

A Role for Iron-Sulfur Clusters in the Regulation of Transcription Factor Yap5-dependent High Iron Transcriptional Responses in Yeast*

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Background: *Saccharomyces cerevisiae* responds to increased cytosolic iron through changes in the activity of the transcriptional activator Yap5.

Results: A genetic screen identified a mutation in *SSQ1* that prevented Yap5-dependent transcription.

Conclusion: Reduced levels of proteins required for mitochondrial iron-sulfur cluster synthesis prevented Yap5-dependent transcription.

Significance: The transcriptional response to low iron or high cytosolic iron is dependent on iron-sulfur clusters.

Yeast respond to increased cytosolic iron by activating the transcription factor Yap5 increasing transcription of *CCC1*, which encodes a vacuolar iron importer. Using a genetic screen to identify genes involved in Yap5 iron sensing, we discovered that a mutation in *SSQ1*, which encodes a mitochondrial chaperone involved in iron-sulfur cluster synthesis, prevented expression of Yap5 target genes. We demonstrated that mutation or reduced expression of other genes involved in mitochondrial iron-sulfur cluster synthesis (*YFH1*, *ISU1*) prevented induction of the Yap5 response. We took advantage of the iron-dependent catalytic activity of *Pseudaminobacter salicylatoxidans* gentisate 1,2-dioxygenase expressed in yeast to measure changes in cytosolic iron. We determined that reductions in iron-sulfur cluster synthesis did not affect the activity of cytosolic gentisate 1,2-dioxygenase. We show that loss of activity of the cytosolic iron-sulfur cluster assembly complex proteins or deletion of cytosolic glutaredoxins did not reduce expression of Yap5 target genes. These results suggest that the high iron transcriptional response, as well as the low iron transcriptional response, senses iron-sulfur clusters.

Yeast respond to changes in the intracellular concentration of transition metal levels by altering transcription of genes that encode proteins required for metal acquisition, storage, or usage. *Saccharomyces cerevisiae* responds to iron deprivation by inducing the expression of about 20 genes collectively referred to as the iron regulon (for review, see Refs. 1 and 2). These genes encode cell surface and vacuolar elemental iron transporters, cell surface siderophore transporters, proteins involved in iron-independent metabolic pathways, and proteins that destabilize transcripts involved in iron storage and metabolism. The expression of these genes is regulated by the transcription factor Aft1 and to a lesser extent by the paral-

gous Aft2. Aft(s) reside in the cytosol and translocate into the nucleus in response to low iron where they occupy the promoters of iron regulon genes. Studies suggest that Aft1 responds to changes in mitochondrial iron-sulfur (Fe-S) cluster production and export, for which iron is an essential substrate (3). Defects in the assembly of cytosolic Fe-S cluster-containing proteins do not activate the iron regulon (4). The precise role of Fe-S clusters in regulating the iron regulon remains to be clarified, but it is thought that the cytosolic glutaredoxins Grx3 and Grx4, which can obtain Fe-S clusters independent of the cytosolic Fe-S machinery (5), may play a role in regulating Aft1 transcriptional activity (6, 7).

S. cerevisiae also respond to high iron conditions by activating the transcription factor Yap5. Yap5 is constitutively present in the nucleus, where it occupies the promoters of at least three genes (*CCC1*, *TYW1*, *GRX4*) (8–10). Most notably, *CCC1*, which encodes the vacuolar iron importer, plays a major role in regulating cytosolic iron concentration (8, 9). The activation domain of Yap5 contains 7 cysteines important for iron sensing as mutation of those cysteines reduces transcription. Iron is known to participate in redox reactions, which can affect cysteine sulfhydryls. We determined that Yap5 was not activated by oxygen radicals as H₂O₂ addition did not activate Yap5 transcription and high iron under anaerobic conditions activated Yap5 transcription (8).

We utilized a genetic screen to identify the mechanism of high iron induction of Yap5. We found that Yap5 activation is dependent on the mitochondrial production of Fe-S clusters as Yap5-dependent transcription does not occur in the absence of mitochondrial Fe-S cluster synthesis. We showed that reductions in mitochondrial Fe-S cluster synthesis do not decrease cytosolic iron levels. We also show that the cytosolic Fe-S assembly complex is not involved for Fe-S cluster dependent Yap5 activation.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Culture Conditions—The wild type strains DY150 or DY1457 (W303 background) and deletion strains were used for most experiments. The Δ *ccc1* and

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TABLE 1

Strains used in this study

Yeast strain	Genotype	Source
DY150	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)</i>	Wild type
$\Delta ccc1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta ccc1::HIS3$</i>	Refs. 8 and 9
$\Delta ccc1/FET3/FTR1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta ccc1::HIS3$ PGK1FET3/FTR1::ade2-1 FET3-lacZ::HO</i>	Ref. 3
$\Delta isu1\Delta ccc1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta isu1::KanMX \Delta ccc1::HIS3$</i>	This study
BY4742	<i>MATa his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0</i>	Wild type
$\Delta glr1$	<i>MATa his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0 $\Delta glr1::KanMX$</i>	Ref. 8
$\Delta grx3\Delta grx4$	<i>MATa his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0 $\Delta grx3::LEU2 \Delta grx4::KanMX$</i>	Ref. 6
$\Delta trr1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta trr1::HIS3$</i>	Ref. 8
<i>Gal-DRE2</i>	<i>MATa ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 leu2-Δ1 GAL1-DRE2::HIS3</i>	Ref. 27
<i>Gal-NAR1</i>	<i>MATa ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,112 GAL1-NAR1::HIS3</i>	Ref. 26
$\Delta yfh1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta yfh1::HIS3$ MET3-YFH1</i>	Refs. 3 and 11
$\Delta ssq1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta ssq1::KanMX$ MET3-SSQ1</i>	This study
$\Delta ssq1\Delta ccc1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta ssq1::KanMX \Delta ccc1::HIS3$ MET3-SSQ1(URA3)</i>	This study
$\Delta yfh1\Delta ccc1$	<i>MATa ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc) $\Delta yfh1::HIS3 \Delta ccc1::LEU2$ MET3-YFH1(URA3)</i>	This study

$\Delta yfh1$ strains were described previously (3, 11). The $\Delta isu1$ strain was generated in a DY150 background by PCR amplifying the deletion construct with the *KanMX* marker from the homozygous diploid deletion collection (Research Genetics, Stanford, CA). Deletion strains of $\Delta grx3\Delta grx4$ were derived from BY4742 (S288C background), and the *GalDRE2* and *GalNAR1* were gifts from Dr. Winge (University of Utah). The double deletion $\Delta ccc1\Delta yfh1$ strain was generated by PCR amplification of the *CCC1* deletion construct and recombination into the background strain. *SSQ1* was deleted in DY1640 diploid cells by PCR amplification of the *KanMX* marker from the heterozygous diploid deletion collection (Research Genetics) and transformed with a plasmid containing *MET3-SSQ1*. Tetrads were dissected, and haploids with a *SSQ1* deletion were isolated. The *MET3-SSQ1* plasmid-expressing $\Delta ssq1$ cells were crossed with $\Delta ccc1$ cells to generate a $\Delta ssq1\Delta ccc1$ double deletion. All yeast strains used in this study are shown in Table 1. The complete minimal (CM)³ medium was composed of yeast nitrogen base without amino acids, dextrose, and the required amino acids. Dextrose was replaced by galactose as specified. Low iron media were made by adding 80 μ M bathophenanthroline disulfonate (BPS) and the specified concentration of FeSO₄ or ferrous ammonium sulfate. For anaerobic experiments, cells were grown in a BBL™ GasPak™ EZ anaerobic container system (Thermo Fisher Scientific).

Yeast Genetic Screen Designation—The parent strain is a $\Delta ccc1$ strain in a DY1457 background with a *KanMX* marker. *CCC1-lacZ* was integrated into the genome at the *HO* locus, and a construct containing the *GAL1* promoter (~1000 up stream of *GAL1* gene) fused to the *HIS3* gene was integrated into the genome at the *URA3* locus. A construct containing a *GAL4-YAP5* chimera, which responds to iron (8), with a *TRP1* marker was transformed into the parent strain. Cells were plated on 200 μ M iron-containing plates and exposed to UV for 12 s. Candidates were selected by a β -galactosidase color assay and –HIS plate spot assay.

β -Galactosidase Assay— β -Galactosidase activity was measured by a kinetic assay (7). Protein determinations were performed using the bicinchoninic acid assay (Pierce), and enzyme

activity is expressed as nmol/min/mg of protein. Blue color colony assays were performed by transferring fresh yeast colonies to a nitrocellulose membrane, cells were permeabilized by freeze/thaw in liquid nitrogen, and color was developed by adding X-gal overnight at 30 °C (12).

Total RNA Extraction and RT-PCR Assay—Total RNA was isolated from cells grown to mid-log phase using an Agilent RNA kit (Agilent). First-strand cDNA synthesis was performed using a SuperScript III kit from Invitrogen. Real-time PCR was performed using primers for the detection of *CCC1* transcription (5'-tcggtggcggttactact-3' and 5'-accaccacaacaaccattt-3'), primers for the detection of *FET3* (5'-tca cag ttt cga tcc gga caa cca-3' and 5'-tcc ac gaga aca aga ccc aaa cct-3'), and primers for the detection of *TYW1* (5'-aac aaa tgg tgg cga aag ac-3' and 5'-tgg atg aac atg cca aag aa-3'). Expression levels were normalized to the internal control *CMD1*, as described previously (8).

Western Blot Analysis—For Western blot assays, cell extracts were prepared by homogenization with glass beads in 25 mM Tris-HCl, pH 7.4, 50 mM NaCl lysate buffer with 1 \times protease inhibitor tablets (Roche) and 0.5 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich). Mouse anti-FLAG antibody and mouse anti-PGK antibody were purchased from Invitrogen. Rabbit anti-Yap5 (1:500) was generated in our laboratory using purified Yap5 as an antigen. Mouse anti-carboxypeptidase Y (1:3000) was obtained from Mitosciences Inc. (Eugene, OR). Mouse anti-HIS-G (1:5000) was obtained from Invitrogen. Peroxidase-conjugated goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:5000) (Jackson ImmunoResearch Laboratories) was used as secondary antibody. Western blots were developed using Western Lightning reagent (PerkinElmer Life Sciences).

Gentisate 1,2-Dioxygenase Assay—Gentisate 1,2-dioxygenase (GDO) plasmid was a generous gift from Dr. Stolz from Stuttgart University, Germany. The gene was cloned into yeast vector pRS426 using SpeI and BamHI restriction sites, and its expression was under the control of *ADHI* promoter. A FLAG tag was added to the carboxyl terminus of GDO. Plasmids were transformed into yeast cells, grown on suitable selective medium to exponential growth phase, and harvested by centrifugation. Transformed cells were washed with deionized water and lysed by glass bead agitation in a lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich)). The cell lysates were then subjected to GDO activity assay as described previously (12). The assay mix-

³ The abbreviations used are: CM, complete minimal; BPS, bathophenanthroline disulfonate; GDO, gentisate 1,2-dioxygenase; PGK, phosphoglucose kinase; CIA, cytosolic iron-sulfur protein assembly; IRP, iron regulatory protein.

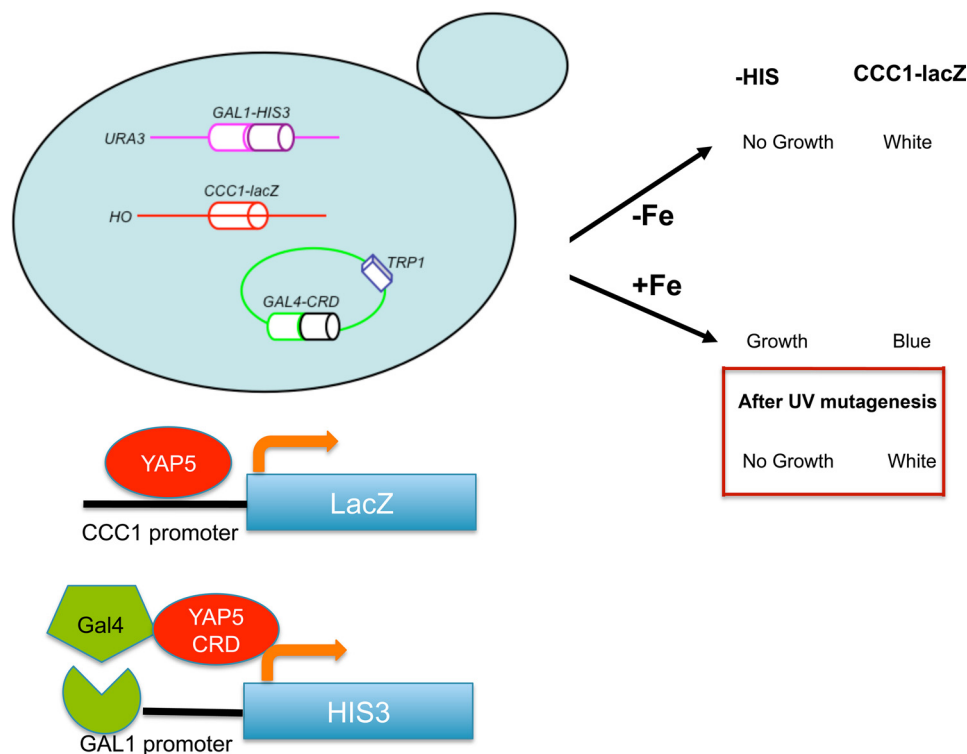


FIGURE 1. **Diagram of selection screen to identify genes involved in the high iron transcriptional response.** A $\Delta ccc1$ yeast strain was generated that contained the promoter region of *CCC1* region fused to a *lacZ* reporter integrated at the *HO* locus. The strain also contained a deletion in the *HIS3* gene and a copy of *HIS3* under the control of the *GAL1* promoter integrated at the *URA3* locus. The strain was transformed with a *TRP1* plasmid containing the *GAL4* DNA binding domain fused to a *YAP5* cysteine-rich activation domain (*CRD*). In the presence of iron, the cells are *HIS3* prototrophs and express β -galactosidase.

ture contained 20 mM Tris-HCl (pH 8.0) and 0.1 mM 2,3-dihydroxy-benzoic acid (gentisic acid) as substrate (Sigma-Aldrich). Absorbance was monitored at 340 nm, and enzyme activity was calculated using an extinction coefficient of $10.2 \text{ cm}^{-1} \text{ mm}^{-1}$. The activity was expressed as nmol of substrate converted per minute per mg of protein.

RESULTS

A Genetic Screen for High Iron Sensing Identifies a Role for Fe-S Clusters—We designed a genetic screen to identify genes involved in iron-dependent Yap5 transcription. We engineered a $\Delta ccc1$ strain that contained two independent Yap5 reporters. The strain was deleted for *HIS3* and contained an integrated *HIS3* gene under the control of a *GAL1* promoter (Fig. 1). This strain also contained a plasmid-based chimeric transcription factor composed of the *GAL4* DNA binding domain and the cysteine-rich activation domain of *YAP5*. The fusion protein encoded by this plasmid responds to iron and will induce *GAL1* promoter-regulated genes (8). Thus, this strain will be a *HIS* auxotroph in the absence of iron-induced Gal4-Yap5 activity. The second reporter was an integrated *CCC1-lacZ* construct containing the *CCC1* promoter fused to the *lacZ* gene. Incubation of the strain in low iron results in cells auxotrophic for histidine, which do not express β -galactosidase. The addition of iron resulted in *HIS* prototrophy and expression of β -galactosidase. The use of two different reporter constructs, which respond to two different transcription factors, ensures that any identified mutation must be in a common iron-sensing system rather than in an individual transcription factor/reporter system. The engineered cells were UV-mutagenized and exposed

to high iron, and LacZ-negative cells (“white” cells) were selected for LacZ activity and then examined for *HIS* auxotrophy. Candidates that showed decreased *CCC1-lacZ* activity and *HIS* auxotrophy were selected for further study. One candidate, UV mutant 109b, showed these phenotypes (Fig. 2A). The phenotypes were recessive as determined by diploid analysis (data not shown).

UV mutant 109b was transformed with a *LEU2* genomic library and selected for cells that could express β -galactosidase and grow on $-HIS$ medium (Fig. 2B). Library plasmid 16 conferred iron-dependent growth on $-HIS$ plates and restored iron-dependent β -galactosidase activity. The library clone 16 plasmid was recovered and sequenced, and subcloning identified *SSQ1* as the gene responsible for complementation. We sequenced the chromosomal copy of *SSQ1* in UV mutant 109b and identified a missense mutation, which resulted in an amino acid substitution L217S. Deletion of *SSQ1* in our parental strain W303 resulted in lethality, suggesting that the missense mutation was hypomorphic. The finding that the mutation was a hypomorph might explain why complementation was partial, although it is possible that there was a second mutation in the strain. The observation that a conditional shut-off of *SSQ1* phenocopies the inhibition of Yap5-mediated transcription, however, ensures that *SSQ1* is the gene responsible for the phenotype.

SSQ1 is a nuclear gene that encodes a mitochondrial protein chaperone that plays an important role in the mitochondrial synthesis of Fe-S clusters (13, 14). Decreased levels of Ssq1 compromise Fe-S cluster synthesis, leading to decreased activ-

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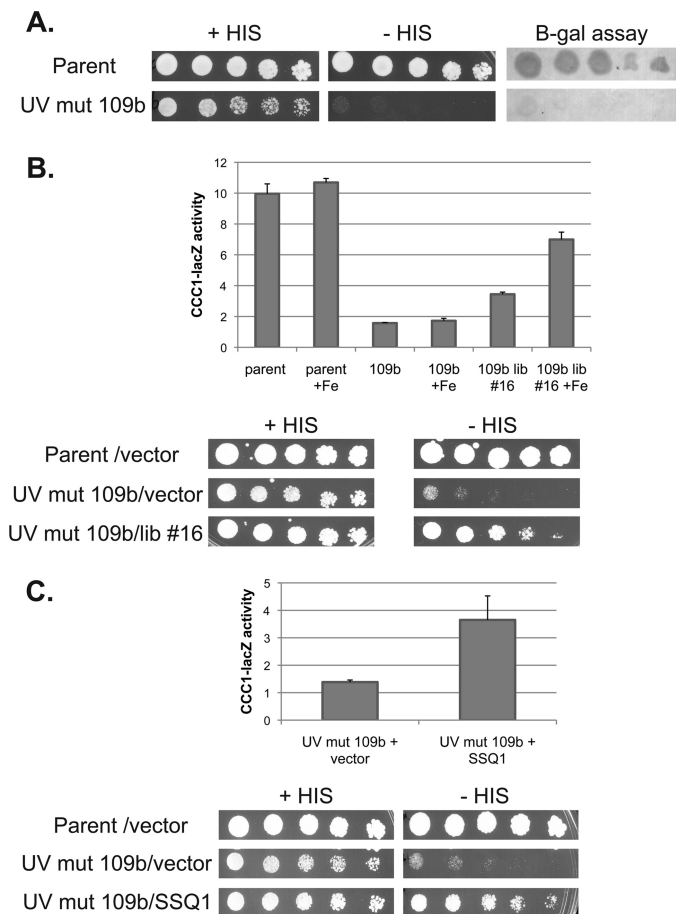


FIGURE 2. Identification of a mutant *SSQ1* that affects Yap5 induction of *CCC1*. *A*, serial dilutions of the parental strain and UV mutant (*UV mut*) 109b cells were spotted on plates in the presence or absence of histidine. Cells were grown for 3 days, and then a β -galactosidase assay was performed. *B*, parental, UV mutant 109b, and UV mutant 109b, transformed with library plasmid 16 (*lib #16*), were grown overnight in low iron medium, 500 μ M iron was added for 2.5 h, and a quantitative β -galactosidase assay performed. Cell protein was determined, and the data are expressed as specific activity. The data are the average of at least three experiments and are presented as the mean \pm S.D. *Bottom panel:* cells from above were spotted in a serial dilution in the presence or absence of histidine and grown for 3 days. *C*, a β -galactosidase assay and a plate spot assay were performed for mutant cells transformed with a control vector or a subcloned *SSQ1* plasmid. Cell protein was determined, and the β -galactosidase assay data are expressed as specific activity. The data are the average of at least three experiments and are presented as the mean \pm S.D.

ities of Fe-S cluster-containing enzymes, an inability to grow on respiratory substrates, and induction of the iron regulon. All three of these phenotypes are seen in mutant 109b (data not shown). The inability to induce *CCC1-lacZ* in mutant 109b and the inability to grow on $-$ HIS medium were complemented by transformation with an *SSQ1*-containing plasmid (Fig. 2C). As shown below, reduced levels of other proteins required for mitochondrial Fe-S cluster synthesis prevent iron-induced expression of Yap5 target genes.

There are a number of explanations for why cells defective in Fe-S cluster synthesis fail to induce Yap5 target genes. Notably, defects in Fe-S cluster synthesis lead to increased mitochondrial iron, and mitochondrial iron sequestration may result in low cytosolic iron, preventing the high iron transcriptional response. Previously, we suggested that cytosolic iron is not low in cells with defects in mitochondrial Fe-S cluster synthesis (3),

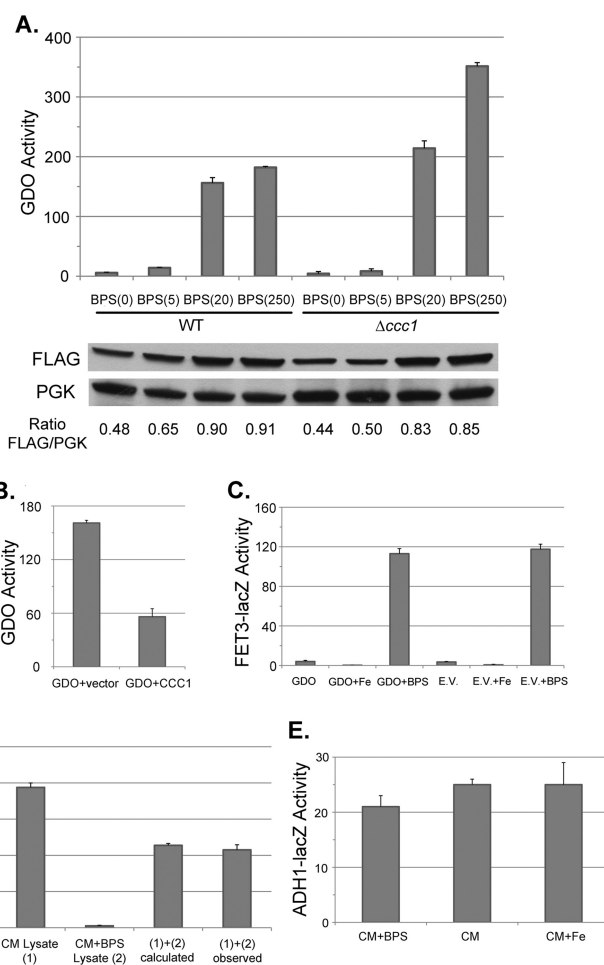


FIGURE 3. The activity of expressed GDO in yeast can be used as a measure of cytosolic iron. *A*, wild type or $\Delta ccc1$ cells were transformed with a plasmid expressing GDO with a carboxyl-FLAG epitope, under the control of the *ADH1* promoter. Cells were grown in CM medium supplemented with 80 μ M BPS and various concentrations of FeSO_4 (i.e. *BPS(5)* indicates 80 μ M BPS with 5 μ M FeSO_4) added back. Cells were grown overnight to mid-log phase and homogenized as described under "Experimental Procedures," and GDO activity and cell protein were determined. GDO activity is expressed as nanomoles of substrate converted per minute per mg of protein. The amount of GDO-FLAG was detected by Western blot with phosphogluco kinase (PGK) as a loading control. The ratio of GDO-FLAG to PGK was determined by densitometry. *B*, wild type cells were transformed with either a control plasmid or a plasmid containing *CCC1* under the control of its endogenous promoter and a plasmid containing GDO under the control of the *ADH1* promoter. Cells were grown overnight in selective medium and homogenized, and GDO activity and cell protein were determined. The data are expressed as specific activity, and the results are the mean of three separate experiments (\pm S.D.). *C*, wild type cells containing a *FET3-lacZ* reporter construct integrated at the *HO* locus were transformed with a GDO expression plasmid or an empty vector (*E.V.*). Cells were grown in CM medium with or without 40 μ M BPS (+BPS) with 100 μ M FeSO_4 added (+Fe); cell lysates were prepared, and β -galactosidase activity and cell protein were determined. The data are presented as specific activity, and the results are the mean of three separate experiments (\pm S.D.). *D*, cells expressing GDO were grown overnight in CM medium without BPS added (*CM Lysate (1)*) or CM medium with 80 μ M BPS added (*CM+BPS Lysate (2)*). Cells were harvested, and the lysates from these two conditions were assayed separately or were mixed together at a 1:1 volume ratio prior to determining GDO activity. The calculated value (*(1)+(2) calculated*) indicates the theoretical GDO activity assuming no activity change occurring upon mixing. The data are presented as specific activity, and the results are the mean of three separate experiments (\pm S.D.). *E*, WT cells (DY150) were transformed with a plasmid containing a *ADH1-lacZ* construct and grown in low iron medium (CM supplemented with 80 μ M BPS, *CM+BPS*), regular medium (CM), or high iron (CM supplemented with 100 μ M FeSO_4 , *CM+Fe*). Cells were harvested and lysed, and β -galactosidase activity and cell protein were determined. The data are presented as specific activity, and the results are the mean of three separate experiments (\pm S.D.).

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a result confirmed by others (15). To measure cytosolic iron, we utilized a mutant enzyme involved in ergosterol synthesis. Erg25 is a methyl sterol oxidase required for the demethylation of 4,4,-dimethylzymosterol to zymosterol. Erg25 is a putative iron-containing enzyme, and the mutant Erg25-2 showed decreased activity when cytosolic iron levels were decreased (16). Loss of Fe-S cluster synthesis did not affect the activity of the Erg25-2, suggesting that cytosolic iron was not decreased. The measurement of Erg25 activity, however, was indirect and was based on the accumulation of sterol intermediates in intact cells. Further, the methyl sterol oxidase enzymatic reaction requires mitochondrial-produced heme, which in turn is dependent on mitochondrial iron. It is possible that decreased Erg25 activity reflected decreased mitochondrial function rather than decreased cytosolic iron. Based on these concerns, we looked for an independent measure of cytosolic iron to assay the effect of decreased Fe-S cluster synthesis on induction of the low and high iron response.

Use of GDO to Measure Cytosolic Iron—*Pseudaminobacter salicylatoxidans* GDO catalyzes the ring fission reaction that degrades gentisate (17). This pathway does not exist in yeast, and yeast do not express GDO. The crystal structure for GDO has been solved, and it shows that Fe(II) is the sole cofactor (18). The iron binding site is solvent-accessible, and GDO has a relatively low affinity for iron as it binds iron with only three ligands (3 His residues). In contrast, *Escherichia coli* iron-superoxide dismutase binds iron with four ligands (3 His residues and 1 Asp residue) (19). GDO-bound iron can be removed by ferrous chelators, and the inactivated enzyme can be incompletely reactivated by the addition of reduced iron but not other metals (20). Based on these observations, we generated a yeast plasmid expressing GDO under the control of the *ADH1* promoter. GDO expressed in yeast showed activity using gentisate as a substrate. Similar levels of activity were seen for both untagged and carboxyl-terminal FLAG-tagged versions of GDO (data not shown). There was low GDO activity in medium made into low iron medium through the addition of the impermeable iron chelator BPS (Fig. 3A). Deletion of *CCC1*, which prevents import of iron to vacuoles, resulted in increased GDO activity in iron-replete medium. The addition of iron to BPS medium resulted in increased GDO activity. Cells overexpressing the vacuolar iron transporter *Ccc1* showed decreased GDO activity (Fig. 3B). Expression of GDO did not lead to induction of *FET3-lacZ*, suggesting that GDO did not consume enough iron to affect iron homeostasis (Fig. 3C). Measurement of GDO activity in combined extracts from iron-replete cells and iron-depleted cells showed that GDO activity was the average of the two (Fig. 3D). These results indicate that cell breakage did not affect GDO activity and that iron was stable within GDO for at least 5 h unless an iron chelator was added, permitting accurate measurements of activity. Further, GDO protein was found in the cytosolic fraction, demonstrating that GDO activity reflected cytosolic iron and not organellar iron (data not shown).

We note that the level of GDO-FLAG was reduced in iron-depleted medium, although the effect of iron on specific activity was more sensitive than the effect of iron on protein level (Fig. 3A). The decrease in GDO level in low iron conditions was not

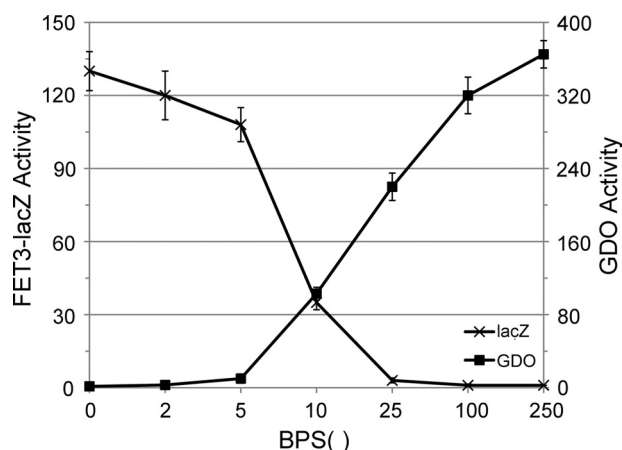


FIGURE 4. **Cytosolic GDO activity responds to changes in medium iron.** A strain was constructed in which $\Delta ccc1$ cells contained a *FET3-lacZ* reporter integrated at the *HO* locus and a *FET3/FTR1* construct under the control of the *PGK1* promoter integrated at the *ADE2* locus. Cells were transformed with a GDO plasmid. The cells were incubated overnight in CM with 80 μM BPS and varying amounts of FeSO_4 . Cells were homogenized, and β -galactosidase, GDO activity, and cell protein were determined. The data are expressed as specific activity of GDO or β -galactosidase. The data are the average of at least three experiments and are presented as the mean \pm S.D.

due to a decrease in expression. GDO is expressed using an *ADH1* promoter, and analysis of *ADH1-lacZ* expression constructs indicated that modulation of cellular iron levels did not affect β -galactosidase activity (Fig. 3E). Together, these results suggest that GDO has reduced stability in the absence of iron.

We examined GDO activity and induction of *FET3-lacZ* as a function of medium iron. To eliminate the variable of induction of high affinity iron transporters we used $\Delta ccc1$ cells that constitutively expressed *FET3/FTR1* under the control of the *PGK1* promoter (21). There was an inverse relationship between external iron and *FET3-lacZ* activity and a direct relationship between external iron and GDO activity (Fig. 4). At 80 μM BPS with 10 μM FeSO_4 , the reduction in GDO was 80%, whereas induction of *FET3-lacZ* was 32% of maximum, leading to a large dynamic range in GDO activity. Although not giving absolute concentrations of cytosolic iron, these data demonstrate that GDO can be used to assess cytosolic iron levels.

Failure to Induce the *Yap5* Transcriptional Response due to Decreased Fe-S Clusters Does Not Result from Vacuolar or Mitochondrial Iron Sequestration—We utilized the GDO assay to examine the effect of decreased Fe-S cluster synthesis on cytosolic iron levels. We generated a *SSQ1* deletion strain that was kept alive by a plasmid that contained a *MET3*-regulated *SSQ1*. The strain was also deleted for *CCC1* to eliminate vacuolar iron sequestration. The strain was transformed with either a *FET3-lacZ* reporter or a *CCC1-lacZ* reporter. The addition of methionine resulted in a time-dependent increase in *FET3-lacZ* expression (Fig. 5A) and a decrease in the activity of the Fe-S cluster-containing protein aconitase (Fig. 5B). We note that the iron regulon was induced even in the absence of *CCC1*, indicating that loss of Fe-S clusters did not result from vacuolar iron sequestration. We then examined the effect of loss of *SSQ1* on induction of the high iron regulon. The addition of iron to cells grown in the absence of methionine resulted in an increase in cytosolic iron as measured by GDO activity (Fig. 5C) and an increase in *CCC1-lacZ* expression (Fig. 5D). In the absence of

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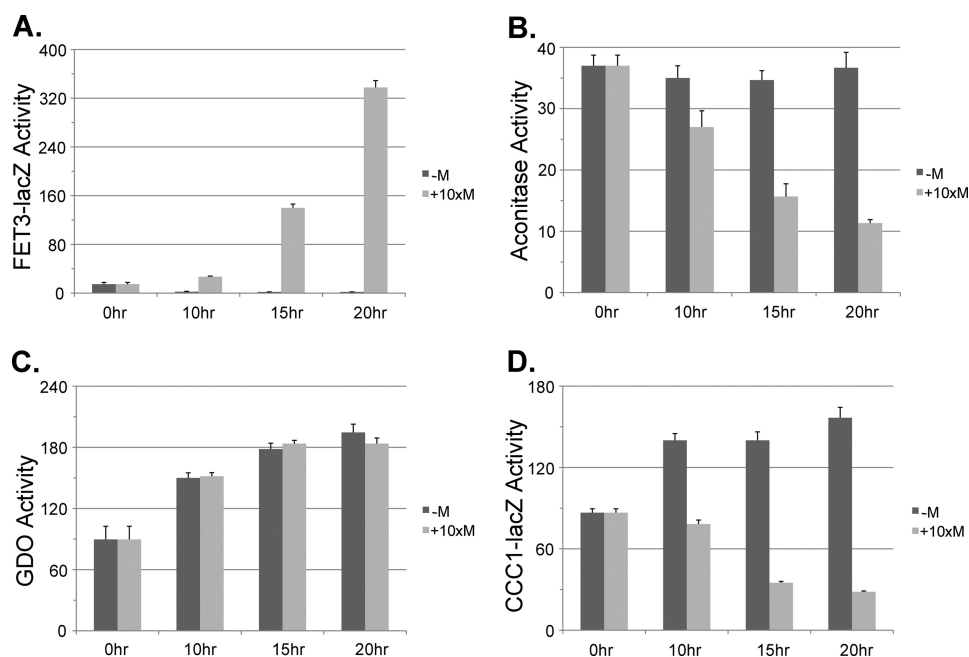


FIGURE 5. Reduction in *SSQ1* affects the induction of both the low and the high iron response without affecting cytosolic iron levels. Cells ($\Delta ssq1\Delta ccc1$) were transformed with plasmids containing a *MET3*-regulated *SSQ1*. The cells were also transformed with a GDO-expressing plasmid, a *FET3-lacZ* reporter construct, or a *CCC1-lacZ* reporter construct. Cells were grown overnight in CM without methionine to mid-log phase. Cells were harvested and placed in growth medium containing $100\ \mu\text{M}$ FeSO_4 with or without $10\times$ methionine for the specified times. Cells were homogenized, and β -galactosidase activity, GDO activity, aconitase activity, and cell protein were determined. *A*, *FET3-lacZ*. *B*, aconitase. *C*, GDO. *D*, *CCC1-lacZ*. The data are presented as specific activity, and the error bars reflect the S.D. of three independent experiments.

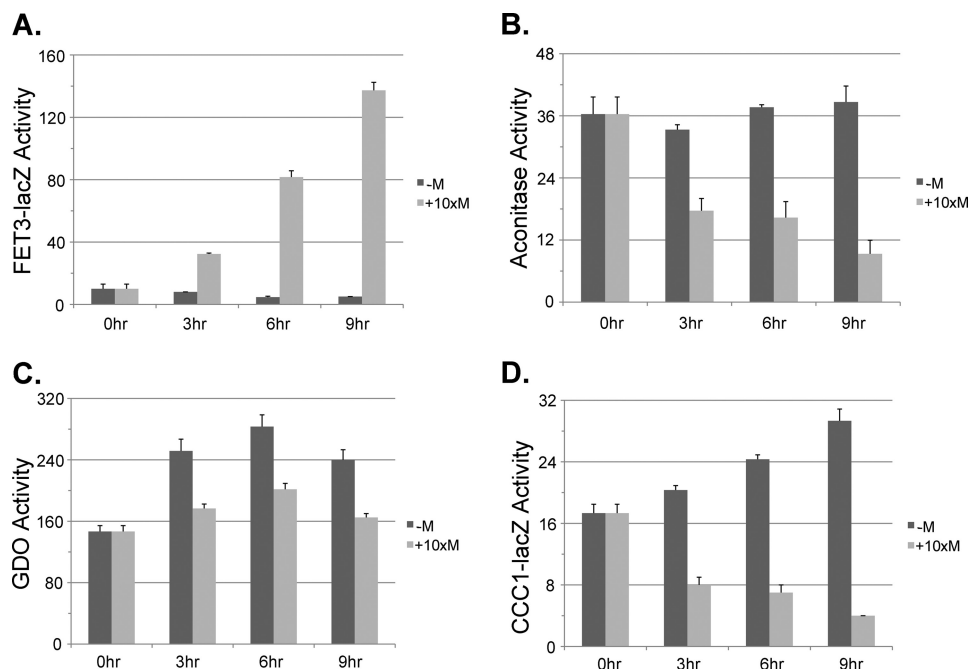


FIGURE 6. Reduction in *YFH1* affects the induction of the low and high iron response without affecting cytosolic iron levels. Cells ($\Delta yfh1\Delta ccc1$) were transformed with a plasmid containing a *MET3*-regulated *YFH1*. The cells were also transformed with a GDO plasmid and either a *FET3-lacZ* reporter construct or a *CCC1-lacZ* reporter construct. Cells were grown overnight in CM without methionine to mid-log phase. Cells were harvested and placed in growth media containing $100\ \mu\text{M}$ FeSO_4 with or without $10\times$ methionine for the specified times. Cells were homogenized, and *FET3-lacZ* (*A*), aconitase (*B*), GDO (*C*), and *CCC1-lacZ* (*D*) activities and cell protein were determined. The data are presented as specific activity, and the error bars reflect the S.D. of three independent experiments.

SSQ1 expression, GDO activity showed a response to increased medium iron, but there was no increase in *CCC1-lacZ* activity in response to added iron; rather there was a decrease in *CCC1-lacZ*. This result indicates that loss of *SSQ1* does not result in

decreased cytosolic iron but does result in decreased *CCC1-lacZ* expression.

Yfh1 is thought to act as an iron chaperone in the synthesis of Fe-S clusters. In the absence of *Yfh1*, there is a severe reduction

in mitochondrial Fe-S cluster synthesis (22). We used $\Delta yfh1$ cells that contained a *MET3*-regulated *YFH1* (23). This strain was also deleted for *CCC1* to rule out effects due to vacuolar iron sequestration. The strain was transformed with either a *FET3-lacZ* reporter or a *CCC1-lacZ* reporter. In the absence of methionine, which permits expression of Yfh1, there was a low level of *FET3-lacZ* (Fig. 6A). The addition of methionine, which prevents expression of Yfh1, resulted in a time-dependent induction of *FET3-lacZ* and a time-dependent decrease in aconitase activity (Fig. 6B). We note that decreased expression of Yfh1 resulted in a more rapid loss of aconitase activity than did decreased expression of Ssq1, which might reflect either a shorter half-life for Yfh1 than for Ssq1 or that the function of Ssq1 requires less protein. GDO activity increased over time in the absence of Yfh1 expression (Fig. 6C). In the presence of methionine, GDO activity showed less of an increase, but stayed above the base line. The absence of methionine resulted in a time-dependent increase in *CCC1-lacZ* activity (Fig. 6D). In contrast, in the presence of methionine, there was a decrease in *CCC1-lacZ* activity, suggesting that Fe-S cluster synthesis is required for the iron-dependent induction of *CCC1*.

Isu1 is one of two Fe-S scaffold proteins, and deletion of *ISU1* results in increased mitochondrial iron and induction of the iron regulon (24). Cells with a deletion of *ISU1* and *CCC1* show increased *FET3-lacZ* activity when compared with $\Delta ccc1$ cells in normal iron or iron-supplemented conditions (Fig. 7A). GDO activity in response to either iron depletion or iron addition was similar in $\Delta ccc1$ and $\Delta ccc1\Delta isu1$ cells (Fig. 7B). Loss of *ISU1* prevented the iron-dependent induction of *CCC1-lacZ* (Fig. 7C). Thus, the decrease in *CCC1-lacZ* in $\Delta isu1\Delta ccc1$ cells cannot be ascribed to decreased cytosolic iron.

Previously, we showed that Yap5 levels were not affected by changes in cytosolic iron (8). It is possible, however, that the levels of Yap5 may be altered under decreased Fe-S synthesis and that decrease could be responsible for changes in Yap5-dependent transcription. We examined the levels of endogenous Yap5 using a polyclonal rabbit anti-Yap5 antibody. We did not observe any significant changes in the levels of Yap5 in wild type cells relative to $\Delta isu1$ cells (Fig. 8A). Similarly, the level of Yap5 showed no difference in either the presence or the absence of *SSQ1* or *YFH1*. To confirm these results, we overexpressed HIS-tagged Yap5 (8) in $\Delta ccc1$ cells containing the *MET3*-regulated *SSQ1* as described above. The level of HIS-Yap5 was not decreased by the absence of *SSQ1*. There was, however, a decrease in *CCC1-lacZ* activity when compared with *SSQ1*-expressing cells (Fig. 8B). We conclude that changes in Yap5 protein levels are not responsible for the Yap5-dependent transcription changes seen when Fe-S cluster synthesis is blocked.

We confirmed that the effect of decreased mitochondrial Fe-S cluster synthesis on the high iron transcriptional response was not an artifact of the *CCC1-lacZ* reporter construct. Loss of *SSQ1* prevented iron induction of *CCC1* transcripts, whereas increased transcripts were seen in iron-incubated cells expressing *SSQ1* (Fig. 9A). Under the same conditions, *FET3* mRNA was increased in the absence of *SSQ1*. We then assayed the levels of *TYW1*, another Yap5 target gene (9). In $\Delta ccc1$ cells, *TYW1* mRNA levels were decreased in the absence of *SSQ1*, *YFH1*, or *ISU1* when compared with $\Delta ccc1$ cells with unim-

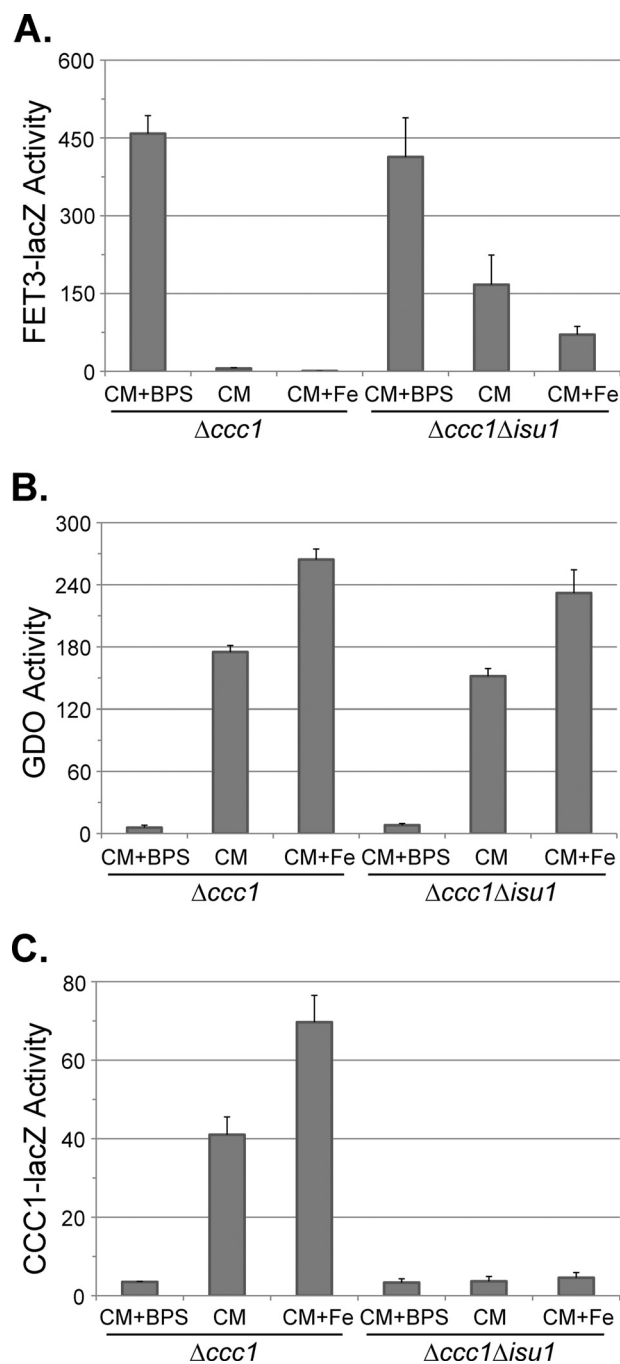


FIGURE 7. Loss of *ISU1* prevents iron-dependent induction of *CCC1-lacZ*. $\Delta ccc1$ or $\Delta ccc1\Delta isu1$ cells were transformed with a GDO plasmid and either *FET3-lacZ* or *CCC1-lacZ* reporter constructs. Cells were grown overnight in CM medium (CM) or CM medium with 80 μ M BPS plus (CM+Fe) or minus 100 μ M $FeSO_4$ (CM+BPS). Cells were homogenized, and *FET3-lacZ* (A), GDO (B), and *CCC1-lacZ* (C) activities and cell protein were determined. The data are presented as specific activity, and the error bars reflect the S.D. of three independent experiments.

paired Fe-S cluster synthesis (Fig. 9B). These results demonstrate that decreased mitochondrial Fe-S cluster synthesis affects the high iron transcriptional response.

Iron Induction of Yap5-regulated Genes Is Independent of the CIA Complex or Grx3 and Grx4—A complex of cytosolic proteins, termed the CIA complex, is required to insert Fe-S clusters on cytosolic or nuclear apoproteins (25). Rutherford *et al.*

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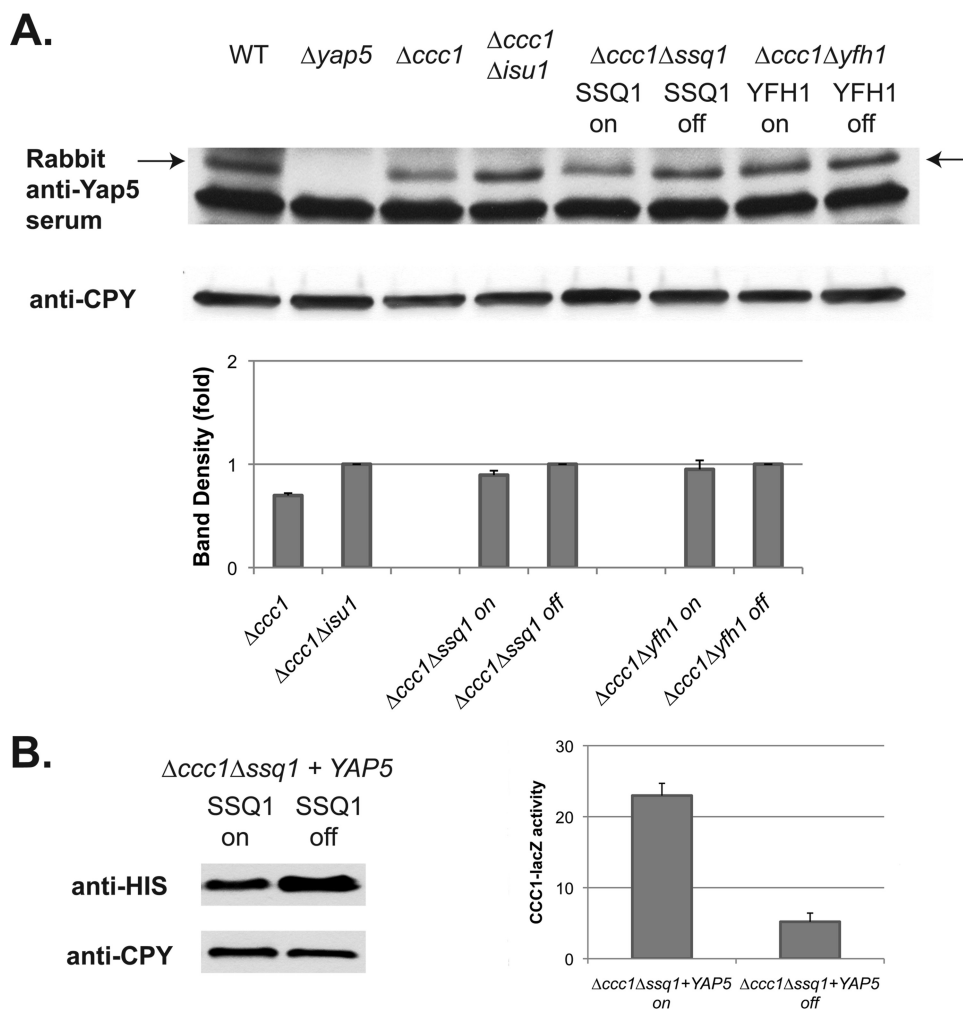


FIGURE 8. Loss of Fe-S cluster synthesis does not lead to decreased Yap5 levels. *A*, cells (wild type, $\Delta yap5$, $\Delta ccc1$, $\Delta ccc1 \Delta isu1$, $\Delta ssq1 \Delta ccc1$ (*pMET3SSQ1*), and $\Delta yfh1 \Delta ccc1$ (*pMET3YFH1*)) were grown in CM medium in the presence (*off*) or absence (*on*) of methionine overnight. Cells were harvested, treated with 10% trichloroacetic acid, and broken with glass beads, and cell extracts were analyzed by Western blot using a polyclonal rabbit anti-Yap5 and a mouse anti-carboxypeptidase Y (*CPY*) antibody. The *arrow* denotes the Yap5-specific band. A representative blot is shown. Western blots were quantified using carboxypeptidase Y as a loading control from three separate experiments. *B*, cells ($\Delta ssq1 \Delta ccc1$ (*pMET3SSQ1*)) containing a *CCC1-lacZ* reporter construct were transformed with a high copy plasmid containing HIS-tagged Yap5 under the control of its endogenous promoter. Cells were grown as in *A*, and HIS-Yap5 levels were determined by Western blot using mouse anti-HIS antibody. *CCC1-lacZ* activity in these cells was determined. The data are presented as specific activity, and the *error bars* reflect the S.D. of three independent experiments.

(4) showed that the CIA complex is not involved in the low iron-signaling pathway as reduced expression of CIA proteins did not result in induction of the iron regulon. To test whether the CIA complex is required for the high iron-signaling pathway, we utilized strains containing regulated expression of *NAR1* ($\Delta nar1$ *pGALNAR1*) or *DRE2* ($\Delta dre2$ *pGALDRE2*), which encode components of the CIA complex (26, 27). Reduced levels of either protein resulted in a decrease in the activity of the cytosolic Fe-S-containing protein Leu1, indicating decreased Fe-S cluster assembly of cytosolic proteins (Fig. 10A). Loss of either protein did not result in induction of the low iron transcriptional response, confirming previous results (4) (Fig. 10B). Loss of either protein did not prevent the iron-induced Yap5 response, and in fact, there was an increase in *CCC1-lacZ* activity (Fig. 10C). These results indicate that although the Yap5 response requires mitochondrial Fe-S clusters, it does not require assembly of Fe-S clusters on cytosolic or nuclear proteins, although there may be CIA-independent routes for Fe-S cluster assembly. These data suggest that if Fe-S clusters are not

incorporated into cytosolic proteins, they may accumulate in the cytosol, resulting in an increased Yap5 response.

The low iron transcriptional response is increased upon deletion of the cytosolic glutaredoxins *GRX3* and *GRX4* in the BY4743 background strain (6, 7). Deletion of both genes resulted in increased expression of *FET3-lacZ* (Fig. 11A). *CCC1-lacZ* was only increased in $\Delta grx3 \Delta grx4$ cells when iron was added (Fig. 11B). There was no increase in *CCC1-lacZ* in the absence of added iron. We conclude that deletion of *GRX3* and *GRX4* does not directly affect Yap5-dependent transcription. We then examined whether overexpression of Grx3 or Grx4 would affect *CCC1-lacZ* expression. Overexpression of either Grx3 or Grx4 had no effect on *CCC1-lacZ* activity in CM medium (data not shown).

The Effect of Decreased Fe-S Cluster Synthesis on Induction of the Yap5 Response Is Not due to Increased Oxidants—It is recognized that loss of mitochondrial Fe-S cluster synthesis leads to increased oxidant damage (15, 28). Previously, we reported that deletion of glutaredoxin 1 (*GLR1*) or thioredoxin reductase

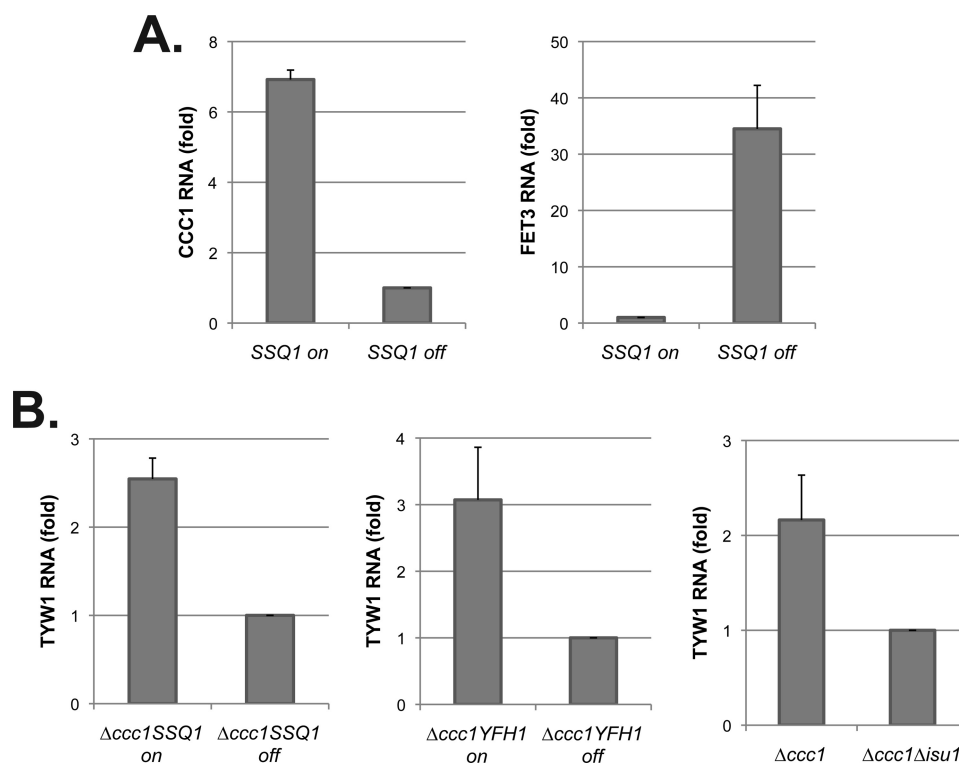


FIGURE 9. Mutations in Fe-S cluster synthesis genes lead to decreased transcripts for Yap5 targets. Total RNA was isolated from cells grown in an iron-replete medium. *A*, $\Delta ssq1$ cells containing a *MET3*-regulated *SSQ1*-expressing plasmid were grown in a medium without methionine (*on*) or with 10 \times methionine (*off*) overnight. *CCC1* mRNA and *FET3* mRNA were measured by real-time PCR. *B*, $\Delta ccc1\Delta ssq1$ cells containing a *MET3*-regulated *SSQ1*-expressing plasmid or $\Delta ccc1\Delta yfh1$ cells with a *MET3*-regulated *YFH1*-expressing plasmid were grown in a medium without methionine (*on*) or with 10 \times methionine (*off*) overnight. $\Delta ccc1$ and $\Delta ccc1\Delta isu1$ were grown in an iron-replete medium overnight. Real-time PCR was performed for *TYW1* transcription (a Yap5-regulated gene). -Fold changes of each gene transcriptional level were quantified based on *CMD1* as an internal control. The error bars reflect the S.D. of two independent experiments.

1 (*TRR1*), which encode antioxidant enzymes, did not lead to induction of *CCC1-lacZ*, suggesting that Yap5-dependent transcription was not due to increased oxidants (8). We used these strains to determine whether increased oxidants would prevent iron-induced expression of the *CCC1-lacZ* reporter construct. In both deletion strains, iron induced β -galactosidase expression to the same extent as in iron-treated wild type cells (Fig. 12A). Second, we had also shown that iron could induce Yap5-dependent transcription under anaerobic conditions (8). In anaerobic conditions, iron had a marginal effect on the expression of *CCC1-lacZ* in cells with a deletion in *ISU1* (Fig. 12B). Similarly, when the levels of *Ssq1* or *Yfh1* were reduced, iron was unable to induce *CCC1-lacZ* under anaerobic conditions. These results support the view that the lack of induction of Yap5-dependent transcription resulted from the loss of Fe-S clusters and not from an oxidant-induced suppression of Yap5 activation.

DISCUSSION

Cells respond to increased levels of cytosolic iron by decreasing iron uptake and increasing iron storage. Most eukaryotes store cytosolic iron in ferritin, a protein whose synthesis is translationally regulated by increased iron. Plants and fungi store cytosolic iron in vacuoles, which requires expression of the vacuolar iron importer *CCC1* (yeast) or *VTA1* (plants). *CCC1* mRNA is destabilized under low iron conditions by binding of Cth1 and Cth2 (29). Transcription of *CTH1* and *CTH2* is

controlled by the low iron transcription factor Aft1. Iron induces the Yap5-mediated transcription of *CCC1* and a limited number of other genes. Yap5 constitutively occupies the *CCC1* promoter and is activated by high iron (8). The mechanism by which Yap5 “senses” iron is unknown. The results of this study suggest that Yap5 transcription is reduced by the loss of Fe-S cluster synthesis even under conditions in which cytosolic iron is increased. This result implies that the high iron transcriptional response, like the low iron transcriptional response, is coordinated by the availability of Fe-S clusters.

Critical to this conclusion was the finding that defects in Fe-S cluster synthesis affected iron-dependent signaling independent of changes in cytosolic iron. Previously, we utilized the iron-dependent activity of a mutant methyl sterol oxidase to establish that point (3). We confirm and expand that conclusion by taking advantage of the iron-dependent activity of GDO. Use of GDO has several strategic and technical advantages when compared with use of the mutant *Erg25-2*. The activity of GDO is not essential, and expression of GDO does not affect cellular iron metabolism. The enzyme is easy and inexpensive to assay. Iron binds to GDO with relatively low affinity, and enzyme activity shows a large dynamic range in response to iron deprivation and excess. GDO has a strict requirement for Fe(II), which is thought to be the predominant form of available iron in cytosol. Using GDO to monitor cytosolic iron, we confirmed our initial observation that reduction

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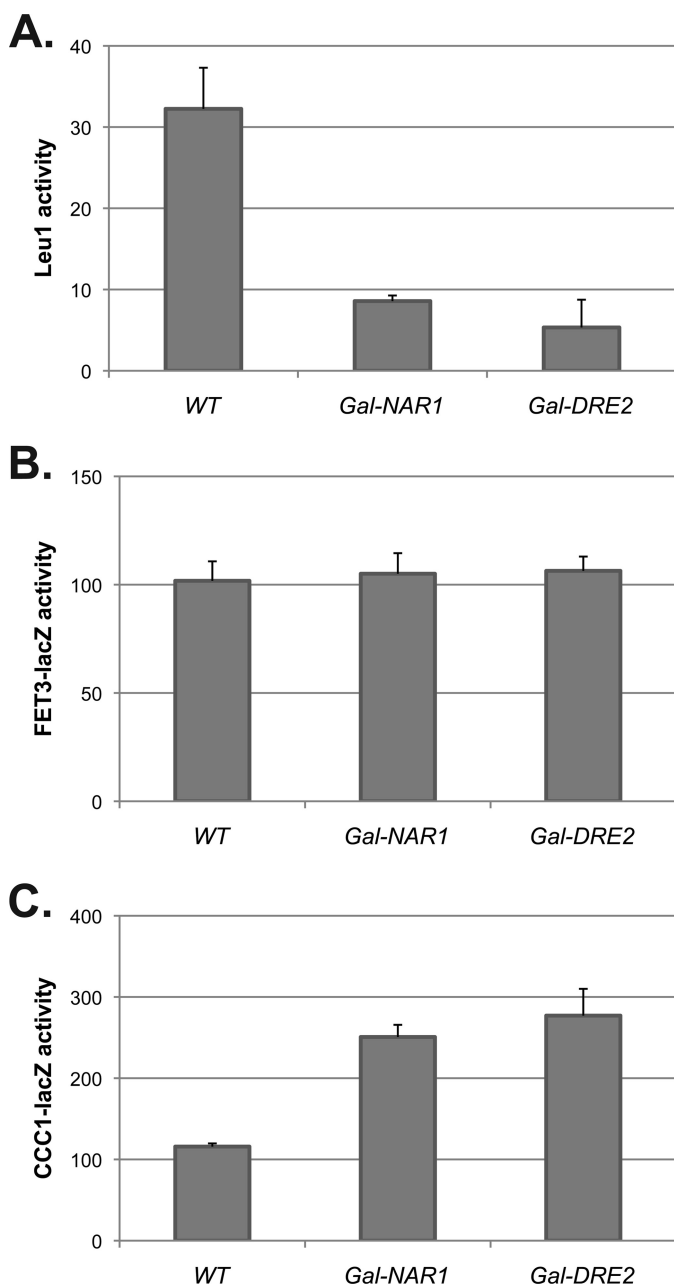


FIGURE 10. Decreased activity of the cytosolic Fe-S machinery does not prevent induction of CCC1. Cells containing a galactose-regulated *NAR1* or a galactose-regulated *DRE2*, transformed with either a *CCC1-lacZ* plasmid or a *FET3-lacZ* plasmid, were grown in galactose medium overnight. Cells were washed and reinoculated into glucose medium for another 16 h to turn off *NAR1* or *DRE2* expression. Cells were homogenized, and Leu1 activity (A), *FET3-lacZ* activity (B), and *CCC1-lacZ* activity (C) and cell protein were assayed. The data are presented as specific activity normalized to cell protein, and the error bars reflect the S.D. of three independent experiments.

in enzymes involved in mitochondrial Fe-S synthesis induced the iron regulon with little decrease in cytosolic iron. This conclusion was supported by others using iron-sensitive fluorescent dyes (15).

Our genetic screen showed that a mutation in *SSQ1*, a gene required for mitochondrial Fe-S cluster synthesis, prevented the high iron transcriptional response. We then determined that reduced expression of other genes involved in mitochondrial Fe-S cluster synthesis also prevented iron induction of the

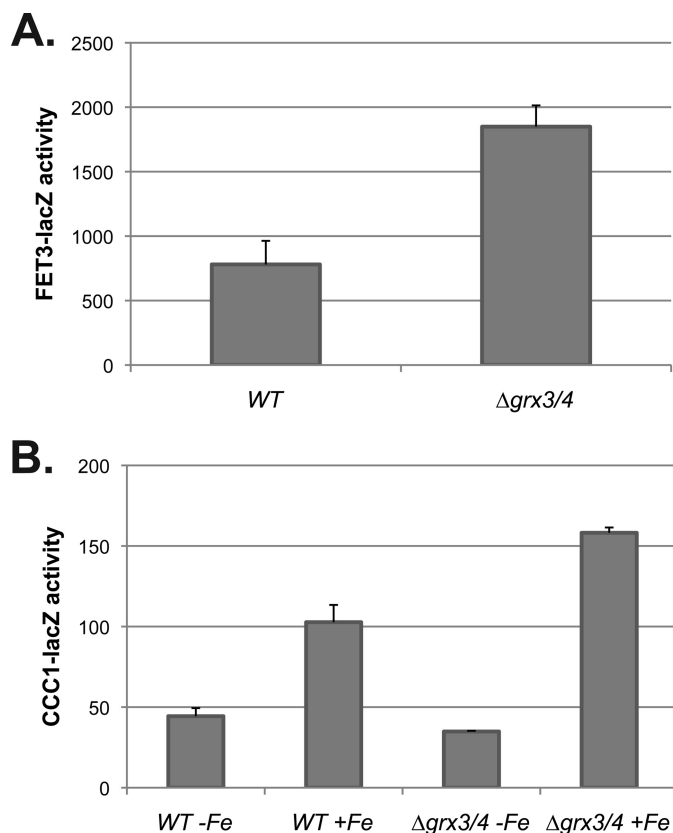


FIGURE 11. Loss of GRX3/GRX4 affects the low iron transcriptional response but does not directly affect the high iron transcriptional response. A and B, wild type and $\Delta grx3/grx4$ cells were transformed with either a *FET3-lacZ* reporter construct (A) or a *CCC1-lacZ* reporter construct (B). The cells were grown overnight in CM, in CM made iron-limited by the addition of 80 μ M BPS, or in high iron (500 μ M). Cells were homogenized, and β -galactosidase activity and cell protein determined. The data are presented as specific activity normalized to cell protein, and the error bars reflect the S.D. of three independent experiments.

Yap5 response even under anaerobic conditions. This finding has two implications. First, it suggests that the lack of Yap5 induction is not due to production of oxidative radicals resulting from defective mitochondrial activities. Second, it provides evidence that the lack of Yap5-dependent transcription was not due to mitochondrial iron sequestration. Under aerobic conditions, defects in mitochondrial Fe-S cluster synthesis result in mitochondrial iron accumulation in which iron accumulates as a detergent-insoluble particulate, referred to as a nanoparticle. Under anaerobic conditions, cells with reduced levels of Yfh1 or Atm1 do not accumulate iron in mitochondria (15, 30). The lack of mitochondrial iron accumulation again rules out mitochondrial iron sequestration as causal to the lack of Yap5-dependent transcription.

Rutherford *et al.* (4) showed that the iron regulon responded to decreased mitochondrial Fe-S cluster synthesis but did not respond to loss of members of the cytosolic machinery that are required to assemble Fe-S clusters on most apoproteins. Reductions in the cytosolic glutaredoxins Grx3 and Grx4 result in induction of the iron regulon, whereas overexpression of either Grx3 or Grx4 leads to termination of the low iron transcriptional response and binding of Grx3 or Grx4 to Aft1 (6, 7). Interestingly, although the CIA complex is required for forma-

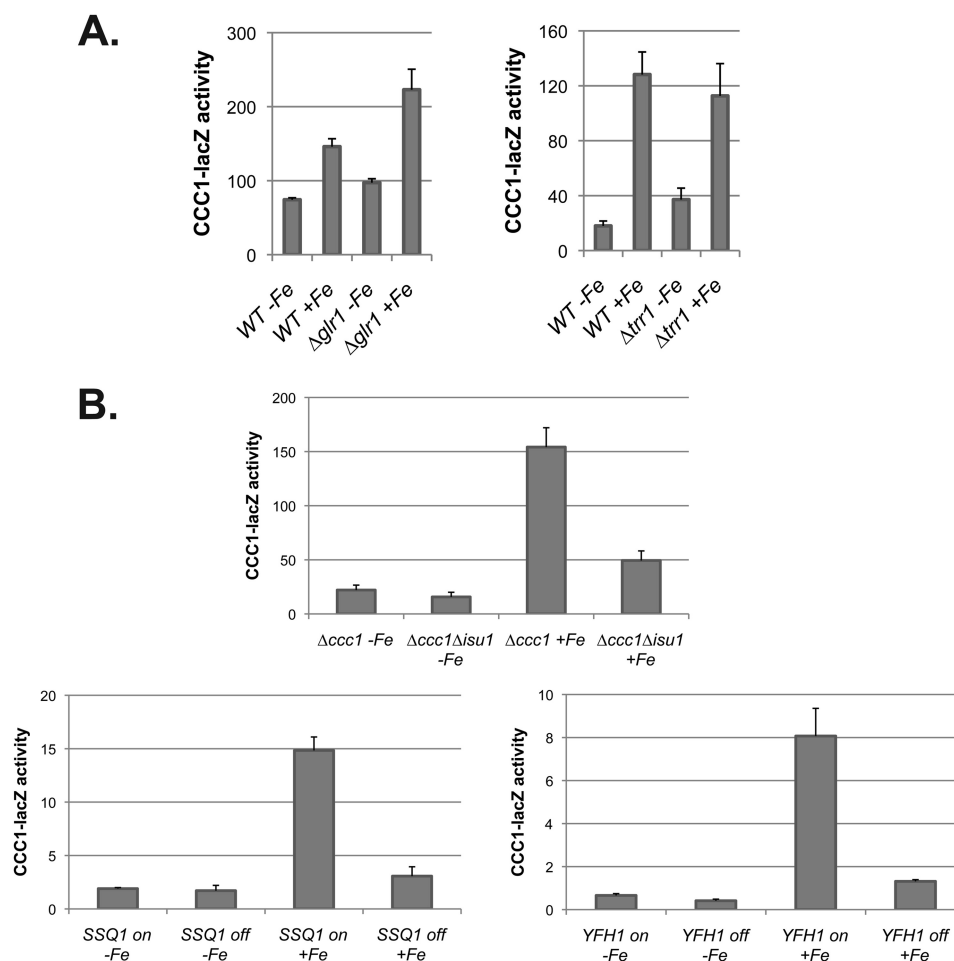


FIGURE 12. Increased oxidants do not suppress Yap5-induced expression of *CCC1*. *A*, wild type, $\Delta glr1$, and $\Delta trr1$ cells were transformed with a *CCC1-lacZ* plasmid. Cells were incubated in low iron medium, and then 500 μM iron was added. After a further 2-h incubation, cells were harvested, and β -galactosidase and cell protein were determined. *B*, cells ($\Delta ccc1$, $\Delta ccc1\Delta isu1$, $\Delta ccc1\Delta ssq1$ transformed with *MET3-SSQ1*, and $\Delta ccc1\Delta yfh1$ transformed with *MET3-YFH1*) were transformed with *CCC1-lacZ*. Expression of *MET3-SSQ1* and *MET3-YFH1* was controlled by the presence (*off*) (10 \times) or absence (*on*) of methionine. Cells were grown in low iron medium anaerobically overnight, and 500 μM iron was added for an additional 2.5 h under anaerobic conditions. Cells were harvested, and β -galactosidase activity and cell protein were determined. The data are presented as specific activity normalized to cell protein, and the error bars reflect the S.D. of three independent experiments.

tion of most Fe-S holoproteins, it is not required for acquisition of iron by Grx3 or Grx4 (5). These data support a role for Grx3/4 in mediating the Fe-S cluster signal from mitochondria to Aft1. We confirmed that reduction in CIA members does not lead to transcription of the low iron regulon and find that it also does not prevent transcription of the high iron regulon. We also confirmed that reduction in Grx3 and Grx4 results in induction of the low iron regulon and that it also induces the high iron regulon. Our data suggested that induction of the high iron regulon by reduced levels of Grx3 and Grx4 is a secondary consequence of increased expression of cell surface iron transporters, which permits increased cellular iron. There was no induction of the high iron regulon in $\Delta grx3\Delta gr4$ cells placed on low iron medium.

Currently, we do not know how Fe-S clusters affect the high iron transcriptional response. Yap5 contains clusters of cysteines, which are critical for iron-induced transcription (8). It might well be that these cysteines can bind Fe-S clusters directly without the requirement for accessory proteins. That possibility is currently being tested. A finding that both the low iron and the high iron transcriptional response are sensitive to the same

metabolite, Fe-S clusters, has precedence. Post-transcriptional regulation of mammalian iron acquisition by regulation of transferrin receptors as well as regulation of cytosolic iron levels through regulation of iron storage (ferritin) and iron export (ferroportin) respond to the binding of iron regulatory proteins (IRPs) to iron-responsive elements in the untranslated regions of mRNAs (31). Loss of RNA binding activity of IRP1 results from conversion of IRP1 into a Fe-S cluster-containing aconitase. Thus, both iron acquisition and iron storage respond to Fe-S cluster synthesis.

The cell surface high affinity iron transport system of *Schizosaccharomyces pombe* is highly homologous to that of *S. cerevisiae*, composed of a ferroxidase and a transmembrane iron permease (32). The regulation of this transport system, as well as other genes involved in adaptation to a low iron response, is quite different in the two yeasts. In *S. cerevisiae*, the low iron-sensing transcription factor Aft1 is an activator, and iron induces Aft1 binding to promoters (33). In *S. pombe*, the transcriptional regulator for genes involved in iron acquisition, Fep1, is a repressor, and iron induces the loss of Fep1 from promoters (34). Iron sensing by Fep1 is dependent on its inter-

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action with the *S. pombe* monothiol glutaredoxin Grx4 (35–37). Deletion of Grx4 results in constitutive repression of the iron regulon. Iron does not promote binding of Grx4 to Fep1, but it does affect Fep1 conformation. Under iron-replete conditions, proteins involved in iron utilization and storage are transcribed; under iron-limited conditions, they are repressed by Php4. Php4 is regulated transcriptionally by Fep1, but it also regulates post-transcriptionally by binding to Grx4 (38). Deletion of Grx4 results in constitutive activation of Php4. Additionally, Php4 transits between nucleus and cytosol, and loss of Grx4 prevents Php4 exit from the nucleus. Although we do not know the mechanistic details of how Fe-S clusters regulate iron-dependent transcription in *S. cerevisiae*, our finding that Fe-S clusters affect both low and high iron sensing shows a conservation of signal transduction in evolutionarily disparate species of yeast and vertebrates.

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