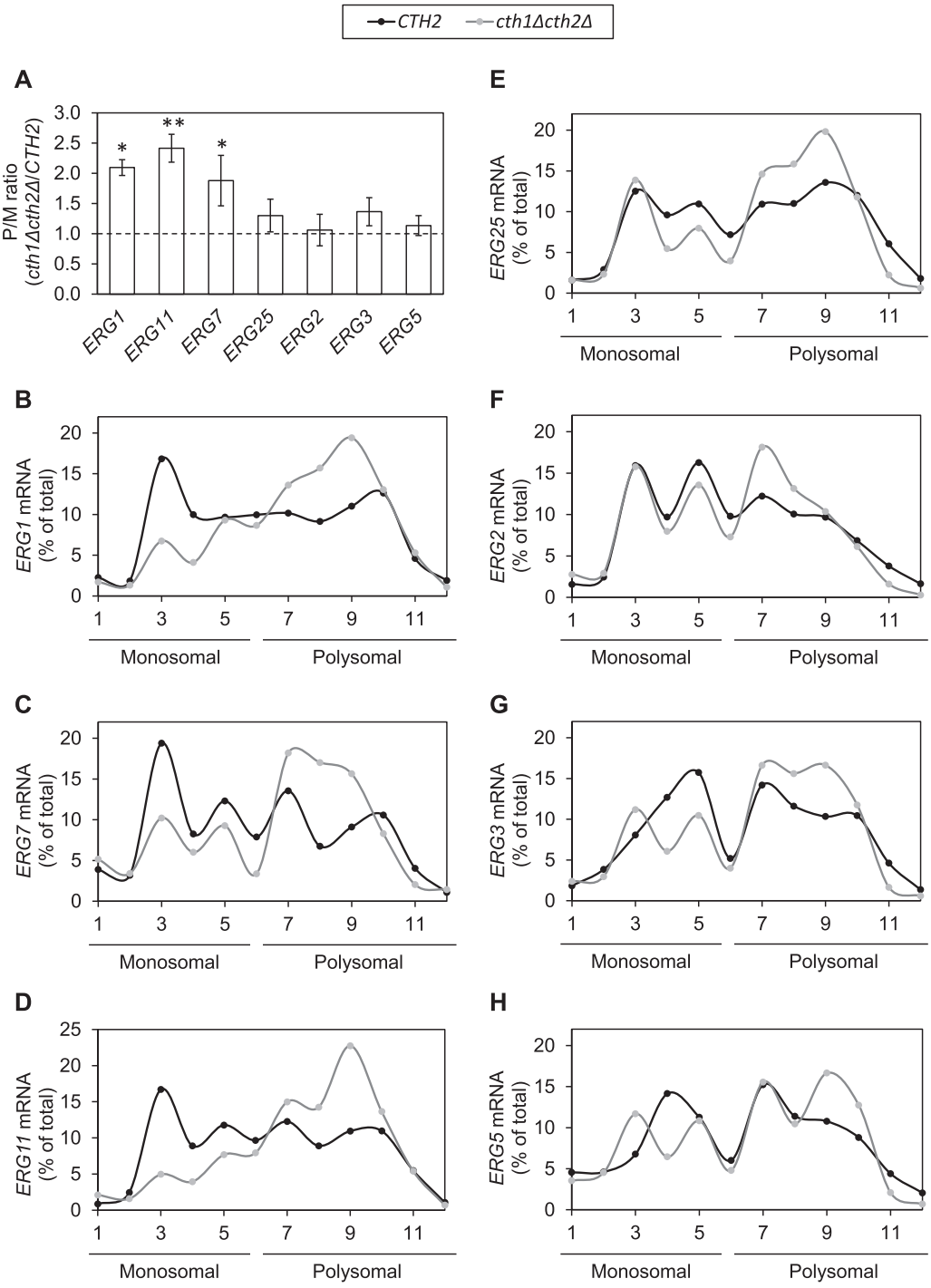

Fig. 4. *CTH2* limits the translation of particular *ERG* genes during adaptation to iron deficiency. Yeast BY4741 *cth1Δcth2Δsdh4Δ* mutant strains co-transformed with pRS416-*SDH4* and either pRS415-*CTH2* (*CTH2*) or pRS415 (*cth1Δcth2Δ*) plasmids were cultivated at 30 °C for 7 h in SC-Ura-Leu with 100 μM BPS. The RNA in individual ribosomal fractions was extracted and *ERG* genes mRNA levels were analyzed by RT-qPCR. (A) The ratio of the mRNA relative abundance in polysomal fractions/monosomal fractions (P/M ratio) in *cth1Δcth2Δ* cells was represented in relation to the ratio in *CTH2*-expressing cells for each *ERG* gene. Data show the average and SD of the values normalized to both *phe* and *lys* mRNA from *Bacillus subtilis* added to the samples before RNA extraction. Asterisks indicate statistically significant differences between both strains (* p-value <0.05; ** p-value <0.01). (B–H) Polysome profiles of the indicated *ERG* genes in *CTH2* (black line) and *cth1Δcth2Δ* (grey line) strains normalized to *phe* mRNA. The position of monosomal and polysomal fractions has been indicated.

CTH2 in media with elevated concentrations of ethanol (EtOH) or sorbitol. Overexpression of *CTH2* (P_{TEF}-*CTH2* cells) under iron-sufficient conditions slightly impaired growth in liquid media (Figs. 5B–C and S1B). Importantly, when cells were treated with 8 % EtOH or when osmotic stress was induced by addition of 2.5 M sorbitol, *CTH2* overexpression fairly disturbed yeast cells growth (Fig. 5B–C). These results suggest that increased *CTH2* expression impairs the resistance of yeast cells to particular stress conditions.

4. Discussion

We have recently reported that Hap1 and Upc2-Ecm22 transcriptional factors modulate the expression pattern of *ERG* genes during the progress of iron deficiency by promoting their initial transcriptional repression and their later activation, respectively [9]. Here, we demonstrate that the post-transcriptional factor Cth2 regulates ergosterol biosynthesis by limiting the expression of *ERG1*, *ERG7* and *ERG11* mRNAs when iron is scarce. Collectively, these results are consistent with a regulatory model in which Cth2 and Hap1 down-regulate *ERG* gene expression during the initial stages of iron depletion to economize

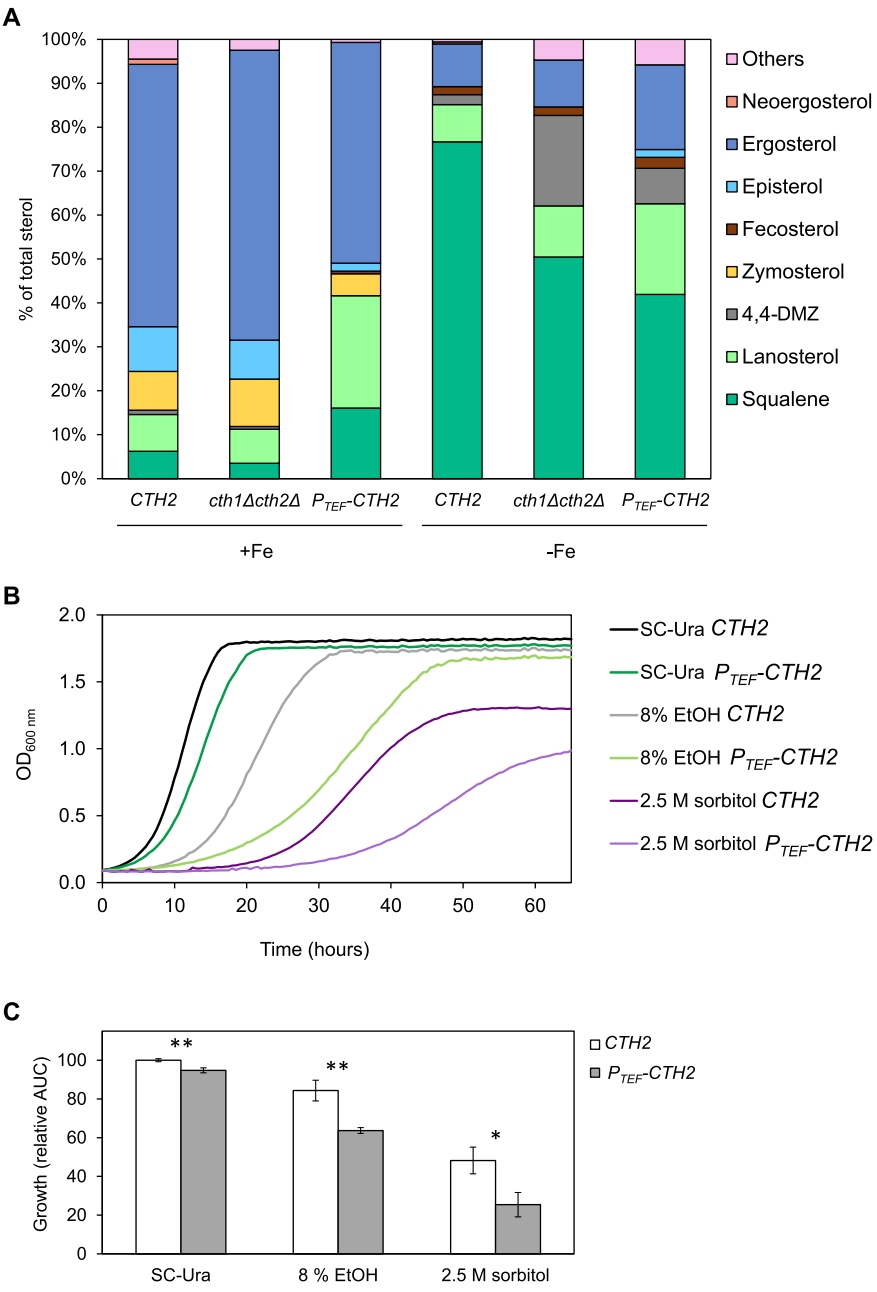

Fig. 5. Altered *CTH2* expression disturbs sterol profile of yeast cells and limits adaptation to environmental stresses. (A) Yeast BY4741 *cth1Δcth2Δ* cells were transformed and cultivated for 9 h as indicated in Fig. 3. The relative abundance of sterol species was determined and represented in a stacked bar chart. Data show the average of four biologically independent experiments. (B) *cth1Δcth2Δ* cells transformed as in Fig. 3 were grown in liquid SC-Ura medium supplemented or not with 8 % ethanol (EtOH) or with 2.5 M sorbitol for 3 days at 28 °C, and OD_{600} was determined every 30 min with a Spectrostar Nano 96 well plate reader. The average growth curve of three biologically independent assays is shown. (C) The area under the growth curve (AUC) was determined at 48 h for each treatment and then divided by the AUC of *CTH2*-expressing cells cultivated in SC-Ura (relative AUC). Data show the average and SD of three biological replicates. Asterisks indicate statistically significant differences (* p-value <0.05; ** p-value <0.01).

iron, whereas Upc2 and Ecm22 activate *ERG* genes expression and ergosterol biosynthesis when iron deficiency persists and ergosterol levels decrease below critical levels.

In this report, we observed that *ERG* genes expression profile upon iron depletion was altered in cells lacking the iron-regulated mRNA-binding proteins Cth1 and Cth2 (Fig. 2). *ERG1* mRNA, which contains two potential AREs within its 3′UTR (specifically at 122 and 133 nt after termination codon), has been recently shown to be up-regulated in response to iron depletion in *cth2Δ* as compared to wild-type cells [16], suggesting a direct regulation of this transcript by the ARE-binding protein Cth2. An additional analysis of *ERG* transcript levels in cells lacking *CTH2* or constitutively expressing it under the *TEF2* promoter strongly suggested that Cth2 promotes the down-regulation of all three initial steps of the late ergosterol pathway, catalyzed by *ERG1*, *ERG7* and *ERG11* gene products (Fig. 3). Interestingly, *ERG7* and *ERG11* also contain putative AREs within their 3′UTR (*ERG7* at 4 and 70 nt, and *ERG11* at 174, 203 and 273 nt, after termination codon), suggesting that Cth2 may also trigger their degradation in response to iron depletion. Furthermore, analysis of the polyribosome profile of *ERG1*, *ERG7* and *ERG11* mRNAs shows that the presence of *CTH2* limits their translation (Fig. 4A–D). We analyzed other *ERG* genes containing putative AREs, such as *ERG28* (at 52 nt) and *ERG12* (at 19 nt), but no dependence of *CTH2* expression was found in their mRNA levels (Fig. S2A). The rest of *ERG* genes analyzed (*ERG25*, *ERG5*, *ERG2* and *ERG3*) lack canonical AREs, and no significant Cth2-dependent changes were detected in either their mRNA levels (Fig. 3E–H) or polyribosome profiles (Fig. 4A and E–H). Nevertheless, *ERG25* and *ERG3* mRNA levels were slightly increased in *cth1Δcth2Δ* when compared to wild-type cells (Figs. 2D–E and 3C), and genome-wide studies pointed to an up-regulation of *ERG25*, *ERG28* and *ERG12* genes in *cth1Δcth2Δ* iron-deficient cells [4,10]. Thereby, an influence of Cth2 on other *ERG* mRNAs cannot be ruled out. In sum, these results strongly suggest that, in response to iron deficiency, Cth2 down-regulates mainly ARE-containing mRNAs *ERG1*, *ERG7* and *ERG11* by promoting their degradation and inhibiting their

translation.

Analysis of multiple sterol species revealed the direct effect of the Cth2-mediated down-regulation of early *ERG* genes on the relative abundance of sterol intermediates. Consistent with the lack of expression of *CTH2* under iron replete conditions, very little differences in the sterol pattern were observed between cells (Figs. 5A and S3). As previously observed for wild-type cells [8,9,19], all yeast strains used in this study accumulate squalene in response to iron deficiency (Figs. 5A and S3). If we assume that iron depletion leads to a decrease in the enzymatic activity of the iron-using enzymes, we would expect iron limitation to cause an increase in lanosterol levels, which is the substrate for Erg11, the first iron-dependent step of the pathway. Instead, a dramatic accumulation of squalene is observed, likely due to the lanosterol-dependent degradation of Erg1 mediated by Doa10 ubiquitin ligase [23]. We have recently shown that the heme-sensing transcription factor Hap1 represses *ERG1* expression upon iron depletion, contributing to increased relative squalene abundance [9].

Expression of *CTH2* under the control of the constitutively expressed *TEF2* promoter leads to its overexpression under iron-sufficient conditions, and to slightly lower expression levels under iron starvation conditions than when controlled by its own promoter (Fig. 3A). The relative abundance of sterol species can be mostly explained considering that Cth2 promotes the down-regulation of the initial stages of the late ergosterol synthesis pathway. Thus, under iron replete conditions, the overexpression of *CTH2*, as compared to *CTH2* or *cth1Δcth2Δ* cells, where *CTH2* expression is either minimal or absent, not only increases the abundance of squalene and decreases ergosterol synthesis, but it also leads to the accumulation of lanosterol, as expected by the *CTH2*-dependent down-regulation of *ERG11* (Figs. 5A and S3). Under iron limitation, the expression of *CTH2* is lower in P_{TEF}-*CTH2* than in *CTH2* cells (Fig. 3A), leading to higher *ERG1*, *ERG7* and *ERG11* expression (Fig. 3B–D). Remarkably, under low iron conditions, cells expressing *CTH2* under the control of *TEF2* promoter accumulate less squalene than *cth1Δcth2Δ* cells (Fig. 5A). We attribute this effect to the fact that, despite the first steps of the ERG pathway are slightly down-regualted in P_{TEF}-*CTH2* as compared to *cth2Δ* cells (Fig. 3B–D), the levels of *ERG2*, *ERG3* and *ERG5* mRNAs are higher in these cells than in *cth2Δ* (Fig. 3F–H), leading to the deviation of part of the squalene and lanosterol to the production of more ergosterol (Fig. 5A).

When the sterol profiles of iron-sufficient P_{TEF}-*CTH2* cells and iron-deficient *CTH2* cells are compared, a more severe down-regulation of ergosterol biosynthesis is observed in the latter one (Fig. 5A). In addition to the effect of Cth2 on *ERG1*, *ERG7* and *ERG11* mRNAs down-regulation, the enzymatic activity of the iron-dependent enzymes Erg11, Erg25, Erg3 and Erg5 decreases in low iron conditions. As a consequence, iron-deficient *CTH2* cells accumulate higher squalene and less ergosterol than P_{TEF}-*CTH2* cells. We can conclude that multiple factors including *ERG* genes regulation by Cth2, Hap1 and Upc2/Ecm22, the enzymatic activity of iron-dependent enzymes within the ergosterol biosynthesis pathway, and the severity of iron deficiency would contribute to the final profile of sterols under low iron conditions.

The concentration of ergosterol at the plasma membrane modulates the adaptation of yeasts and other fungi to multiple environmental stresses [2,21,22,24]. For instance, during wine and brewing strains of *S. cerevisiae* produce high levels of ethanol, which increase the fluidity of cellular membranes and disrupt their structure, a lipid interdigitated phase is formed and membrane thickness diminishes, which can lead to alterations in membrane protein function and cellular inactivation [25–27]. To antagonize the rise in fluidity caused by the elevated concentrations of ethanol, yeast cells synthesize higher levels of ergosterol, which contributes to enhance membrane rigidity and diminishes lipid interdigitation [21]. Since *CTH2* expression disturbs ergosterol synthesis, we postulated here that it could also influence ethanol tolerance. Indeed, *CTH2* overexpression limits growth in the presence of high ethanol concentrations (Fig. 5B–C). On the other hand, previous studies have linked ergosterol levels to hyperosmotic stress response. Some *ergΔ* mutants show less tolerance to high sorbitol concentrations [22] and ergosterol is important for maintaining Ssk22 protein levels, a kinase involved in osmosensing [28]. Other studies have suggested that downregulation of ergosterol levels is physiologically important for osmostress adaptation [17], and overexpression of ergosterol pathway confers increased susceptibility to salt stress [17,29]. Here we show that disturbance of ergosterol biosynthesis by *CTH2* overexpression limits growth in media with high sorbitol concentrations (Fig. 5B–C). These results indicate that *CTH2* may influence adaptation of yeast cells to environmental stresses beyond iron deficiency.

5. Conclusions

Previous work has shown that Hap1 and Upc2/Ecm22 transcriptional factors modulate *ERG* genes expression and ergosterol biosynthesis during iron deficiency [9]. Here, we demonstrate that Cth2, which directly responds to iron deprivation via Aft1 transcriptional factor, contributes to ergosterol biosynthesis by post-transcriptionally limiting the expression of *ERG* genes involved in the initial steps of the late ergosterol pathway. The combined regulation by these activating and repressing factors, at different stages of iron depletion, allow 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 and compromises cellular adaptation.

CRediT authorship contribution statement

Tania Jordá: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing – original draft. **Nicolas Rozès:** Investigation, Methodology, Formal analysis. **María Teresa Martínez-Pastor:** Project administration, Supervision, Writing – review & editing. **Sergi Puig:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data presented in this study are openly available in Digital CSIC (https://digital.csic.es) at DOI: https://doi.org/10.20350/digitalCSIC/15420

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Appendix A. Supplementary data

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