The glucose sensor Snf1 and the transcription factors Msn2 and Msn4 regulate transcription of the vacuolar iron importer gene *CCC1* and iron resistance in yeast

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The budding yeast *Saccharomyces cerevisiae* stores iron in the vacuole, which is a major resistance mechanism against iron toxicity. One key protein involved in vacuolar iron storage is the iron importer Ccc1, which facilitates iron entry into the vacuole. Transcription of the *CCC1* gene is largely regulated by the binding of iron–sulfur clusters to the activator domain of the transcriptional activator Yap5. Additional evidence, however, suggests that Yap5-independent transcriptional activation of *CCC1* also contributes to iron resistance. Here, we demonstrate that components of the signaling pathway involving the low-glucose sensor Snf1 regulate *CCC1* transcription and iron resistance. We found that *SNF1* deletion acts synergistically with *YAP5* deletion to regulate *CCC1* transcription and iron resistance. A kinase-dead mutation of Snf1 lowered iron resistance as did deletion of *SNF4*, which encodes a partner protein of Snf1. Deletion of all three alternative partners of Snf1 encoded by *SIT1*, *SIT2*, and *GAL83* decreased both *CCC1* transcription and iron resistance. The Snf1 complex is known to activate the general stress transcription factors Msn2 and Msn4. We show that Msn2 and Msn4 contribute to Snf1-mediated *CCC1* transcription. Of note, *SNF1* deletion in combination with *MSN2* and *MSN4* deletion resulted in additive effects on *CCC1* transcription, suggesting that other activators contribute to the regulation of *CCC1* transcription. In conclusion, we show that yeast have developed multiple transcriptional mechanisms to regulate Ccc1 expression and to protect against high cytosolic iron toxicity.

Regulation of cellular iron toxicity in eukaryotes is effected by regulation of iron acquisition and/or iron storage. Fungi and plants store iron in the vacuole, and vacuolar iron storage is critical for maintaining cytosolic iron levels. In the budding yeast, *Saccharomyces cerevisiae*, iron export from the vacuole is mediated by iron transporters Fet5/Fth1 and Smf3. These genes are regulated by the low iron-sensing transcription factors Aft1 and Aft2 (for reviews, see Refs. 1 and 2). The expression of these transporters, in concert with cell surface iron transporters, leads to iron influx into the cytosol. In contrast, when iron levels are replete, the low-iron transcription response is curtailed, and there is an induction of a high-iron transcriptional response. The number of genes responding to high iron is more limited than the number responding to low iron. Most notable is *CCC1*, which encodes the vacuolar iron importer (3). Yeast with a functioning Ccc1 are highly resistant to iron toxicity, whereas loss of Ccc1 results in a dramatic increase in iron toxicity in both aerobic and anaerobic conditions.

Ccc1 is highly regulated both by transcriptional activation and by mRNA degradation. The activity of Ccc1 is affected by changes in oxidant levels in which oxidants, in a manner still yet to be defined, can increase Ccc1 transport activity (4). At the post-transcriptional level, the stability of *CCC1* mRNA is reduced through the binding of Cth1 and Cth2 to elements in the *CCC1* mRNA 3′-untranslated region. *CTH1* and *CTH2* are transcriptionally induced by activation of the low-iron transcriptional sensors Aft1 and Aft2 (5). Thus, under low-iron conditions, import of iron from cytosol to vacuole is reduced, leading to an increase in cytosolic iron. This coordination of the low- and high-iron responses also extends to the transcriptional regulation of *CCC1*, which occurs through the transcriptional activator Yap5 (6). Yap5 is a member of the basic leucine zipper stress response family of transcriptional activators (7). Although Yap5 is not related to Aft1/Aft2 by sequence, like Aft1/Aft2, transcription mediated by Yap5 is regulated by Fe–S clusters. In the presence of high levels of mitochondrially produced Fe–S clusters, promoter-bound Yap5 is activated, whereas Aft1 is evicted from the promoters of target genes and is exported to the cytosol (8). In the absence of Fe–S cluster synthesis, resulting in part from decreased iron availability, Aft1 is translocated to the nucleus and induces gene transcription, whereas Yap5-induced transcription is terminated. Thus, both the low- and high-iron transcriptional activators coordinate their response to the same small iron-dependent product. That is, Aft1/Aft2 is activated by the absence of Fe–S clusters (9), whereas Yap5 is activated by the presence of Fe–S clusters (10).

Examination of both iron toxicity and *CCC1* transcription indicates, however, that there are other transcriptional activators besides Yap5 that affect *CCC1*. Deletion of *YAP5* decreases iron resistance of yeast but not to the same extent as deletion of *CCC1* (6, 10, 11). Furthermore, iron-dependent transcription of *CCC1* occurs in the absence of Yap5, albeit to a decreased

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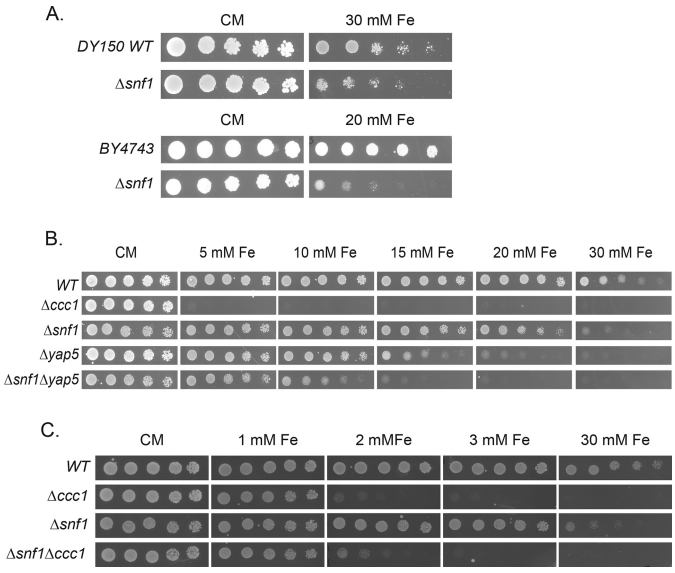

Figure 1. Deletion of *SNF1* affects iron resistance in yeast. *A*, serial dilutions of wild-type yeast (DY150 or BY4743) or Δ*snf1* cells in each background were spotted on plates with different concentrations of iron and grown for 2 days, and images were captured. *B*, serial dilutions of wild-type yeast (DY150) and cells deleted for *CCC1*, *SNF1*, *YAP5*, or *SNF1* and *YAP5* were grown for 2 days on plates with the designated concentrations of iron. *C*, serial dilutions of wild-type yeast (DY150) and cells deleted for *CCC1*, *SNF1*, or *SNF1* and *CCC1* were grown for 2 days on plates with the designated concentrations of iron.

extent. Here we report that the low-glucose sensor Snf1 plays a role in the regulation of *CCC1* transcription. Deletion of *SNF1* renders cells sensitive to iron toxicity by affecting *CCC1* transcription. Although some of that effect can be ascribed to the transcription factors Msn2 and Msn4, deletion of *SNF1* has an additive effect with deletion of *MSN2*/*MSN4* indicating the existence of further transcription factors involved in *CCC1* transcription.

Results

Deletion of SNF1 results in increased iron toxicity

The vacuolar iron importer Ccc1 is the principal component of high-iron resistance in yeast as deletion of *CCC1* results in a dramatic decrease in cellular iron resistance. Yap5 is a major transcriptional activator of *CCC1* (6). Deletion of *YAP5* results in a decrease in iron resistance but as shown here and by others (10, 11) not to the same extent as does deletion of *CCC1*. This result indicates that *CCC1* can be regulated by other factors. To determine what other genes could affect *CCC1* expression, we considered the fact that the low- and high-iron regulons are regulated coordinately, albeit inversely (8). Conditions that increase the low-iron regulon (iron availability and Fe–S cluster synthesis) decrease the high-iron regulon and vice versa. Haurie *et al.* (12) reported that the Aft1-dependent transcription was affected by Snf1, a major component of the glucose-sensing apparatus in yeast. Based on this observation, we examined whether deletion of *SNF1* affected iron toxicity. Deletion of *SNF1* decreased iron resistance in two different yeast backgrounds (W303 and S288c) (Fig. 1*A*). Deletion of *SNF1* in a Δ*yap5* strain had an additive effect and led to a further decrease in iron resistance, although we note that the decrease was not to the same extent as deletion of *CCC1* (Fig. 1*B*). The effect of *SNF1* on iron resistance, however, was dependent on expression of *CCC1* as an *SNF1* deletion in a Δ*ccc1* strain did not show effects different from deletion of *CCC1* (Fig. 1*C*).

SNF1 is required for iron-dependent transcription of CCC1

We then examined the effect of *SNF1* on transcription of *CCC1* using a β-galactosidase reporter construct containing the *CCC1* promoter (*CCC1-lacZ*). As shown previously, deletion of *YAP5* decreased the iron-dependent transcription of *CCC1* compared with iron-treated wild-type cells (6). Deletion of *SNF1* in Δ*yap5* cells resulted in a lowering in iron-dependent expression of the *CCC1-lacZ* activity, which was restored by transformation with an *SNF1*-containing plasmid (Fig. 2*A*). We confirmed the effect of *SNF1* on *CCC1* transcription by RT-PCR. Deletion of both *YAP5* and *SNF1* resulted in a severe cutback in *CCC1* mRNA (Fig. 2*B*). Transformation of Δ*snf1*Δ*yap5* cells with an *SNF1* plasmid resulted in increased *CCC1* mRNA. Changes in *CCC1* mRNA resulted in concomitant changes in Ccc1 protein levels. The levels of Ccc1 protein were greatly reduced in Δ*yap5* cells (Fig. 2*C*) and were near undetectable in Δ*yap5*Δ*snf1* cells, whereas in Δ*yap5*Δ*snf1* cells transformed with a *SNF1* plasmid, Ccc1 levels were low but detectable (Fig. 2*D*).

There are eight homologous *YAP* genes in which *YAP1* is most noted for being an oxidant-response gene (7). Overexpression of some of the *YAP* genes can induce *CCC1* expression in Δ*yap5* cells (10). It is possible that *SNF1* is affecting the expression of the other *YAP* genes, which may bind to the Yap site in the *CCC1* promoter and induce transcription. To test this possibility, we transformed cells with a plasmid containing a mutated *CCC1* promoter that lacks the critical Yap-binding site, as deletion of the ATG-proximal site abrogated Yap5-induced transcription (6). Transformation of wild-type cells with a Yap-mutated *CCC1-lacZ* reporter construct resulted in a decrease in iron-dependent *CCC1-lacZ* activity (Fig. 3*A*). The level of activity was similar to that seen in Δ*yap5* cells expressing the same Yap-mutated *CCC1-lacZ*. In contrast, *CCC1-lacZ* activity was decreased in Yap-mutated *CCC1-lacZ*-containing Δ*snf1* cells compared with Δ*yap5* cells, and there was only a slight decrease in activity in *yap5*Δ*snf1* cells. These results suggest that Snf1-mediated transcription of *CCC1* is independent of Yap5 or is independent of the Yap-binding sites. The finding that deletion of *SNF1* and *YAP5* dramatically restricted iron-dependent *CCC1* expression suggests that the other Yap homologues expressed at endogenous levels have little effect on iron-dependent transcription of *CCC1*.

Many of the *YAP* genes are involved in resistance to oxidant conditions. *CCC1* is required for iron resistance under both aerobic and anaerobic conditions (13), and deletion of *YAP5* affects both aerobic and anaerobic iron resistance. Based on this result, we examined whether Snf1-mediated *CCC1* expression was necessary to protect cells from iron toxicity under anaerobic conditions. Similar to Yap5, *SNF1* was required under anaerobic conditions to effect iron resistance (Fig. 3*B*). This observation rules out a role for oxygen-based signaling in the *SNF1*-mediated regulation of *CCC1* transcription.

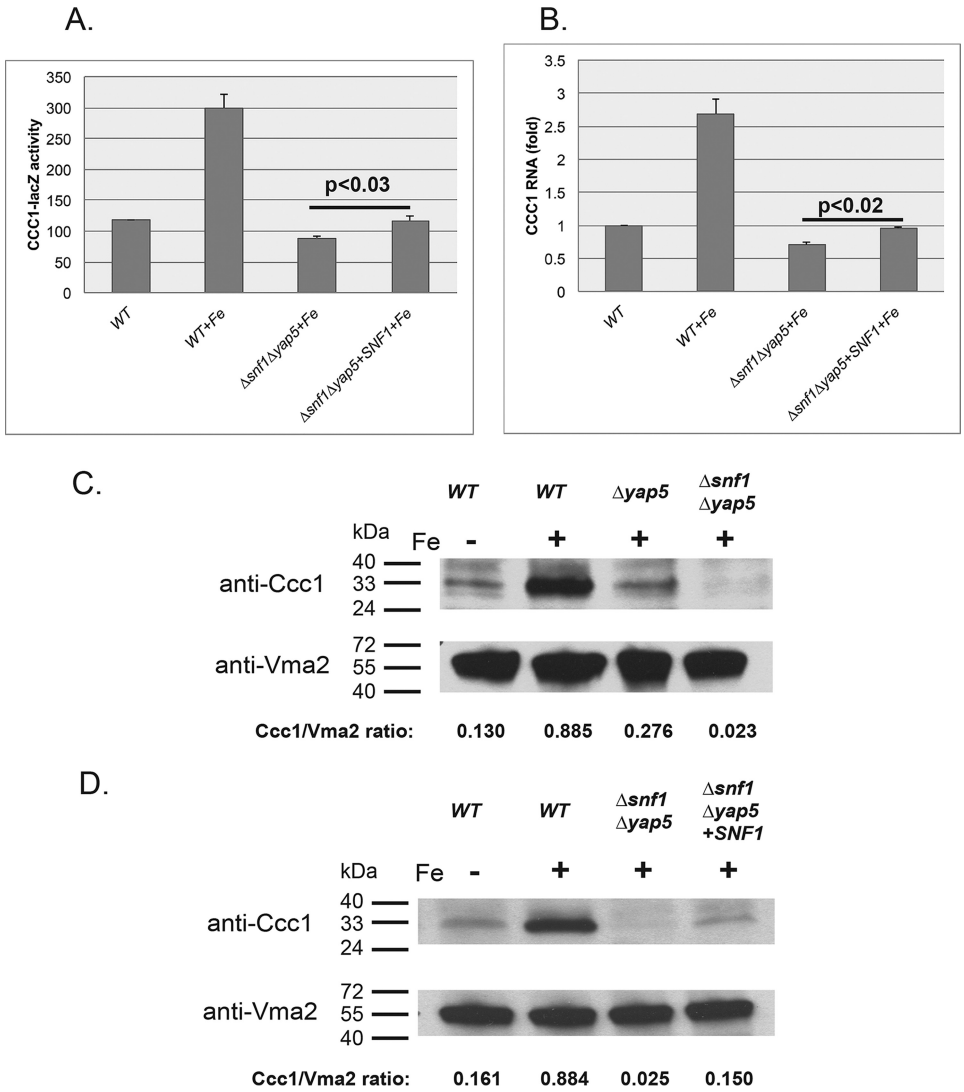

Figure 2. Deletion of *SNF1* reduces *CCC1* expression. *A*, wild type (*WT*) with control vector, Δ*snf1*Δ*yap5* with control vector, or Δ*snf1*Δ*yap5* with *SNF1* cells transformed with a *CCC1-lacZ* plasmid were incubated in CM plus 5 mM iron for 4 h. Cells were homogenized, and β-galactosidase activity and cell protein were determined. *Error bars* represent S.D. *B*, cells grown as in *A* were processed for quantitative RT-PCR to determine both *CCC1* and *ACT1* mRNA levels. The data are expressed as the -fold change in *CCC1* mRNA with *ACT1* as a control. *Error bars* represent S.D. *C*, Western blot analysis was performed on WT, Δ*yap5*, and Δ*yap5*Δ*snf1* cells grown in the presence or absence of iron using rabbit anti-Ccc1 or mouse anti-Vma2 antibody. *D*, Western blot analyses for Ccc1 and Vma2 protein levels were performed on samples as in *A*. Representative blots for *C* and *D* are shown and were quantified using Bio-Rad ImageLab™ software using Vma2 as a loading control.

Snf1 and Yap5 differentially affect transcription of CCC1

The transcriptional activation of *CCC1* by Yap5 requires that Yap5 bind Fe–S clusters (8, 10). Mutations that affect mitochondrial Fe–S cluster synthesis reduce the transcription of *CCC1* and dramatically increase iron sensitivity. To determine whether loss of *SNF1*-dependent iron resistance is similarly affected by Fe–S cluster synthesis, we examined the effect of a deletion of *ISU1*. Isu1 is a component in the mitochondrial Fe–S cluster synthetic pathway. Isu1 is one of two Fe–S scaffold proteins, and deletion of *ISU1* results in decreased Fe–S cluster synthesis, induction of the low-iron transcriptional response (14), and decreased *CCC1* transcription due to reduced Yap5-mediated activation (8). Deletion of *ISU1* resulted in decreased iron resistance (Fig. 4*A*). We note that the decrease in iron resistance due to loss of *ISU1* was relatively severe. There are several reasons for this, including decreased Yap5-induced expression of *CCC1* and of *TYW1*, which encodes a Fe–S cluster-containing protein that like other Fe–S cluster-containing proteins can act as an "iron sink" (15). Furthermore, decreased Fe–S cluster synthesis induces the low-iron Aft1 transcriptional response that can affect iron toxicity through expression of Cth1 and Cth2, which destabilizes *CCC1* mRNA (5). Finally, expression of the low-iron regulon leads to increased expression of cell surface and vacuolar iron transporters, resulting in additional iron entering the cytosol. Deletion of *SNF1* in Δ*isu1* cells resulted in a further decrease in iron resistance and decreased *CCC1-lacZ* activity (Fig. 4*B*). This additive effect of loss of *ISU1* and *SNF1* again supports that Yap5 and Snf1 effects on *CCC1* are independent of each other.

Further support for Yap5 and Snf1 exhibiting independent regulation of the high-iron regulon comes from their effects on transcription of *TYW1*. Under normal growth conditions, there

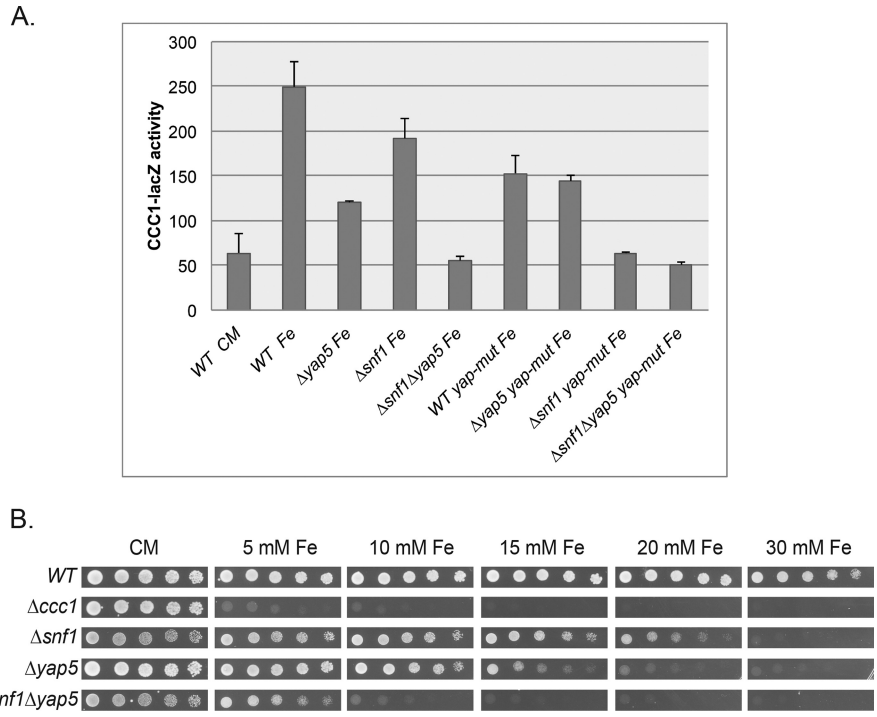

Figure 3. Transcription of *CCC1* and iron resistance is dependent on Yap5 but is independent of the other Yap proteins. *A*, wild-type, Δ*yap5*, Δ*snf1*, and Δ*snf1*Δ*yap5* cells were transformed with either a *CCC1-lacZ* construct or a *CCC1-lacZ* plasmid lacking the Yap-binding sites (*yap-mut*). The cells were incubated with 5 mM iron for 4 h, and β-galactosidase activity or cell protein was determined. *Error bars* represent S.D. *B*, serial dilutions of wild-type, Δ*yap5*, Δ*snf1*, and Δ*snf1*Δ*yap5* cells were plated on different concentrations of iron and grown for 3–4 days under anaerobic conditions.

was little change in the expression of the reporter construct *TYW1-lacZ* in the presence or absence of *SNF1* or *YAP5* (Fig. 4*C*). Under high iron conditions, *TYW1-lacZ* activity was increased in wild-type and Δ*snf1* cells, whereas deletion of *YAP5* resulted in a decrease in *TYW1-lacZ* reporter expression. Although the mechanism leading to the increased expression in Δ*snf1* cells is unknown, the finding that expression of *TYW1* responds differently to deletion of *SNF1* and *YAP5* again suggests that they are mechanistically different.

Snf1 kinase activity is important for preventing iron toxicity

The Snf1 complex is composed of three subunits: the catalytic Snf1 α subunit; the Snf4 γ subunit; and one of three β subunits, Sip1, Sip2, and Gal83 (16). Deletion of the regulatory subunit *SNF4* phenocopied deletion of *SNF1* with respect to iron toxicity (Fig. 5*A*). Many of the effects of Snf1 are due to its activation as a protein kinase. To determine whether the kinase activity of Snf1 was responsible for the change in iron resistance, we transformed Δ*snf1* cells with a missense mutant of *SNF1* (K84R) that has lost kinase activity (17). The Snf1 mutant kinase poorly restored iron resistance and did not restore low-glucose tolerance (Fig. 5*B*). In contrast, transformation of Δ*snf1* cells with a wild-type *SNF1* restored both iron resistance and low-glucose growth. To determine whether *SIP1*, *SIP2*, and *GAL83* are involved in iron toxicity, we deleted each of them separately or in combination. Deletion of each gene individually had little effect on iron toxicity (Fig. 5*C*). Deletion of all three genes showed a consistent decrease in iron resistance, although the effect on iron resistance was not as great as deletion of *SNF1*. Furthermore, deletion of all three genes in a Δ*yap5* strain showed decreased iron resistance (Fig. 6*A*), decreased *CCC1-lacZ* reporter expression (Fig. 6*B*), and decreased Ccc1 protein levels (Fig. 6*C*). These results confirm that *SNF1* affects transcription of *CCC1* and that the components of the Snf complex, Sip1, Sip2, and Gal83, participate in *CCC1* transcription. The degree of sensitivity to iron toxicity or low glucose between Δ*snf1* and Δ*gal83*Δ*sip1*Δ*sip2* was different (Fig. 6*D*), suggesting that other genes contribute to the *SNF1* effect.

Snf1 activity has been shown to be regulated by phosphorylation (16), and the phosphorylation state of Snf1 may be important in iron sensing. Changing glucose levels from 2.0 to 0.05% resulted in a clear increase in Snf1 phosphorylation as reported previously, but incubation with iron did not alter the phosphorylation status of Snf1 (Fig. 7).

Msn2 and Msn4 are involved in Ccc1-dependent iron resistance

The Snf complex is known to activate the general stress transcription factors Msn2 and Msn4 (18, 19). Furthermore, iron has been shown to induce the transcription of *MSN4*, and it has been shown that there are Msn4/Msn2-binding sites on the *CCC1* promoter (20). We confirmed that overexpression of *MSN2* affects *CCC1* transcription by measuring *CCC1-lacZ* activity in cells transformed with a plasmid containing *MSN2* under the control of the *ADH2* promoter. In CM[2] medium, vector-transformed yeast showed a low level of *CCC1-lacZ* activity, and *MSN2*-transformed cells showed an increase in *CCC1-lacZ* activity (Fig. 8*A*). Addition of iron resulted in increased *CCC1-lacZ* activity in both vector alone- and *MSN2-*

[2] The abbreviation used is: CM, complete minimal.

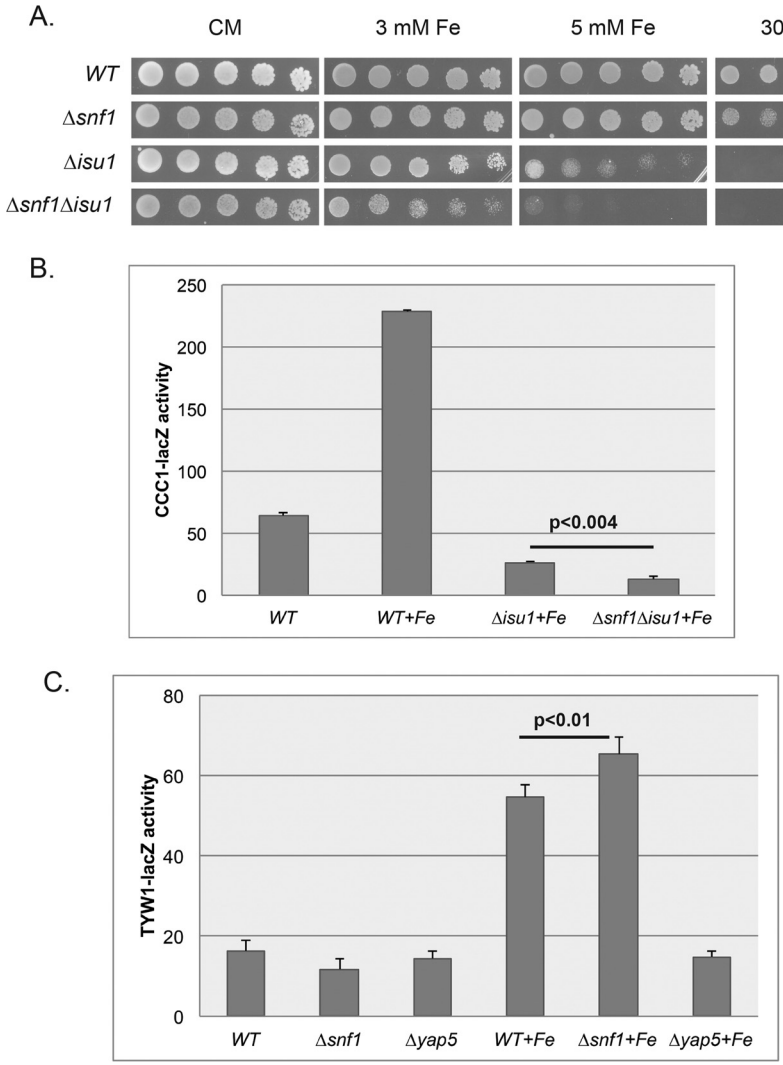

Figure 4. Snf1-dependent transcription of *CCC1* is independent of Fe–S clusters and does not affect other members of the high iron regulon. *A*, serial dilutions of wild-type, Δ*snf1*, Δ*isu1*, or Δ*snf1*Δ*isu1* cells were plated on different concentrations of iron and grown for 2 days. *B*, wild-type, Δ*isu1*, and Δ*snf1*Δ*isu1* cells transformed with a *CCC1-lacZ* plasmid were grown in CM plus 5 mM iron for 4 h, and β-galactosidase activity and protein determinations were performed. *Error bars* represent S.D. *C*, wild-type, Δ*snf1*, and Δ*yap5* cells were transformed with a *TYW1-lacZ* reporter construct. Cells were grown in CM medium in the presence or absence of 5 mM iron. Cells were harvested, and β-galactosidase activity and cell protein were determined. *Error bars* represent S.D.

transformed cells. As expected, deletion of *YAP5* decreased iron-dependent expression of *CCC1-lacZ*; however, Δ*yap5* cells transformed with *MSN2* showed an increase in iron-dependent *CCC1-lacZ* expression. Deletion of *YAP5* and *SNF1* severely decreased expression of *CCC1-lacZ* activity, which could be restored by expression of *MSN2*.

Overexpression of *MSN2* in wild-type cells resulted in a small but measureable increase in iron resistance (Fig. 8*B*). Similarly, overexpression of *MSN2* in Δ*yap5* cells restored iron resistance, albeit not to wild-type levels. Deletion of both *YAP5* and *SNF1*, as shown previously, compromised iron resistance. Overexpression of *MSN2* in Δ*yap5*Δ*snf1* cells restored iron resistance although not to the level seen in wild-type cells. These results suggest that Msn2 can affect transcription of *CCC1-lacZ* independently of either Yap5 or Snf1.

To confirm a role for Msn2/Msn4 in regulating iron toxicity, we generated single and double deletion strains and grew those cells on high-iron plates. Deletion of either *MSN2* or *MSN4* singly had no effect on iron sensitivity, whereas Δ*msn2*Δ*msn4* cells showed a decrease in iron resistance (Fig. 9*A*). To determine whether Snf1 plays a role in the activation of Msn2/Msn4, we examined iron resistance in strains deleted for *SNF1* and *MSN2*/*MSN4*. Deletion of *SNF1* decreased iron toxicity relative to wild-type cells as did deletion of *MSN2*/*MSN4* (Fig. 9*B*). There was little difference between the effects of deletion of *SNF1* or *MSN2*/*MSN4*; however, Δ*snf1*Δ*msn2*/*4* showed decreased resistance to iron toxicity. The decreased resistance to iron toxicity could be correlated with decreased transcription of *CCC1*. Deletion of *YAP5* and *SNF1* reduced the iron-dependent expression of *CCC1-lacZ* (Fig. 9*C*). The level of *CCC1-lacZ* expression was lowered even further when *MSN2* and *MSN4* were deleted in the Δ*yap5*Δ*snf1* strain. The level of *CCC1-lacZ* expression was higher in Δ*yap5*Δ*msn2*/*4* than in Δ*yap5*Δ*snf1*Δ*msn2*/*4* cells. These results suggest that Snf1 has effects on expression of *CCC1* beyond that of activating Msn2/Msn4 and that other transcription factors and signal transduction systems must be involved in Ccc1-mediated iron resistance.

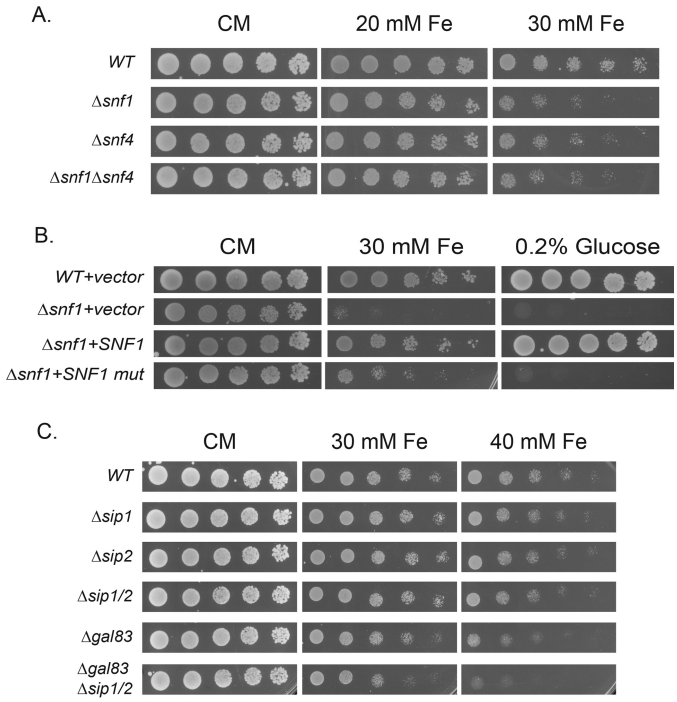

Figure 5. Iron resistance requires other components of the Snf complex. *A*, serial dilutions of wild-type, Δ*snf1*, Δ*snf4*, or Δ*snf1*Δ*snf4* cells were plated on CM or CM containing high concentration of iron and grown for 2 days. *B*, wild-type and Δ*snf1* cells transformed with either a control vector, an *SNF1*-containing plasmid, or a plasmid containing *SNF1* mutated (*mut*) in the phosphorylation site (Thr-172) were plated on either CM, CM medium containing 30 mM iron, or CM containing 0.2% low glucose. Cells were grown for 2 days. *C*, wild-type, Δ*sip1*, Δ*sip2*, Δ*sip1/2*, Δ*gal83*, or Δ*gal83*Δ*sip1*Δ*sip2* cells were plated on CM containing different concentration of iron and grown for 2 days.

Discussion

Iron homeostasis in all eukaryotes is dominated by the fact that iron is both required and biologically rare; that is, iron is abundant but most often is present in a biologically unavailable condition. Once accumulated by organisms, there is no physiologically regulated excretion of iron. Thus, along with regulation of iron uptake, regulation of iron storage is a critical mechanism protecting eukaryotes from iron toxicity. Fungi (3), plants (21), and *Plasmodium* (22) do not contain cytosolic ferritin, but rather they store iron in the vacuole. In yeast, Ccc1 is the only identified vacuolar iron importer, and its expression tightly regulates cytosolic iron concentration. Yeast expressing a functional Ccc1 are extremely iron-resistant, and overexpression of *CCC1* can result in mitochondrial and cytosolic iron depletion. The level of Ccc1 is highly regulated transcriptionally, post-transcriptionally, and at the level of activity. Yap5 was identified as a major iron-dependent transcriptional activator of *CCC1* and of a limited number of other genes, including *TYW1* and *GRX4* (6, 11, 15). Iron-mediated activation of Yap5 can be ascribed to the binding of mitochondrially produced Fe–S clusters to either of two cysteine-rich domains in Yap5, which results in the transcriptional activation of promoter-bound Yap5 (6, 10). The binding of Fe–S clusters to the low-iron-sensing transcription factor Aft2 (1) (and presumably Aft1) decreases transcription and increases the high-iron response, suggesting that these programs are coordinated (9, 23). It has been recognized, however, that Yap5 is not the only transcriptional regulator of *CCC1*. Loss of *YAP5* leads to increased sensitivity to iron that is not as severe as loss of *CCC1*. In the absence of *YAP5*, there is still some transcription of *CCC1*, albeit at different levels in different yeast strains (6, 8). It was suggested that *YAP5*-independent expression of *CCC1* could be ascribed to other *YAP* homologues as overexpression of a number of them led to activation of a *CCC1* promoter gene (10). However, deletion of the Yap-binding site in a *CCC1* reporter construct did not prevent iron from inducing expression in Δ*yap5* cells. This result implies that *CCC1* is not responding to expression of the other *YAP* genes at their endogenous levels.

Here we provide evidence that *SNF1* plays a role in the regulation of *CCC1*. We determined that the induction of *CCC1* by Snf1 is independent of Yap5, is independent of Fe–S clusters, and is specific to *CCC1* as loss of *SNF1* does not affect transcription of another high-iron target gene, *TYW1*. We further show that the Ccc1-dependent iron resistance and *CCC1* transcription by Snf1 require the kinase activity of Snf1 and the subunits Gal83, Sip1, and Sip2 of the Snf1 complex. We were unable to detect direct evidence that increased iron affected the phosphorylation status of Snf1. Snf1 has been shown to mediate resistance to other metals, including calcium (24), lithium (25), cadmium, and arsenate (26). In some instances, evidence of metal-induced phosphorylation of Snf1 could be found. For example, calcium addition clearly increased Snf1 phosphorylation on Thr-210 (24). In the case of cadmium, mutation of Snf1Thr-210 prevented cadmium resistance, but the authors could not detect cadmium-dependent phosphorylation of Snf1 (26). Thus, although metals can induce phosphorylation of Snf1, depending on the metal, the effects are weak in comparison with glucose, which results in robust phosphorylation of Snf1 (12).

In considering the downstream transcription factor(s) affected by activation of Snf1, we considered Msn2 and Msn4 as candidates based on published studies that showed that Msn2 and Msn4 were activated by Snf1 (27) and that Msn2/Msn4 activation resulted in increased iron-dependent growth and induction of *CCC1* (20). We confirmed that Msn2 and Msn4 contributed to Snf1-mediated regulation of *CCC1* transcription. That yeast still show some regulation of *CCC1* transcription in the absence of *MSN2* and *MSN4* suggests that the Snf complex activates other transcription factors to protect cells from iron toxicity that are yet to be identified.

The data presented here show that transcription of the gene encoding the single vacuolar iron importer, Ccc1, is regulated by different stimuli, and iron resistance is regulated by different stimuli. In contrast, iron entry into the cytosol from the cell surface is mediated by different transporters (*Fet3*/*FTR1*, *SMF1*, and *FET4*), all of which are regulated by different transcriptional activators, permitting different responses to environmental conditions. For example, the elemental iron transporter Fet3/Ftr1 does not function under anaerobic conditions where the low-affinity iron (metal) transport system Fet4 does. This differential expression is accomplished by both transcriptional repressors for *FET4* (*e.g.* Yap1) (28) and by transcriptional repression under anaerobic conditions of the Aft1 response by Tup1/Cti6 (29). These latter repressors do not

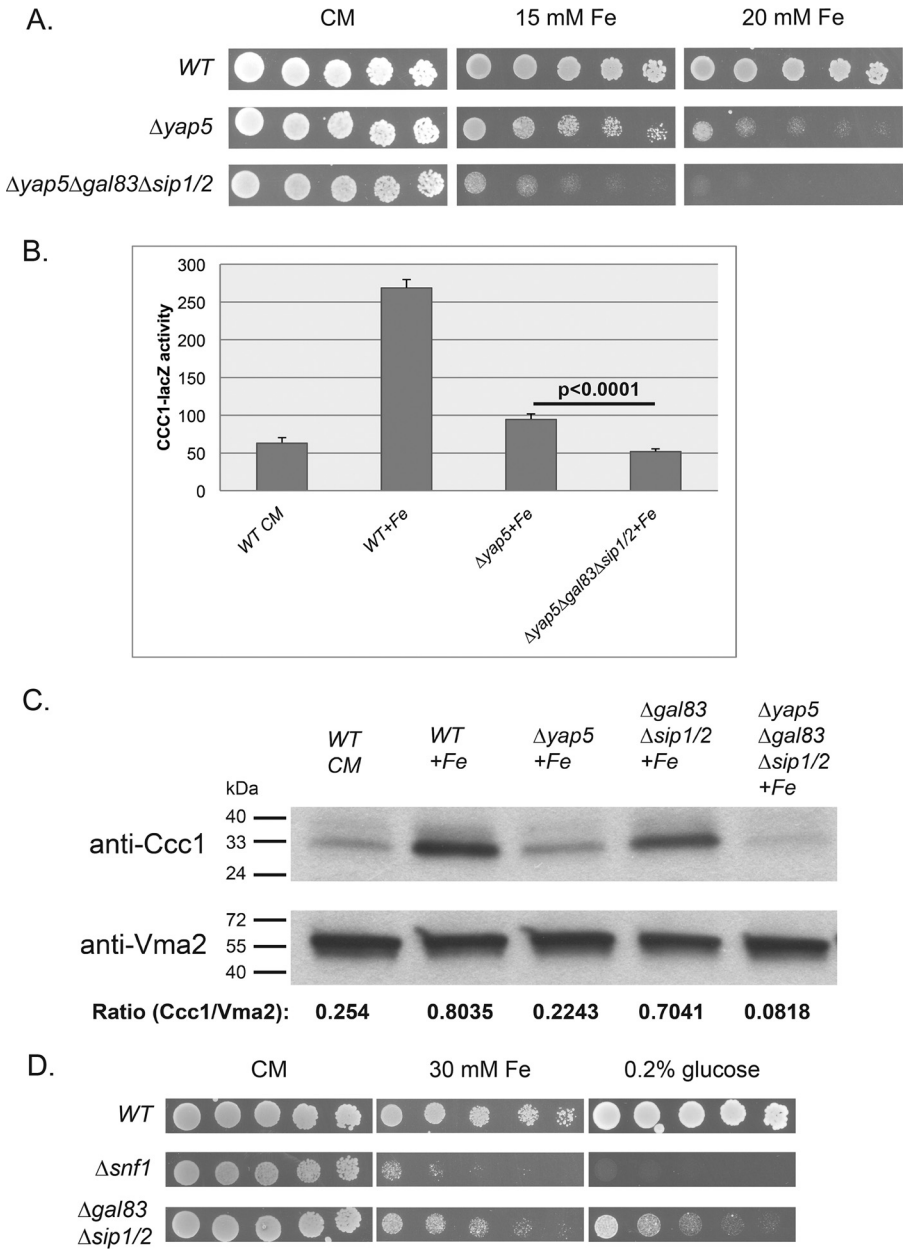

Figure 6. Deletion of *SIP1*, *SIP2*, and *GAL83* affects *CCC1* expression. *A*, serial dilutions of wild-type, Δ*yap5*, and Δ*yap5*Δ*gal83*Δ*sip1*Δ*sip2* cells were plated on different concentrations of iron and grown for 2 days. *B*, wild-type, Δ*yap5*, and Δ*yap5*Δ*gal83*Δ*sip1*Δ*sip2* cells transformed with a *CCC1-lacZ* construct were grown in iron-containing medium (5 mM) for 4 h, and then β-galactosidase activity and cell protein were determined. *Error bars* represent S.D. *C*, wild-type, Δ*yap5*, Δ*gal83*Δ*sip1*Δ*sip2*, or Δ*yap5*Δ*gal83*Δ*sip1*Δ*sip2* cells were grown in iron-containing medium (5 mM) for 4 h, and then Ccc1 and Vma2 levels were determined by Western blot analysis. *D*, wild-type, Δ*snf1*, or Δ*gal83*Δ*sip1*Δ*sip2* cells were plated on high iron or 0.2% low glucose and grown for 2 days.

affect Aft1-mediated siderophore gene expression, permitting expression of transporters that are not oxygen-dependent (siderophore) and inhibition of those that are oxygen-dependent (*e.g.* Fet3/Ftr1).

In contrast to iron entry into the cytosol, iron exit from the cytosol into the vacuole only occurs through Ccc1. Perhaps because there is only one iron importer that transporter is subject to regulation through different inputs. The activity of the major regulator Yap5 is regulated by Fe–S clusters, which permits it to be coordinately regulated, albeit inversely with the low-iron-sensing transcription factors Aft1/Aft2. We speculate that the additional mechanisms that regulate *CCC1* transcription are in place to increase Ccc1 levels and protect cells from high-iron toxicity when the Fe–S cluster Yap5-mediated sensing is saturated. The ability to regulate *CCC1* expression independently of Aft1 or other members of the high-iron regulon permits a metabolic and environmental flexibility to the modulation of cytosolic iron concentration.

Experimental procedures

Strains, plasmids, and growth conditions

Genotypes of strains used in this study are listed in Table 1. The wild-type strains used for most experiments were from the W303 background. Single deletion of *SNF1*, *GAL83*, *SIP1*, or *SIP2* was generated in DY1640 diploid cells by PCR amplifica-

tion of the KanMX marker from the diploid deletion collection (Research Genetics). Marker switch of deletion strains was carried out by using "marker swap" plasmids obtained from David Stillman (University of Utah). Double, triple, or quadruple deletions were achieved by mating, sporulation, and dissecting the tetrads.

SNF1-8Xmyc plasmid was a gift from Marian Carlson (Columbia University). The Snf1 kinase-dead mutant K84R was a gift from Julianne H. Grose (Brigham Young University).

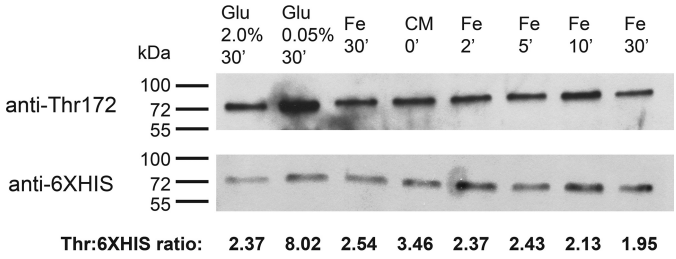

Figure 7. Addition of iron does not lead to measureable changes in Snf1 phosphorylation. Wild-type cells grown in CM with 2 or 0.05% glucose or in CM with additional 5 mM iron were harvested at the designed times. Cells were then treated as described under "Experimental procedures," loaded onto a 4–20% minigel, and transferred to nitrocellulose, and Western blot analysis with rabbit anti-Thr-172 antibody and mouse anti-His_6 antibody was performed. ′, minutes.

ADH-MSN2-GFP was a gift from James R. Broach (Penn State College of Medicine). *CCC1-lacZ*, its truncation reporter constructs, and *TWY1-lacZ* were as used previously (15).

Cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or CM (0.67% yeast nitrogen base, 0.12% dropout amino acid mixture, 2% dextrose). Media were made high iron at specific concentrations by adding ferrous ammonium sulfate to growth medium. Anaerobic growth was carried out in a BD GasPak™ EZ Anaerobe Container System.

β-Galactosidase assay

β-Galactosidase activity was performed by a 10-min kinetic assay in a SpectraMax 190 machine from Molecular Devices described as previously (29). Protein determinations were measured using the bicinchoninic acid assay (Pierce), and enzyme activity is expressed as nmol/min/mg of protein.

Total RNA purification, cDNA synthesis, and quantitative 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 in a LightCycler from Roche

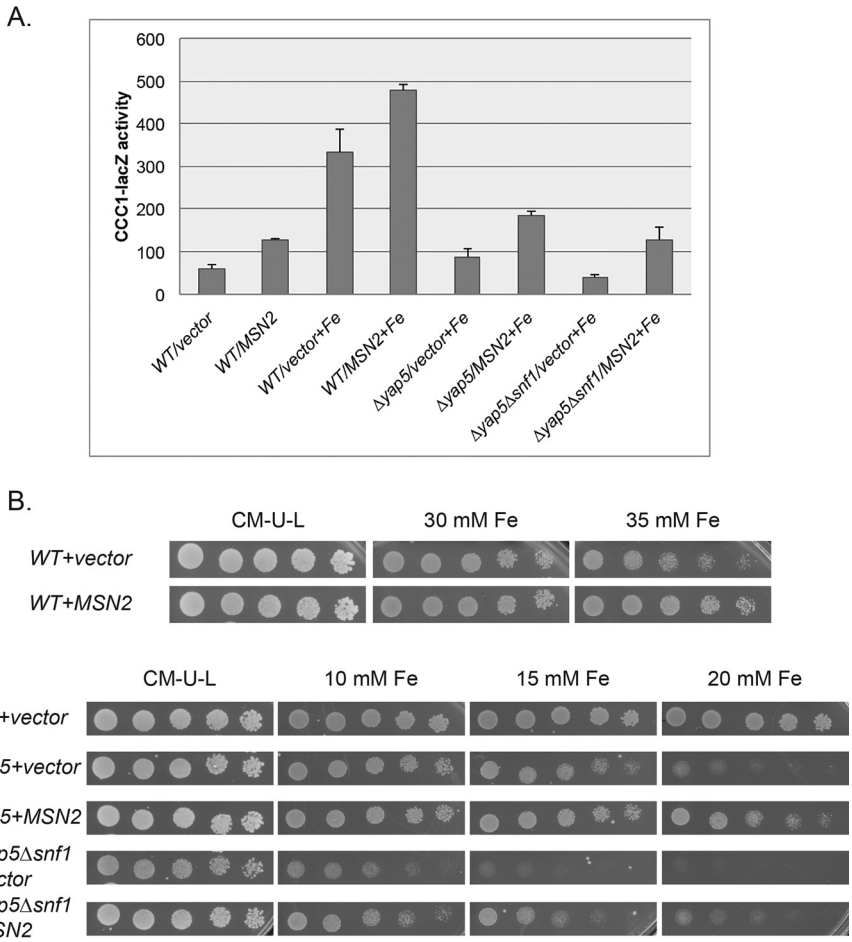

Figure 8. Overexpression of *MSN2* affects *CCC1*-dependent transcription and iron resistance. *A*, wild-type, Δ*yap5*, or Δ*yap5*Δ*snf1* cells containing *CCC1-lacZ* were transformed with either a control vector or a plasmid containing *MSN2* under the control of the *ADH* promoter. Cells were incubated in CM medium or CM medium with iron for 4 h. Cells were then processed for cell protein and β-galactosidase activity, and the data were expressed as *CCC1-lacZ* specific activity. *Error bars* represent S.D. *B*, cells as in *A* transformed with an empty vector or *MSN2*-containing plasmid were plated at serial dilutions on different concentrations of iron and grown for 2 days.

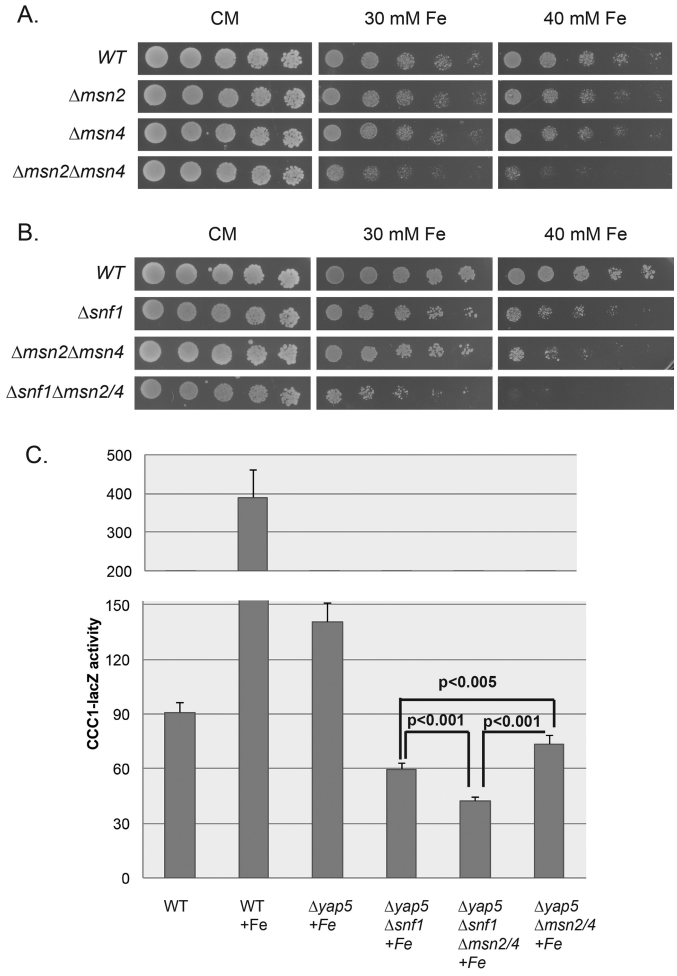

Figure 9. Deletion of *MSN2*/*MSN4* affects *CCC1-lacZ* expression and iron resistance. *A*, serial dilutions of wild-type, Δ*msn2*, Δ*msn4*, or Δ*msn2*Δ*msn4* cells were plated on high concentrations of iron and grown for 2 days to reveal iron sensitivity. *B*, serial dilutions of wild-type, Δ*snf1*, Δ*msn2*Δ*msn4*, or Δ*snf1*Δ*msn2*Δ*msn4* cells plated on high concentration of iron were grown for 3 days. *C*, wild-type, Δ*yap5*, Δ*yap5*Δ*snf1*, Δ*yap5*Δ*snf1*Δ*msn2/4*, or Δ*yap5*Δ*msn2/4* cells transformed with a *CCC1-lacZ* reporter construct were incubated with iron (5 mM) for 4 h. Cells were harvested, and cell protein and β-galactosidase activity were assayed. *Error bars* represent S.D.

Applied Science. Expression levels were normalized to the internal control, ACT1, as described previously.

Western blot and Snf1 phosphorylation analyses

For Western blot assays, a crude vacuolar fraction was prepared by glass bead preparation. Rabbit anti-Ccc1 (1:500) was generated in our laboratory using purified Ccc1 as an antigen. Mouse anti-Vma2 (1:2500) was obtained from Invitrogen. Rabbit anti-human AMP-activated protein kinase Thr-172 antibody (Cell Signaling Technology) successfully detects the corresponding yeast Snf1 Thr-210 phosphorylation site (30). 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).

The protocol for the Snf1 phosphorylation assay was adapted from a previous publication (30). Briefly, cells grown in CM (2% glucose), CM with 5 mM iron, or CM with 0.05% glucose for different times were rapidly frozen using liquid nitrogen. Cells were resuspended in phosphorylation inhibition buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 0.5% Triton X-100, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and 1× Complete protease inhibitor mixture (Roche Applied Science), glass beads were added, cells were vortexed for 2 min followed by 2 min on ice 10 times, and beads and intact cells were pelleted. Cell lysates were separated by 4–20% SDS-PAGE, proteins were transferred to nitrocellulose, and Western blotting was performed using rabbit anti-phospho-AMP-activated protein kinase α (Thr-172) (1:1000) (Cell Signaling Technology) and mouse anti-His_6 antibody (1:500) (Clontech) as a loading control followed by corresponding peroxidase-conjugated goat anti-rabbit or -mouse IgG.

Statistical analyses

All experiments were performed a minimum of two times with two independent clones, or three independent experi-

Table 1
Yeast Strains

Yeast strain	Genotype	Source/Ref.
DY150 WT	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc)*	3
Δ*ccc1*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δccc1::HIS3*	3
Δ*snf1*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsnf1::KanMX*	This study
Δ*snf4*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsnf4::His3*	This study
Δ*snf1*Δ*snf4*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsnf1::KanMx, Δsnf4::HIS3*	This study
Δ*snf1*Δ*ccc1*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δccc1::HIS3, Δsnf1::KanMx*	This study
Δ*yap5*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δyap5::KanMx*	6
Δ*snf1*Δ*yap5*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δyap5::KanMx, Δsnf1::KanMx*	This study
Δ*isu1*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δisu1::KanMx*	8
Δ*snf1*Δ*isu1*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsnf1::KanMx, Δisu1::KanMx*	This study
Δ*sip1*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsip1::KanMx*	This study
Δ*sip2*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsip2::KanMx*	This study
Δ*sip1*Δ*sip2*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsip1::KanMx, Δsip2::KanMx*	This study
Δ*gal83*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δgal83::KanMx*	This study
Δ*gal83*Δ*sip1*Δ*sip2*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δgal83::KanMx, Δsip1::HIS3, Δsip2::LEU2*	This study
Δ*yap5*Δ*gal83*Δ*sip1/2*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δyap5::KanMx, Δsnf1::KanMx, Δsip1::HIS3, Δsip2::LEU2*	This study
Δ*msn2*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δmsn2::KanMx*	This study
Δ*msn4*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δmsn4::KanMx*	This study
Δ*msn2*Δ*msn4*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δmsn2::KanMx, Δmsn4::KanMx*	This study
Δ*snf1*Δ*msn2/4*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsnf1::KanMx, Δmsn2::HIS3,Δmsn4::KanMx*	This study
Δ*yap5*Δ*msn2/4*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δyap5::LEU2, Δmsn2::HIS3, Δmsn4::KanMx*	This study
Δ*yap5*Δ*snf1*Δ*msn2/4*	*MATa ade2–1 his3–11 leu2–3.112 trp1–1 ura3–52 can1–100(oc) Δsnf1::KanMx, Δyap5::LEU2, Δmsn2::HIS3, Δmsn4::KanMx*	This study

ments with a single clone were performed. Statistics were calculated using a two-tailed unpaired Student's *t* test.

Author contributions—J. K., L. L., and D. M. W. designed the experiments. L. L. performed the experiments and generated the figures. L. L., J. K., and D. M. W. analyzed the data and wrote the manuscript.

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