The Role of the Yap5 Transcription Factor in Remodeling Gene Expression in Response to Fe Bioavailability

Catarina Pimentel¹, Cristina Vicente¹, Regina Andrade Menezes¹, Soraia Caetano¹, Laura Carreto², Claudina Rodrigues-Pousada¹*

¹ Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal, ² Department of Biology, Center for Environmental and Marine Studies, Universidade de Aveiro, Aveiro, Portugal

Abstract

The budding yeast Saccharomyces cerevisiae has developed several mechanisms to avoid either the drastic consequences of iron deprivation or the toxic effects of iron excess. In this work, we analysed the global gene expression changes occurring in yeast cells undergoing iron overload. Several genes directly or indirectly involved in iron homeostasis showed altered expression and the relevance of these changes are discussed. Microarray analyses were also performed to identify new targets of the iron responsive factor Yap5. Besides the iron vacuolar transporter CCC1, Yap5 also controls the expression of glutaredoxin GRX4, previously known to be involved in the regulation of Aft1 nuclear localization. Consistently, we show that in the absence of Yap5 Aft1 nuclear exclusion is slightly impaired. These studies provide further evidence that cells control iron homeostasis by using multiple pathways.

Introduction

Iron (Fe) is an essential metal to most forms of life. The function of Fe in biological processes, such as respiration, oxygen transport, DNA synthesis and repair, among others, relies on its capacity to be reversibly oxidized and reduced. However, the same chemical properties that make Fe such a central element for life also make it a strong pro-oxidant that can generate powerful reactive oxygen species (ROS) through Fenton type reactions [1]. Organisms accurately regulate the concentration of Fe through regulation of Fe uptake, storage and mobilization [2,3,4]. Disturbances of Fe homeostasis affect the pathogenesis of infectious diseases and have severe clinical consequences like Fe-deficiency anemia, Friedreich’s ataxia and hereditary haemochromatosis [5,6,7].

The budding yeast Saccharomyces cerevisiae is able to grow under a wide magnitude of Fe available environments and can survive large fluctuations in Fe bioavailability. Yeast cells respond to Fe deficiency by triggering a complex rearrangement of gene expression that culminates with the activation of Fe transport systems (with the consequent increase of Fe uptake and mobilization from intracellular stores) and the adjustment of metabolism in order to divert iron from Fe-dependent metabolic pathways [8,9,10,11]. The vast majority of these genes are regulated by the Fe-responsive transcription factor, Aft1, to a lesser extent by its parologue, Aft2, constituting the iron regulon [2,3]. Two of the Aft1 targets code for the RNA-binding proteins Cth1 and Cth2, that posttranscriptionally downregulate many mRNAs involved in Fe-dependent processes [9,12]. Aft1 shuttles between the cytosol and the nucleus, accumulating in the latter under Fe depletion and activating transcription of the Fe regulon [13,14]. Aft1 activation does not respond directly to cytosolic iron but rather to the production of mitochondrial iron-sulfur clusters via a signaling pathway that requires the activity of the monothiol glutaredoxins Grx3/Grx4 and the regulatory proteins Fra1/Fra2 [13,15,16,17,18,19,20].

Much less is known regarding the response to increased Fe levels in the environment. Unlike humans, but similar to plants, the yeast cell vacuoles function as iron reservoirs. In yeast, iron storage is mediated by Ccc1, a vacuolar transporter that effects the accumulation of iron in the vacuoles [21]. CCC1 mRNAs are destabilized by Cth2 and Cth1 under iron depleted conditions [9,12]. In a high-Fe milieu, CCC1 deletion is lethal [21] and its expression is regulated by Yap5 [22], one of the eight members of the Yap Activator Protein (Yap) family [23].

Herein, we analyzed the transcriptional response of S. cerevisiae subjected to high-concentrations of Fe. Microarrays analyses of the yap5 mutant strain in the presence of Fe excess, allowed us to identify GRX4 as a Yap5 target. Given the role of Grx4 in Aft1 sub-cellular localization, we analyzed the effect of Yap5 deletion on Aft1 movement to and from the nucleus as a function of cellular iron status. We showed that the absence of Yap5 affects Aft1 localization.
**Results**

Genome-wide transcriptional analysis of *S. cerevisiae* exposed to high iron conditions

Although iron can be toxic, little is known about how iron excess affects metabolic pathways on a global scale in eukaryotic cells.

In order to investigate the response of *S. cerevisiae* to iron excess, we compared the mRNA expression profile of wild-type cells upshifted to Fe-enriched medium (2 mM FeSO₄, 20 and 60 min) to cells grown under Fe-adequate conditions (0.044 mg/L, as measured by inductively coupled plasma atomic emission spectroscopy).

Iron excess leads to an increase of the mRNA steady-state levels of 117 genes and to the repression of 92 genes (Table S1). Functional categories in the dataset are depicted in Figure 1. The transcript levels of genes included in the category Ribosome biogenesis have shown to be decreased (Table S1), whereas those of Stress response, Protein/peptide degradation, Respiration, Lipid/Fatty acids and Carbohydrate metabolism were found to be increased (Figure 1 and Table S1). These categories are altered whenever the environmental conditions change abruptly and cells rapidly and transiently need to reprogram their profile of gene expression [24,25].

With respect to changes in the iron regulon, the expression of the Aft1/2 targets *FET3, FTR1* and *SMF3* was found to be downregulated (Table 1). *FET3* encodes a plasma membrane multicopper oxidase and *FTR1* codes for an iron permease, constituting a complex that belongs to the high-affinity Fe transporter family [27] and whose gene is induced in high-Fe (Table 1). Functional categories in the Ribosome biogenesis category (n = 48) were not included. A list of all the genes included in each functional category is shown in Table S1.

**Figure 1. Transcriptional response to Fe overload in yeast.** BY4742 wild-type cells were grown to exponential phase in SC medium and challenged with 2 mM of FeSO₄. RNAs of Fe-treated and untreated cells were isolated and analyzed with DNA microarrays as detailed in Materials and Methods. Genes up or downregulated were sorted into functional categories according to MIPS database. Downregulated genes comprised in the Ribosome biogenesis category (n = 48) were not included. A list of all the genes included in each functional category is shown in Table S1.

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Yap5 only partially regulates the expression of CCC1

In the presence of iron, Yap5 becomes activated, leading to an increased expression of CCC1 and allowing for re-establishment of iron homeostasis [22]. However, the growth defect exhibited by ccc1 cells challenged with increased iron concentrations is markedly higher compared to the one displayed by the yap5 mutant (Figure 2A). While the ccc1 sensitivity to high-Fe was already observed when cells were grown in 5 mM of FeSO4, cells lacking Yap5 only showed a slight impairment of growth above concentrations of 15 mM (Figure 2A). Accordingly, while CCC1 expression is dramatically reduced in the yap5 mutant under Fe-adequate conditions, the CCC1 induction upon a shift to high Fe is not completely compromised, suggesting that CCC1 is regulated by other metal- or stress-responsive transcription factor. Consistent with Yap5 regulation of CCC1 expression in Fe-adequate growth medium, we showed that Yap5 is able to activate transcription under this condition (Figure S3).

Next, we examined whether the Yap5-independent levels of CCC1 expression were enough to cope with toxic Fe concentrations. As such, we generated a construct harboring the CCC1 coding region and its promoter without the two YREs (Yap Responsive Elements), located at -398 bp and -303 bp upstream from the initiation codon, ATG (Figure 2C). The construct designated by spCCC1 was then used to transform the ccc1 mutant cells and growth was assayed in plates containing Fe. We verified that ccc1 cells carrying the truncated version of CCC1 promoter were able to rescue ccc1 growth phenotype (Figure 2D). These data indicate that the YRE elements are not the only cis-elements implicated in the transcriptional response of CCC1 upon exposure to iron excess and corroborate the hypothesis that another not yet identified factor may also mediating CCC1 gene expression.

Table 1. Genes involved in iron homeostasis with expression altered in Fe excess.

| Systematic Name | Gene Name | Fold Change | Description |
|-----------------|-----------|-------------|-------------|
| YMR058w         | FET3      | -6.9        | Multicopper oxidase required for high-affinity Fe uptake |
| YER145c         | FTR1      | -3.3        | High affinity Fe permease |
| YLR034c         | SMF3      | -1.4        | NRAMP homolog Fe transporter |
| YNL240c         | NAR1      | -1.4        | Component of the cytosolic iron-sulfur (FeS) protein assembly machinery |
| YLL027w         | ISA1      | 1.5         | Protein involved in biogenesis of the iron-sulfur cluster of Fe-S proteins |
| YLR220w         | CCC1      | 2.7         | Transporter that mediates vacuolar Fe storage |
| YER174c         | GRX4      | 3.7         | Monothiol glutaredoxin |

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Figure 2. Yap5 is not the only regulator of CCC1 gene. (A) Exponentially growing cells from wild-type (WT), yap5 mutant (yap5) and ccc1 mutant (ccc1) strains were harvested, serially diluted and spotted onto control SC plates or SC plates containing the designated FeSO4 concentrations. (B) WT and yap5 strains were upshifted to high-Fe medium, by supplementation of SC medium with 2 mM of FeSO4, and harvested at the indicated time-points. The expression of CCC1 was assessed by qRT-PCR as described in Materials and Methods. Values are the mean of biological triplicates ± s.d. (C) Schematic representation of the truncated promoter version of CCC1 gene (spCCC1). (D) ccc1 strain was transformed with a plasmid harboring spCCC1 gene (ccc1<spCCC1>) or the plasmid alone (ccc1<vector>). Wild-type strain was transformed with the empty plasmid (WT<vector>). Exponentially growing cells were harvested, serially diluted and spotted onto control SD plates or SD plates containing 9 mM of FeSO4.

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Yap5 targets under high-iron conditions

Recently it was described that Yap5 regulates the expression of *TTW1* gene that codes for an iron-sulfur cluster enzyme that participates in the synthesis of wybutosine modified t-RNA, whose overexpression alleviates the *ccc1* high Fe sensitivity [33].

Aiming at identifying other Fe-dependent targets of Yap5, we characterized the global changes in the transcriptome of yap5 mutant cells in the presence of high levels of iron. DNA microarrays analyses were conducted by comparing RNAs isolated from the yap5 mutant vs. wild-type strains subjected to high-Fe concentrations for 20 and 60 min (Tables S2, S3). The total number of genes with altered expression in a yap5 strain, upon growth shift to high-Fe medium for 20 or 60 minutes, was 55 and 199, respectively.

Genes differentially expressed were sorted into functional categories according to MIPS. Our data showed that some categories were significantly more represented in yap5 mutant compared to wild-type cells (Figure 3). Interestingly, genes associated with Cell Cycle category were downregulated. It has been described that SBF (Swi4-Swi6 cell cycle box binding factor) binds the promoter of Yap5 [34], and this might explain the downregulation of genes involved in cell cycle.

We searched for genes direct or indirectly involved in iron homeostasis, whose expression is altered under the tested experimental conditions. We found out that in addition to *CCC1* and *TTW1*, Yap5 regulates the expression of *GRX4* and *FET3* genes (Table 2). We focused our studies on *GRX4*, which encodes a glutaredoxin known to be involved in the regulation of Aft1 [19,20].

**GRX4** is transcriptionally regulated by Yap5

To investigate the transcriptional regulation of *GRX4* by Yap5, we followed by qRT-PCR Grx4 mRNA levels in the presence of several iron concentrations, in the wild-type and mutant yap5 strains (Figure 4A). Upon growth shift to high Fe, there was an increase of *GRX4* mRNA levels that was absent in the yap5 mutant. We also monitored the protein expression of a HA-tagged version of Grx4 driven by its native promoter and observed that according to gene expression data, protein levels were decreased in the mutant strain (Figure 4B).

Using YEASTRACT [35], we found that *GRX4* possesses in its promoter region 2 potential YREs, placed at −253 bp and −320 bp from the ATG (Figure 4C). To investigate whether both YREs are functional, we cloned *GRX4* gene and its promoter in a centromeric plasmid and mutated each or both YRE(s) (Figure 4C). The resulting constructs were used to transform the *grx4* mutant and *GRX4* expression was monitored by qRT-PCR (Figure 4D). In the presence of high Fe, there was a remarkable decrease in the expression of *GRX4* when the YRE located at −253 bp or both YREs were mutated (Figure 4D). Mutation of the −320 bp YRE had no effect in *GRX4* expression, suggesting that the YRE closer (−253 bp) to ATG is necessary and sufficient for Yap5 iron-regulated expression of *GRX4*.

We carried out chromatin immunoprecipitation (ChiP) analyses, using a HA-tagged version of Yap5, and detected a significant binding of Yap5 to *GRX4* promoter even in the absence of Fe (Figure 4E). As depicted in Figure 4E, after immunoprecipitation of chromatin bound to Yap5-HA (IP), an enrichment of *GRX4* promoter harboring the YRE located at −253 bp was observed, as compared to the promoter of Actin gene (ACT) that served as a negative control of binding. The same pattern of transcriptional regulation was observed by others regarding Yap3 regulation of *CCC1* expression [22].

Yap5 binding specificity to *GRX4*, in the Yap5-HA ChiP, was further confirmed by comparing the fold enrichment of *GRX4* with two non-harboring YREs genes-*SCR1* and *ARN2*- coding for an mRNA and a siderophore transporter, respectively (Figure 4F). *GRX4* exhibits significant threefold enrichment compared to *SCR1* and *ARN2* in the Yap5-HA ChiP, as illustrated in Figure 4F.

Our studies corroborate a previous genome-wide location analysis in which is shown Yap5 bound to *GRX4* promoter region [36].

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**Figure 3. Functional categories of genes differentially expressed in yap5 mutant cells.** BY4742 wild-type and yap5 mutant strains were grown to exponential phase in SC medium and challenged with 2 mM of FeSO₄ for 60 min. RNA of Fe-treated cells was isolated and analyzed with DNA microarrays as detailed in Materials and Methods. Genes up- or downregulated were sorted into functional categories according to MIPS database. Dubious open reading frame unlikely to encode a protein (100) were not considered. A list of all the genes included in each functional category is shown in Table S3.

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Together these data suggest that the binding of Yap5 to GRX4 is Fe-independent and that Yap5 is directly regulating the iron-dependent transcription of GRX4 gene.

Aft1 localization is affected in the yap5 strain under Fe-adequate and Fe overload growth conditions

Because GRX4 transcription is dependent on Yap5 (Figure 4) and given that Gxl4 together with Grx3 controls Aft1 nuclear localization, we next questioned whether Aft1 localization is affected in the yap5 mutant strain.

We first examined the localization of a plasmid overexpressing Aft1 fused to GFP [20] in the wild-type, yap5 and grx4 mutant strains, by in vivo fluorescence microscopy. Cells exhibiting nuclear fluorescence were counted before (SD medium) and after (+FeSO4) Fe addition to the culture. Under Fe-adequate condition (SD) yap5 was the strain that exhibited less Aft1 in the nucleus (Figure 5A and 5B). On the contrary, when cells were shifted to Fe-enriched medium (+FeSO4), there was more cells with Aft1 in the nucleus in the yap5 mutant compared to the isogenic parent strain (Figure 5A and 5B). The latter effect might be a consequence of the Fe-dependent Yap5 regulation of GRX4 (Figure 4A), since grx4 mutant cells also exhibited a higher content of Aft1 in the nucleus upon Fe addition to the culture (Figure 5A and 5B).

To further confirm these results, we assayed the dependence on Yap5 of the Aft1 target FET3 through qRT-PCR (Figure 5C).

In addition, we used another indirect approach to test the influence of Yap5 in Aft1 localization. Wild-type and yap5 strains were transformed with a plasmid (CTH2-LacZ) containing the consensus Aft1 binding sequences of CTH2 promoter fused to a lacZ reporter [12]. CTH2 and CTH1 are gene targets of Aft1, both expressed in iron deficiency. Corroborating the above assays, we observed that under Fe adequacy (SD) the β-galactosidase activity was higher in the wild-type compared to the mutant strain, but upon a growth-shift to high Fe yap5 mutant exhibited the highest β-galactosidase expression (Figure 5D).

Together these data indicate that Yap5 interferes with Aft1 nuclear localization under Fe adequacy and Fe overload, in opposite ways. Under Fe-adequate conditions there is less Aft1 in the nucleus in the yap5 mutant, whereas under high Fe conditions there is more Aft1 in the nucleus of the mutant cells.

Discussion

Disruption of the iron homeostasis dynamic process originates significant damage in cells. Therefore, organisms and yeasts in particular have evolved sophisticated mechanisms that, on one hand avoid the drastic consequences of iron scarcity and, on the other hand circumvent the toxic effects of iron overload. It is relevant to understand how cells cope with high Fe levels, since hemochromatosis caused by Fe overload is one of the most common human genetic diseases.

The work here reported shows that, as transcriptional primary response to iron excess, S. cerevisiae represses genes involved in iron uptake, FET3 and FTR1, as well as in iron mobilization from the vacuole, SMF3; it also induces genes implicated in Fe storage, CCC1, and in Aft1 export from the nucleus, GRX4. Apparently, the alteration in the expression pattern of these genes is enough to control the intracellular iron concentration that would lead to Fe-induced toxicity. CCC1 induction as well as FET3 and FTR1 repression, when in the presence of high-Fe, have been also reported by other authors [21,22,26]. Surprisingly, only three genes of the Fe-regulon were found to be downregulated (see Table 1, FET3, FTR1 and SMF3). Because under iron overload conditions Aft1 is exported from the nucleus, one would expect to see a greater number of Aft1-dependent genes being down-regulated. Yun et al. noticed that Aft1-dependent transcription is repressed in Fe-sufficient medium [37]. Furthermore, under these conditions, RNAse III Rnt1-mediated RNA surveillance is required to prevent iron toxicity [38]. Rnt1 degrades several Aft1-dependent targets, but not FET3 or FTR1 mRNAs; this may explain why we did not detect other Aft1 targets.

The novelty of our analysis is the induction of GRX4 in a Fe-dependent manner. The physiological function of Grx4/3 has been extensively studied and its role in Aft1 activity inhibition was proposed to be due to the sequester of this transcription factor in the cytosol under iron overload conditions [19,20]. Hence, it is likely that cells increase Grx4 levels when facing high-Fe concentrations in order to guarantee that Aft1 is efficiently removed from the nucleus. Grx4/3 may also participate in intracellular iron trafficking, as the double mutant grx4grx3 elicits severe defects in the maturation of cellular Fe-S proteins, heme-containing and di-iron enzymes, despite the constitutive Aft1 activation and the consequent cytosolic iron accumulation [39,40]. Shakoury-Elizeh et al. observed that under high-Fe pathways involving biotin biosynthesis and nitrogen assimilation via glutamate synthase (Glt1) were activated [11]. These pathways requiring Fe-dependent enzymes may possibly lead to the increase of Grx4 levels that correlates with the shift to a most Fe-consuming metabolism. Grx3 and Grx4 bind a Fe-S center together with glutathione which is crucial for their function [39,40,41]. Therefore another possibility is that Grx4, itself, may buffer the increased cytosolic iron concentrations under such conditions.

Under iron overload conditions, the transcription factor Yap5 is activated and increases the expression of CCC1 that transports iron into the vacuole, leading to the consequent decrease of the cytosolic iron pool [22]. We have now shown that although Yap5

| Table 2. Genes involved in iron homeostasis whose expression is altered in the yap5 mutant. |
|---|---|---|---|---|
| **Systematic Name** | **Gene Name** | **FC* 20 min** | **FC* 60 min** | **Description** |
| YMR058w | FET3 | 1.5 | 2.4 | Multicopper oxidase required for high-affinity Fe uptake |
| YLR220w | CCC1 | -2.9 | -1.7 | Transporter that mediates vacuolar Fe storage |
| YER174c | GRX4 | -2.7 | -1.9 | Monothiol glutaredoxin |
| YPL207w | TTY1 | - | -1.5 | Protein required for the synthesis of wybutosine |

*FC-Fold Change.

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does indeed regulate CCC1 transcription, another yet unidentified Fe-responsive factor drives the expression of CCC1 up to sufficient levels to overcome Fe toxicity. The putative existence of another Fe-responsive factor could justify the almost-normal growth displayed by the yap5 strain in high-Fe medium, as well as the rescue of the poor-growth phenotype of the ccc1 strain by a plasmid harboring the CCC1 gene without YREs (Figure 2). Furthermore, we showed that, under these conditions, in addition to CCC1, 

Figure 4. GRX4 gene expression is dependent on Yap5. (A) Exponentially growing cells from wild-type (WT) and yap5 mutant (yap5) strains were upshifted to high-Fe medium, by supplementation of SC medium with the indicated FeSO₄ concentrations, and harvested at the indicated time-points. The expression of GRX4 was assessed by qRT-PCR as described in Materials and Methods. Values are the mean of biological triplicates ± s.d. (B) WT and yap5 strains were transformed with a plasmid carrying GRX4-HA, treated with 5 and 15 mM of FeSO₄ for 20 min and analyzed by Western blot with an anti-HA antibody. Pgk1 protein levels were used as loading control. (C) Representation of the GRX4 constructs without (a) or with (b,c,d) mutations in the YREs. (D) Mutant grx4 cells expressing the constructs depicted in (C) were grown under Fe-adequate or Fe overload (5 mM FeSO₄) conditions, and the expression of GRX4 was assessed by qRT-PCR as described in Materials and Methods. Values are the mean of biological triplicates ± s.d. (E) yap5 cells were transformed with HA-tagged Yap5 and grown in SD medium not supplemented (−Fe) or supplemented (+Fe) for 15 min with 2 mM of FeSO₄, before being processed for ChIP. ChIP analysis was performed using probes specific for GRX4. (F) ChIP analyses combined with qRT-PCR, were used to determine the fold enrichment of GRX4, SCR1 and ARN2. The sequence enrichment in the ChIP (i.e. IP/IN) was normalized using the ACT gene as a reference. 

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Yap5 is also regulating the expression of GRX4 (Figure 4). Consistently with Grx4 dependence on Yap5, we demonstrated, using different approaches, that Yap5 affects Aft1 localization under Fe overload: *yap5* cells have more Aft1 in the nucleus and consequently exhibit an upregulation of FET3 (Figure 5). We cannot however exclude the possibility that Yap5 might as well play a role in iron trafficking, or in buffering iron excess, due to the recent findings on the role of glutaredoxins Grx3/Grx4 in iron metabolism [39,40].

Moreover, we demonstrated that Yap5 also plays a role under Fe-adequate growth environments, as in such condition, Yap5 is the major regulator of CCC1 (Figure 2A). As such, in the *yap5*
mutant strain, **CCC1** levels are severely compromised, leading to an increase of cytosolic iron with the consequent inhibition of Aft1 (that localizes mainly in the cytosol) and the concomitant downregulation of its target **FET3** (Figure 5).

In light of the data described herein, we propose that under Fe-adequacy Yap5 is activated leading to the upregulation of **CCC1** with the consequent accumulation of Fe in the vacuole and deprivation of the cytosolic iron pool. As a result, Aft1 translocates to the nucleus and upregulates cytosolic iron uptake genes. Under Fe overload conditions, Yap5 transactivation potential increases leading to the upregulation of **GRX4** gene and thus inhibiting Aft1 nuclear localization. Simultaneously, it seems that another iron responsive factor is activated, contributing to the **CCC1** induction and most probably bypassing Yap5 regulation (Figure 6). Further work is in progress in order to clarify the mechanism that controls the Yap5-independent regulation of **CCC1**.

### Materials and Methods

#### Strains, growth conditions and sensitivity analysis
Most of the experiments were performed with the wild-type BY4742 (MAT alpha; his3Δ1; leu2Δ0;lys2Δ0) and its isogenic derivates. The yap1, yap, grx4 and ccc1 strains were purchased from Euroscarf. To produce the double mutant strain yap1,leu2Δ0(cit1), leu2Δ0; ccc1Δ0; yap1::His, TLR220W;xanMX), the complete coding region of **YAP1** gene was deleted by the microhomology PCR method [42] from the **ccc1** strain. Deletion was confirmed by PCR analysis of genomic DNA using upstream and downstream **YAPI** specific primers. EGY48 (Mat alpha ura3-52 his3 LEXALexA-LexA2 <pSH18-34 URA3 2 μ>) was used for transactivation potential assay. Strains were grown in synthetic media (SC: 0.67% ammonium sulphate-yeast nitrogen base without amino acids [Difco], 2% glucose, supplemented with the appropriate selective amino acids) or SC lacking specific requirements (SD). Phenotypic growth assays were carried out by spotting 5 μl of early exponential phase cultures (A600 = 0.4) sequentially diluted (approximately 5×10³ to 10 cells) in medium containing up to 20 mM of FeSO₄. Growth was recorded after 2 days at 30°C. Anaerobiosis assays were performed with cultures transferred to a glove box, for at least 24 h, where the O₂ concentration was kept below 1 ppm. The strains were grown in liquid SD until early exponential phase and phenotypic growth assays were performed as described above. Esherichia coli strain XL1-Blue mcr1 lacD1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacIqZ857 M15 Tn10 (TetR)] (Stratagene) was used as the host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation.

### Plasmids
The **pCCC1** plasmid was generated by blunt cloning into the vector pRS416 (Stratagene) the **CCC1** gene amplified by PCR using BY4742 genomic DNA as template and the primers 5’TACAGGACACACCCCTCAG3’ and 5’GCCCTCTTGGCAGTATAGAAA3’. The construct **pCCC1** was obtained by digesting **pCC1** with XhoI (Fermentas).

Yap5-HA contains a hemagglutinin (HA) epitope inserted immediately upstream the stop codon, and is expressed from the centromere pRS416 plasmid. The following three-step PCR strategy was used to HA-tagged Yap5: **YAP5** promoter region and coding sequence were amplified using the primers CAAAATGTGGTGGTAGTGATTAAAGGACTGGGACCCTAT/CATTAAGGACTGGGACCCTAT and used as the template for a second PCR using the pair CCGGTCCATCATCCACATGGAACGATCATCCA-GATTTACCGTGAAGAGAGAGACTTCTGTAAGTGCAAATGATCG-AFT1-GFP plasmid [20] was a gift from Dr. M. Toledano. The plasmid Yap5-lexA used in this work has been described previously [43].

Plasmids containing consensus (pCM64-CTH2-FeRE-CYC1-LacZ) or mutant (pCM64-CTH2-FeRE-CYC1-LacZ M3) Aft1 binding sequences from **CTH2** promoter fused to the **CYC1** minimal promoter-LacZ reporter [12] were a gift from Dr. D. Thiele.

AFT1-GFP plasmid [20] was a gift from Dr. M. Toledano. **GRX4** gene and its promoter were amplified by PCR with the primers GTTAAACGCTTTTATTGTAAGTG/ACGAAGAAGACCCCTATTCC to clone into pRS416. The first upstream YRE in **GRX4** promoter region was mutated by site-directed mutagenesis as detailed in [44] with two primers: ggaatatgtagcagaat/tgaatgcataaatc. The second YRE in **GRX4** promoter region was mutated using the same strategy, but using the primer pair: tcggagatcaggaga/cgctcaagcttacatgctctacagatg.
Microarray and qRT-PCR analyses

DNA microarray analysis was carried out using in-house spotted DNA-microarrays of **Saccharomyces cerevisiae**, and standard protocols of public domain software used by the NFDM. For transcript profiling, total RNA from early log-phase cultures, either untreated or exposed to 2 mM of FeSO₄, was purified using the RNeasy kit (QIAGEN), followed by the RNA clean up procedure. Forty micrograms of RNA were used to generate labeled cDNA, which were hybridized on the DNA arrays. Images of the microarray hybridizations were acquired using BRB-ArrayTools v3.4.0 software [45], using the Agilent G2565AA microarray scanner. The fluorescence intensities were quantified with QuantArray v3.0 software (PerkinElmer). Using BRB-ArrayTools v3.4.0 software [45], manually flagged bad spots were eliminated and the background was subtracted before averaging the replicate features on the array. Log₂ intensity ratios (M values) were then Median normalized to correct for differences in labeling efficiency between samples.

The relative hybridization signal of each ORF was derived from the average of two dye-swap hybridizations performed for each strain. The normalized log₂ ratio (M value) was considered as a measure of the relative abundance of each ORF relatively to that of the reference strain BY4742. Deviations from the 1:1 hybridization ratio were taken as indicative of changes in gene expression.

T-test analyses, with p value<0.05 were performed using the algorithm implemented in MeV from TM4 software [46]. The individual hybridizations were used as the input data, in a total of two dye-swap hybridizations for each strain. Functional annotations and GO terms association was done following the SGD annotations (http://www.yeastgenome.org/).

The array design, spotting protocol, raw data and pre-processed data from all hybridizations were submitted to the ArrayExpress database and can be accessed using the accession number E-MEXP-3193.

Searches for putative Yap binding sites were carried out using the YEASTRACT database [35]. Gene clustering was performed according to the Munich Information Center for Protein Sequences database (MIPS) functional catalogue (http://mips.helmholtz-muenchen.de/proj/functDB/).

For qRT-PCR experiments, RNA was extracted from early log-phase cultures that were either untreated or exposed to the indicated FeSO₄ concentrations and harvested at the indicated time points. DNA was removed by on-column DNAse I digestion (RNase-Free DNase Set; Qiagen). Total RNA (1 μg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). Gene primer sequences used in the analyses were as follows: **CCL1**: AACCGAAGTGGTGAACCTTAT/TCCTGCGGTGAATTTCTACCC, **GRX4**: TGCCCTACCTAGC-GAACAAAT/AGGTTCGTGAGGGTCCTCTT and **FET3**: ACGGTTGTAATACGCGCTT/TTGGAAAGCGTGACTAT. Primer sequences were the following: **SCR1**: gcgtctctgctgtgagccgcttctgccgctcatcatac; **ARV2**: agggccccagttagatgctgtaggagttggagccgctcatcatac; **GRX4**: ggccacagccctagctagttggagccgctcatcatac. The latter primer pair was used to amplify the region of the GRX4 promoter flanking the YRE located at −253 bp from the ATG. **GRX4** primers were designed in order to exclude the promoter region harboring the YRE located at −320 bp.

Measurements of β-galactosidase activity

The strain EGY48 carrying pSH18-34 (2 μ plasmid carrying a lacZ reporter gene under the control of eight lexA operators) was transformed with Yap5-LexA. BY4742 and its isogenic strain yap5 were transformed with the plasmids pCM64-CTH2-FrERE-CYCI-LacZ and pCM64-CTH2-FrERE-CYCI-LacZ. Cells were grown in SD liquid medium to early log phase, in the presence of 100 μM of BPS (Pathophenolinsulonic acid disodium salt hydrate) or exponentially grown in SD and challenged with 2 mM of FeSO₄ and harvested after 30 min. Relative β-galactosidase activity was monitored as in [48]. Enzyme activity was assayed by following the degradation of the colorimetric substrate ONPG (o-nitrophenyl-b-D-galactopiranoside) at A₅₉₀ and normalized against total protein concentration. The results are the average of at least three biological replicates (n=3).

Fluorescence microscopy

BY4742 and yap5 strains transformed with a plasmid containing AFT1 fused to yEGFP3 [20], were grown to early log phase and induced with 2 mM of FeSO₄ for 30 minutes. 4.6-Diamino-2-phenylindole (DAPI) was added as a DNA marker at a final OD₆₀₀ 1±0.1 were fixed with 1% formaldehyde for 30 min at room temperature, with occasional agitation. The cross-linking was stopped by the addition of glycine to a final concentration of 340 mM. Cells were collected by centrifugation and were disrupted with a FastPrep®-24 instrument (MP Biomedical) in lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% Na-deoxycholate) containing a protease inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. The cell extracts were collected and subjected to sonication to yield DNA fragments in a size range between 100 to 1,000 bp with an average of 500 bp. The cross-linked chromatin was separated from the insoluble debris by centrifugation for 10 min at 10000 g at 4°C. HA-tagged Yap5 was immunoprecipitated by incubating the cross-linked chromatin with the HA-antibody prebound to 50 μl of Dynabeads Pan Mouse IgG (Invitrogen) for 16 h at 4°C. Immune complexes were washed twice in lysis buffer containing 360 mM NaCl, once in wash buffer (10 mMTris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40 and 0.5% Na-deoxycholate) and once in TE. Immunoprecipitated proteins were eluted from the beads by heating the samples for 20 min at 65°C in elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 0.5% SDS), with agitation at 12000 rpm, and fixation was reversed by heating the eluates for 16 h at 65°C. Aliquots of total chromatin input (IN) and immunoprecipitated (IP) chromatin were simultaneously processed for subsequent normalization. After treatment of samples with proteinase K and RNase A the DNA was purified, using the DNA Clean & Concentrator system (ZYMOS RESEARCH), and was eluted in 30 μl TE. Quantification of specific DNA targets (GRX4, ACT1 and SCR1) in the IN and IP samples was performed by real-time PCR. A standard curve, generated with a dilution series of the IP sample, was used to assess the PCR efficiency and the relative enrichment of a specific locus in the immunoprecipitate was determined using the ΔΔCT method through the calculation of log2 (IP/IN). The primer sequences were the following: **SCR1**: cgctctctgctgtgagccgcttctgccgctcatcatac; **ARV2**: agtgatgctgtaggagttggagccgctcatcatac; **GRX4**: gcctagagccctgtagttattgtac. The latter primer pair was used to amplify the region of the GRX4 promoter flanking the YRE located at −253 bp from the ATG. **GRX4** primers were designed in order to exclude the promoter region harboring the YRE located at −320 bp.
Immunoblot Assays

Western blot analyses were performed using early exponential phase cells, challenged with 5 mM or 15 mM of FeSO4 and harvested at the indicated time-points.

Protein extracts were generated from cell cultures using cell lysis buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM KCl, 10% glycerol, 0.1% NP40) supplemented with protease inhibitors (Roche). Protein concentrations were determined using the Bradford assay and 80–100 μg of protein was resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Protein levels were detected using Anti-HA-Peroxidase, High Affinity from rat IgG1 (Roche) and Anti-α-Sba1 or Anti-α-Pgk1. Sba1 and Pgk1 were used as loading controls [49].

Supporting Information

Table S1 Genes whose mRNA steady-state levels are altered in Fe excess (2 mM FeSO4).

Table S2 Genes dependent on Yap3 whose mRNA steady-state levels are altered upon cells incubation with 2 mM of FeSO4 for 20 min.

Table S3 Genes dependent on Yap3 whose mRNA steady-state levels are altered upon cells incubation with 2 mM of FeSO4 for 60 min.

Figure S1 Copper metabolism in yeast is affected upon cells incubation with 2 mM of FeSO4 for growth shift to high-Fe medium.

Figure S2 In the absence of Ccc1, Yap1 is required for cells to overcome Fe-induced oxidative stress. Exponentially growing cells from wild-type (BY4742), yap1, yap1ccc1 and ccc1 strains were harvested, serially diluted and spotted onto control SC plates or SC plates containing the indicated FeSO4 concentrations under (A) aerobicosis and (B) anaerobiosis.

Figure S3 Yap5 transactivation potential in different media used in this work. (B) The transactivation potential of Yap5 in SD medium not supplemented (SD) or supplemented with 100 μM of BPS (SD-Fe), or 2 mM of FeSO4 (SD+Fe), was assayed. EGY48 strain carrying pSH18-34 (a plasmid carrying a lacZ reporter gene) was transformed with Yap5-LexA and β-galactosidase activity was monitored as described in Experimental procedures. Values are the mean of triplicate samples of the same experiment ± s.d.

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Figure S1 Copper metabolism in yeast is affected upon growth shift to high-Fe medium. BY4742 wild-type cells were transformed with a plasmid containing CCS1 (copper chaperone for Cu/Zn superoxide dismutase) HA-tagged and exponentially grown in SD medium. (A) Cells were treated with 2 mM of FeSO4, harvested at the indicated time-points and examined by Western blot with an anti-HA antibody. (B) Ccs1-HA response to high-Cu (9 mM CuSO4, 60 min) was monitored by Western blot and served as Ccs1-HA functional control. Sh1 protein levels were used as loading control.

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Author Contributions

Conceived and designed the experiments: CP CRP. Performed the experiments: CP CV RAM SC. Analyzed the data: CP CRP LC. Wrote the paper: CP CRP.

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