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Expression of the Yeast Cation Diffusion Facilitators Mmt1 and Mmt2 Affects Mitochondrial and Cellular Iron Homeostasis

*EVIDENCE FOR MITOCHONDRIAL IRON EXPORT**

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Background: Mmt1 and Mmt2 are homologous mitochondrial proteins that belong to the family of cation diffusion facilitators.
Results: Overexpression of *MMT1&2* has effects on transition metal homeostasis, cellular oxidants and response to H_2O_2.
Conclusion: Changes in Mmt1/Mmt2 levels can affect the level of cytosolic iron.
Significance: Mmt1/Mmt2 are mitochondrial iron exporters.

Mmt1 and Mmt2 are highly homologous yeast members of the cation diffusion facilitator transporter family localized to mitochondria. Overexpression of *MMT1/2* led to changes in cellular metal homeostasis (increased iron sensitivity, decreased cobalt sensitivity, increased sensitivity to copper), oxidant generation, and increased sensitivity to H_2O_2. The phenotypes due to overexpression of *MMT1&2* were similar to that seen in cells with deletions in *MRS3* and *MRS4*, genes that encode the mitochondrial iron importers. Overexpression of *MMT1&2* resulted in induction of the low iron transcriptional response, similar to that seen in Δ*mrs3*Δ*mr4* cells. This low iron transcriptional response was suppressed by deletion of *CCC1*, the gene that encodes the vacuolar iron importer. Measurement of the activity of the iron-dependent gentisate 1,2-dioxygenase from *Pseudaminobacter salicylatoxidans* expressed in yeast cytosol, showed that changes in Mmt1/2 levels affected cytosol iron concentration even in the absence of Ccc1. Overexpression of *MMT1* resulted in increased cytosolic iron whereas deletion of *MMT1/MMT2* led to decreased cytosolic iron. These results support the hypothesis that Mmt1/2 function as mitochondrial iron exporters.

Mitochondria house the iron-consuming processes of heme and iron-sulfur cluster synthesis. Iron, the substrate for both of these processes must be imported into mitochondria by transporters. The budding yeast *Saccharomyces cerevisiae* contains two homologous mitochondrial high affinity iron transporters Mrs3 and Mrs4 that are members of the mitochondrial carrier facilitator transporter family (1–4). Homologues of these genes, termed mitoferrins, are found in all eukaryotes and mutations in these genes result in defective mitochondrial iron homeostasis in a wide range of species, including *Oryza* (5) *Drosophila* (6), *Danio rerio* (7), and *Mus musculus* (8). Studies in *S. cerevisiae* also identified the mitochondrial carrier family member Rim2 as an iron transporter (9, 10). Rim2 was also identified as a pyrimidine exchanger; however, the relationship between iron transport and pyrimidine exchange is unclear (11). The vacuolar iron transporter Ccc1 protects cells from iron toxicity by transporting iron from the cytosol to the vacuole, resulting in vacuolar iron storage (12). In the absence of *CCC1*, yeast become more sensitive to iron toxicity and overexpression of *RIM2*, *MRS3*, or *MRS4* suppressed iron toxicity (13). These results led to two conclusions: 1) iron toxicity resulted from accumulation of iron in the cytosol, and 2) mitochondria could store iron in a non-toxic form.

The finding that mitochondria could function as an iron storage organelle was unexpected. One implication of mitochondria acting as an iron reservoir is that stored iron might be exported. Transporters that export iron from mitochondria are less well defined than mitochondrial iron importers. Iron can exit the mitochondria as heme and in mammals a mitochondrial form of feline leukemia virus subgroup C receptor has been suggested to be a mitochondrial heme exporter (14); however, it is restricted to mammals and it is unclear how other eukaryotes export heme from mitochondria. Mitochondria export Fe-S clusters, and Atm1 has been implicated in that export (15), although a recent study has suggested otherwise (16). In mammalian cells ABCB8, an ATP-fueled mitochondrial transporter, has been shown to export both glutathione and iron but a yeast equivalent has not been identified (17). Studies have suggested that Mmt1 and Mmt2, which are homologous members of the cation diffusion facilitator family (CDF),[2] might function as mitochondrial iron exporters (18). These proteins are found in fungi and plants, but there is no defined vertebrate homologue.

In this work, we explore the role of Mmt1/Mmt2 in cellular and mitochondrial iron homeostasis. We show that overex-

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[2] The abbreviations used are: CDF, cation diffusion facilitator family; BPS, bathophenanthroline disulfonate; CM, complete medium; *MMT1&2*, *MMT1/MMT2*; c-GDO, cytosolic gentisate 1,2-dioxygenase; ICP-OES, inductively coupled plasma optical emission spectrometry; YPD, yeast extract/peptone/dextrose; DCF, 2′,7′-dichlorodihydrofluorescein.

TABLE 1
Yeast strains

Yeast strain	Genotype	Source
DY150	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)*	Ref. 3
Δ*mmt1*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*mmt1::KanMX*	This study
Δ*mmt2*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*mmt2::KanMX*	This study
Δ*mmt1*Δ*mmt2*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*mmt1::KanMX* Δ*mmt2::HIS3*	This study
Δ*ccc1*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*ccc1::HIS3*	Ref. 3
Δ*ccc1*Δ*mmt1*Δ*mmt2*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*ccc1::KanMX* Δ*mmt1::URA3* Δ*mmt2::HIS3*	This study
Δ*fra1*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*fra1::HIS3*	Ref. 9
Δ*fra1*Δ*ccc1*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)*Δ*fra1::HIS3* Δ*ccc1::LEU2*	This study
Δ*mrs3*Δ*mrs4*	*MAT a ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*mrs3::KanMX* Δ*mrs4::KanMX*	Ref. 3
Δ*ccc1*Δ*mrs3*Δ*mrs4*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*ccc1::HIS3* Δ*mrs3::KanMX* Δ*mrs4::KanMX*	Ref. 3
Δ*aft1*	*MAT***a** *leu2, his3, ura3, lys2, ade2, trp1* Δ*aft1::TRP1*	Ref. 3
Δ*fet5*Δ*smf3*	*MAT***a** *ade2-1 his3-11 leu2-3.112 trp1-1 ura3-52 can1-100(oc)* Δ*fet5::HIS3* Δ*smf3::KanMX*	This study

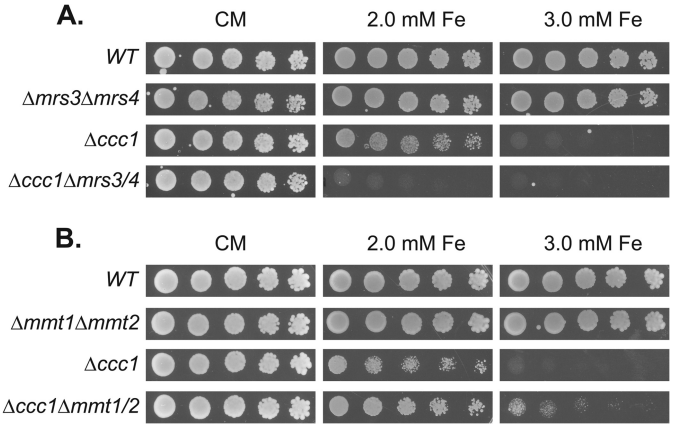

FIGURE 1. **Deletion or overexpression of mitochondrial iron transporters affects the sensitivity of Δ*ccc1* cells to high iron.** *A*, serial dilutions of cells (WT, Δ*mrs3*Δ*mrs4*, Δ*ccc1*, Δ*ccc1*Δ*mrs3*Δ*mrs4*) were plated on CM containing the specified concentrations of iron (*Fe*). *B*, serial dilutions of cells (WT, Δ*mmt1*Δ*mmt2*, Δ*ccc1*, Δ*ccc1*Δ*mmt1*Δ*mmt2*) were plated on CM containing the specified concentrations of iron.

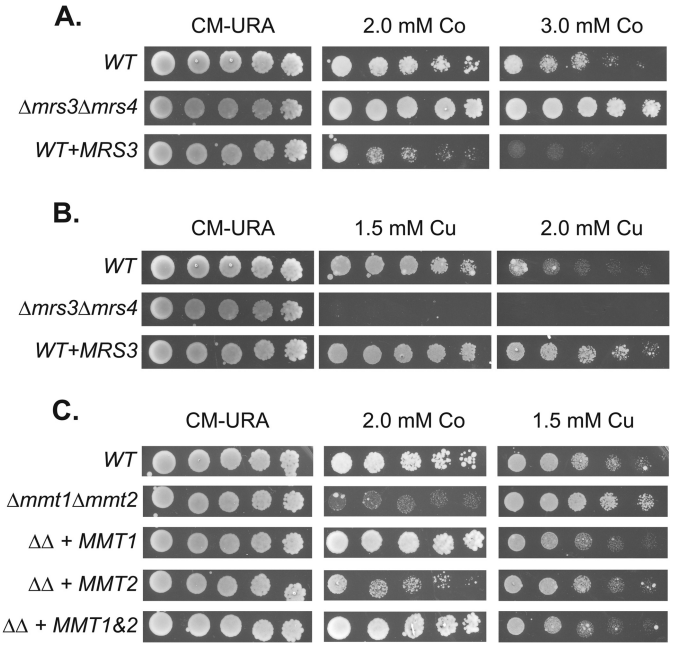

FIGURE 2. **Deletion or overexpression of mitochondrial iron transporters affects transition metal sensitivity.** *A*, WT or Δ*mrs3*Δ*mrs4* cells were transformed with either a control plasmid (pTf63) or a plasmid expressing *MRS3* and plated in serial dilutions on CM-URA plates containing the specified concentrations of cobalt (*Co*). *B*, cells as in A were plated on the specified concentrations of copper (*Cu*). *C*, WT and Δ*mmt1*Δ*mmt2* (ΔΔ) cells were transformed with a control plasmid (pTf63) or a plasmid expressing *MMT1*, *MMT2*, or both *MMT1&2*. Cells were plated on CM-URA containing the specified concentrations of cobalt or copper.

pression of *MMT1*/*MMT2* (*MMT1&2*) results in a set of phenotypes, including increased iron toxicity, increased copper sensitivity, decreased cobalt sensitivity, increased oxygen radical production, increased sensitivity to H_2O_2, and induction of the iron regulon, which are similar to that seen in cells deleted for both mitochondrial iron importers *MRS3* and *MRS4*. Furthermore, we show that the effects of overexpression of *MMT1&2* are suppressed by deletion *CCC1*, similar to that seen when *CCC1* is deleted in Δ*mrs3*Δ*mrs4* cells. We then show that independent of *CCC1*, Mmt1 and Mmt2 are mitochondrial iron exporters.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Growth Conditions—The wild type strains and deletion strains used were derived from the W303 background. Most of the single deletion or multiple deletion strains were generated by PCR amplifying the *KanMX* deletion marker from the homozygous diploid deletion collection (Research Genetics, Stanford, CA). Strains with multiple deletions were generated by marker swapping, mating and sporulation. The strains used in the paper are described in Table 1.

Genomic high copy *MMT1*, *MMT2*, and *MRS3* plasmids were described previously (3, 18). A plasmid containing both *MMT1* and *MMT2* (*MMT1&2*) was made by inserting the *MMT2* gene with its own promoter (1,000 bp) into a *MMT1* high copy plasmid (pTf63). To generate a *MET3*-regulated *MMT1* construct, the *MET3* promoter was cloned into the YEP112 (*TRP1*) vector upstream of the *MMT1* open reading frame containing a carboxyl terminus FLAG epitope. The generation of a plasmid expressing bacterial gentisate 1,2-dioxygenase (c-GDO-FLAG) in yeast cytosol was described previously (19).

Complete minimal (CM) medium was composed of yeast nitrogen base without amino acids, dextrose, and the required amino acids. Low iron media were made by adding 80 μM bathophenanthroline disulfonic acid disodium salt (BPS) and the specified concentration of $FeSO_4$.

High Metal and H_2O_2 Sensitivity Spot Assay—The specified concentrations of $(NH_4)_2Fe(SO_4)_2$, $CoCl_2$, $CuSO_4$, or H_2O_2 were added into CM-selective medium agar plates. Freshly cultured yeast cells were washed with water several times, and 1:3 serial dilutions were made from a stock of 10^6 cells. 10 μl of each dilution of cells was spotted onto plates, and plates were incubated at 30 °C for 2–3 days prior to imaging. Growth on plates

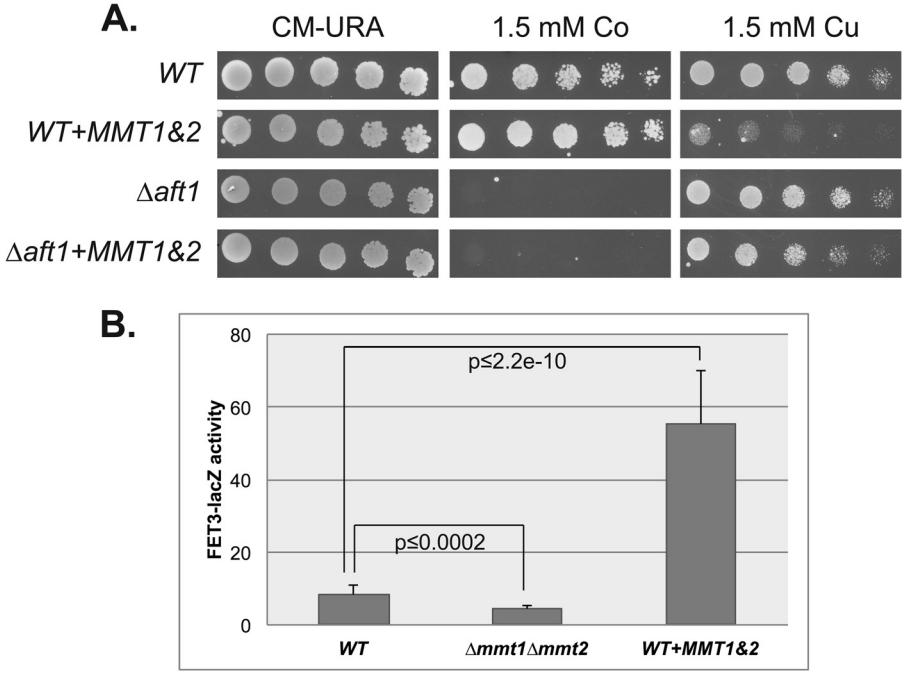

FIGURE 3. **Overexpression of *MMT1/2* leads to induction of the low iron transcriptional response.** *A*, wild type or Δ*aft1* cells were transformed with a control vector or with a plasmid expressing *MMT1&2*. Serial dilutions of cells were plated on medium containing the specified concentrations of cobalt (*Co*) or copper (*Cu*). *B*, wild type and Δ*mmt1*Δ*mmt2* cells were transformed with either a control vector (pTf63) or a plasmid expressing *MMT1&2* and with a *FET3-lacZ* reporter plasmid. Cells were grown to log phase and then assayed for β-galactosidase activity. The data are expressed as the mean specific activity (units/mg protein/min) ± S.D. of three separate experiments.

was captured using a scanner and figures generated using Adobe Illustrator.

β-Galactosidase Assay—The *FET3*-lacZ reporter construct and measurement of β-galactosidase activity was described previously (3). Protein concentrations were performed using the bicinchoninic acid assay (Pierce) detection reagent from Thermo Fisher Scientific.

Cytosolic Gentisate 1,2-Dioxygenase Assay—To measure c-GDO, cell lysates were made by glass bead homogenization, and GDO activity was assayed as described previously (19). The assay mixture contained 20 mM Tris-HCl (pH 8.0) and 0.1 mM 2,3-dihydroxy-benzoic acid (gentisic acid) as a substrate (Sigma-Aldrich). Absorbance was monitored at 340 nm, and enzyme activity was calculated using an extinction coefficient of 10.2 cm^{-1} mm^{-1}. The activity was expressed as nmol of substrate converted per minute per mg of protein.

Other Procedures—The production of reactive oxygen species was measured using 2′,7′-dichlorodihydrofluorescein diacetate to DCF conversion (Invitrogen) as described (9). Western blot analysis was performed as described (19). β-Galactosidase specific activity is reported as nmol/min/mg protein. Aconitase was determined as described previously (20). Iron levels were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-OES) as described previously (3). *p* values were determined using a two-tailed Student *t* test.

RESULTS

Metal-dependent Phenotypes Due to Changes in MMT1/MMT2 Levels—Previously, we determined that overexpression of *MRS3/4*, genes that encode mitochondrial iron importers, protected Δ*ccc1* cells from iron toxicity by transporting cytosolic iron into mitochondria (3, 13). Deletion of *MRS3/4* in Δ*ccc1* cells resulted in the opposite effect and increased iron sensitivity (Fig. 1*A*). These results support the view that mitochondria can act as an iron reservoir and that in the absence of either mitochondria or vacuolar iron sequestration, iron toxicity is exacerbated by increased cytosolic iron. Previously, we determined that overexpression of *MMT1* or *MMT2* in Δ*ccc1* resulted in increased iron toxicity (3). Based on this finding, we then examined the effect of deletion of *MMT1/MMT2* on iron toxicity in Δ*ccc1* cells. Deletion of both *MMT1* and *MMT2* had no effect on iron toxicity in wild type cells but showed modest suppression of toxicity in Δ*ccc1* cells (Fig. 1*B*). These results suggest that changes in *MMT1/2* levels can affect the ability of mitochondria to sequester iron.

Deletion of *MRS3/4* has significant effects on the cellular response to other transition metals (1, 3). Deletion of *MRS3/4* resulted in decreased cobalt sensitivity, while overexpression of *MRS3* resulted in increased cobalt sensitivity (Fig. 2*A*). In contrast, Δ*mrs3*Δ*mrs4* cells showed increased copper sensitivity while overexpression of *MRS3* resulted in decreased copper sensitivity (Fig. 2*B*). Changes in the levels of *MMT1/MMT2* also affected metal sensitivity. Deletion of *MMT1/2* resulted in increased cobalt sensitivity and decreased copper sensitivity (Fig. 2*C*, see also Fig. 3*A*). Overexpression of *MMT1* and/or *MMT2* suppressed these phenotypes. We note that overexpression of *MMT1* suppressed the cobalt sensitivity better than *MMT2* and that expression of both was not additive. Collectively, the data on both iron and transition metal toxicity show that the effects of *MMT1/2* are opposite those of *MRS3/4*; dele-

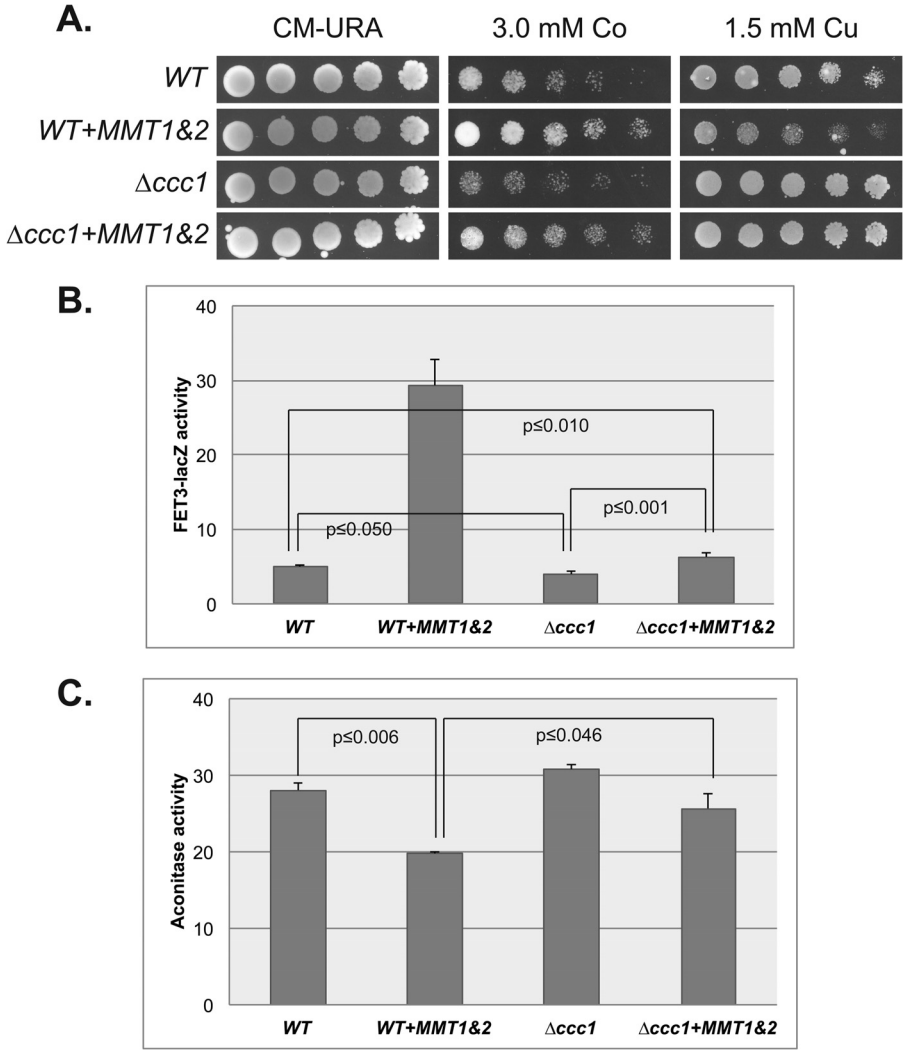

FIGURE 4. **Deletion of *CCC1* suppresses many of the effects of *MMT1&2* overexpression.** *A*, wild type or Δ*ccc1* cells were transformed with either a control plasmid (pTf63) or a *MMT1&2*-expressing plasmid. Serial dilutions of cells were plated on CM-URA medium containing the specified concentrations of cobalt (*Co*) or copper (*Cu*). *B*, wild type or Δ*ccc1* cells were transformed with a *FET3-lacZ* reporter construct and either a control plasmid (pTf63) or a *MMT1&2* expressing plasmid. Cells were grown to log phase, and β-galactosidase and cell protein were determined. The data are expressed as the mean specific activity (units/mg protein/min) ± S.D. of a representative experiment with two independent transformants ($n = 3$). *C*, aconitase activity was determined in cells treated as described in *B*. The data are expressed as the mean specific activity (units/mg protein/min) ± S.D. of a representative experiment ($n = 3$).

tion of *MMT1/2* is phenotypically similar to overexpression of *MRS3/4* and overexpression of *MMT1/2* is phenotypically similar to deletion of *MRS3/4*.

Changes in MMT1/2 Levels Affect Induction of the Low Iron Transcriptional Response—We demonstrated that Δ*mrs3*Δ*mrs4* cells showed increased expression of the low iron regulon, which is mediated by the transcription factor Aft1 (3). Many of the altered transition metal phenotypes in Δ*mrs3*Δ*mrs4* cells were due to Aft1-mediated transcription, as deletion of *AFT1* suppressed both increased copper sensitivity and decreased cobalt sensitivity. Increased transcription via Aft1 results in increased expression of the plasma membrane copper transporter *CTR1*, resulting in increased copper uptake (3). Cobalt resistance has also been ascribed to increased Aft1 activity, although the mechanism behind increased resistance is still unclear (21). Based on those results, we examined whether Aft1 was involved in the metal sensitivity of Δ*mmt1/2* cells and in cells overexpressing *MMT1&2*. Deletion of *AFT1* resulted in an increase in cobalt sensitivity that was not affected by overexpression of *MMT1&2* (Fig. 3*A*). This result is consistent with the established requirement for Aft1-mediated transcription in cobalt resistance. Deletion of *AFT1* in *MMT1/2* overexpressing cells suppressed the increased sensitivity to copper seen in wild type cells overexpressing *MMT1&2*. Deletion of both *MMT1* and *MMT2* led to a reduction in the expression of the Aft1-responsive *FET3-lacZ* reporter construct (Fig. 3*B*). In contrast, overexpression of *MMT1&2* led to increased expression of the *FET3-lacZ* reporter. These results suggest that the levels of Mmt1 and Mmt2 affect Aft1-mediated transcription.

Induction of the Iron Regulon by MMT1&2 Overexpression Is Dependent on the Presence of CCC1—Decreased mitochondrial iron accumulation resulting from deletion of *MRS3/MRS4* induced the iron regulon via increasing the activity of the vacuolar iron transporter Ccc1 (3, 9). This conclusion was based on the fact that the level of Ccc1 does not increase in Δ*mrs3*Δ*mrs4* cells, but deletion of *CCC1*

abrogates the Δ*mrs3*Δ*mrs4* phenotypes (3, 12). Deletion of *MRS4* in *Candida albicans* also results in altered sensitivity to Li^+ and Cu^{2+}, which is suppressed by deletion of *CCC1* (22). As the metal phenotypes seen in Δ*mrs3*Δ*mrs4* cells are similar to that seen in *MMT1&2* overexpressing cells, we examined whether Ccc1 plays a role in the phenotypes observed when *MMT1&2* is overexpressed. Overexpression of *MMT1* led to decreased cobalt sensitivity and increased copper sensitivity. Deletion of *CCC1* by itself led to a slight increase in cobalt sensitivity and a decrease in copper sensitivity. Deletion of *CCC1* in *MMT1&2*-overexpressing cells resulted in increased cobalt sensitivity, but decreased copper sensitivity compared with wild type cells overexpressing *MMT1&2* (Fig. 4*A*). Similarly, deletion of *CCC1* reduced the increase in *FET3-lacZ* activity seen in *MMT1&2*-overexpressing cells (Fig. 4*B*). Induction of *FET3-lacZ* is often the result of a decrease in mitochondrial Fe-S cluster synthesis, which can be monitored by measurement of the activity of the mitochondrial Fe-S containing enzyme aconitase. Overexpression of *MMT1&2* resulted in decreased aconitase activity (Fig. 4*C*). Deletion of *CCC1* resulted in a slight increase in aconitase activity. We hypothesize that this is due to the inability to transport iron from cytosol to vacuole leading to increased cytosolic and mitochondrial iron. Overexpression of *MMT1&2* in Δ*ccc1* cells results in a decrease in aconitase activity compared with Δ*ccc1* cells; however, the level of aconitase activity is still higher than in wild type cells expressing *MMT1&2*. Thus, the level of aconitase tracks with the induction or lack of induction of the iron regulon.

Overexpression of MMT1&2 Affects Cellular Oxidants—We reported that Δ*mrs3*Δ*mrs4* cells had increased levels of cellular oxidants, as shown by increased fluorescence of DCF, a reporter for oxygen radicals (9). Based on this result, we examined whether increased expression of *MMT1&2* resulted in increased oxidant levels. We detected a small increase in DCF fluorescence in cells overexpressing *MMT1&2* (Fig. 5*A*). *FRA1* encodes a protein, which binds to antioxidant effectors Grx3, Grx4 (23), Tsa1 (9), and Tsa2 (24) and suppresses oxidant damage. Deletion of *FRA1* in Δ*mrs3*Δ*mrs4* cells led to an increase in DCF fluorescence, which was suppressed by deletion of *CCC1* (9). Relative to wild type cells, deletion of *FRA1* had no effect on DCF fluorescence, whereas overexpression of *MMT1&2* in Δ*fra1* cells resulted in a significant increase in DCF fluorescence although much less than deletion of *FRA1* in Δ*mrs3*Δ*mrs4* cells (9). To amplify the effects of oxidant stress, we added H_2O_2 to cells to increase cellular oxidant levels. Addition of H_2O_2 had little effect on DCF fluorescence in either wild type cells, wild type cells overexpressing *MMT1&2* or Δ*fra1* cells (Fig. 5*B*, note change in scale). In contrast, Δ*fra1* cells overexpressing *MMT1&2* cells showed a dramatic increase in DCF fluorescence. Deletion of *CCC1* suppressed the increase in DCF fluorescence in overexpressing *MMT1&2* Δ*fra1* cells. These results show that changes in cellular oxidants, due to altered mitochondrial iron transporters, are affected by the presence of Ccc1.

Increased activity of Ccc1 results in induction of the Aft1-dependent low iron transcriptional response. As part of that response, there is increased expression of the vacuolar iron

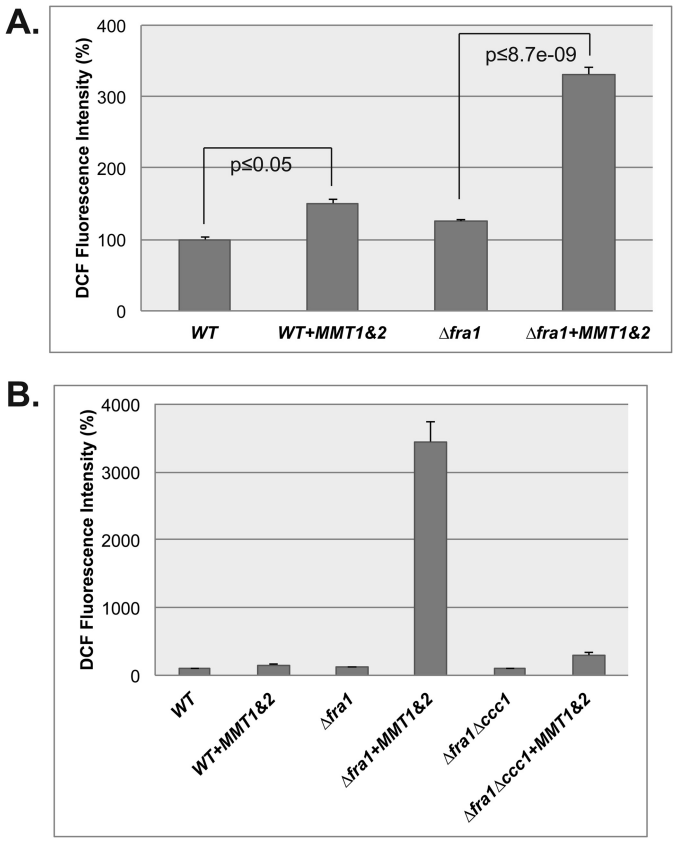

FIGURE 5. **Overexpression of *MMT1&2* leads to increased cellular oxidants.** *A*, wild type or Δ*fra1* cells were transformed with a control plasmid (pTf63) or a *MMT1&2* expressing plasmid. Cells were grown overnight and then incubated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate for 1 h, and fluorescence intensity was assayed. The data are expressed as the mean percent DCF fluorescence compared with wild type cells normalized to 100% ± S.D. of two independent transformants and is a representative experiment ($n = 2$). *B*, wild type, Δ*fra1*, or Δ*fra1*Δ*ccc1* cells were transformed with a control plasmid (pTf63) or a *MMT1&2* expressing plasmid. Cells were grown overnight, 0.00125% H_2O_2 was added for 15 min followed by addition of 10 μM 2′,7′-dichlorodihydrofluorescein diacetate for 1 h, and fluorescence intensity was determined. The data are expressed as the mean percent DCF fluorescence compared with wild type cells normalized to 100% ± S.D. of a single transformant. Three independent transformants were analyzed, and a representative experiment is shown.

exporters Smf3 and Fet5/Fth1, which might counter the effect of Ccc1 by exporting vacuolar iron to the cytosol. Deletion of both *SMF3* and *FET5* had little effect on DCF fluorescence or growth even in the presence of H_2O_2 (Fig. 6*A*). In contrast, Δ*fet5*Δ*smf3* cells overexpressing *MMT1&2* showed a large increase in oxidant generation in the presence of H_2O_2. Growth effects of these manipulations are consistent with the biochemical data. Deletion of both *FET5* and *SMF3* had a small effect on growth of cells plated with H_2O_2 (Fig. 6*B*). In contrast, Δ*fet5*Δ*smf3* cells overexpressing *MMT1&2* showed an increase in H_2O_2 sensitivity. These results suggest that prevention of iron export from vacuoles exacerbates both oxidant generation and cell growth defects resulting from overexpression of *MMT1&2*.

Changes in Mitochondrial Iron Transporters Affect Cytosolic Iron Levels—We reported that deletion of *MRS3* and *MRS4* resulted in increased expression of Fet3/Ftr1 and increased cellular iron as assayed by ICP-OES, both of which were suppressed by deletion of *CCC1* (3). We also showed that overexpression of *MRS3* or *MRS4* led to reduced cellular iron, whereas

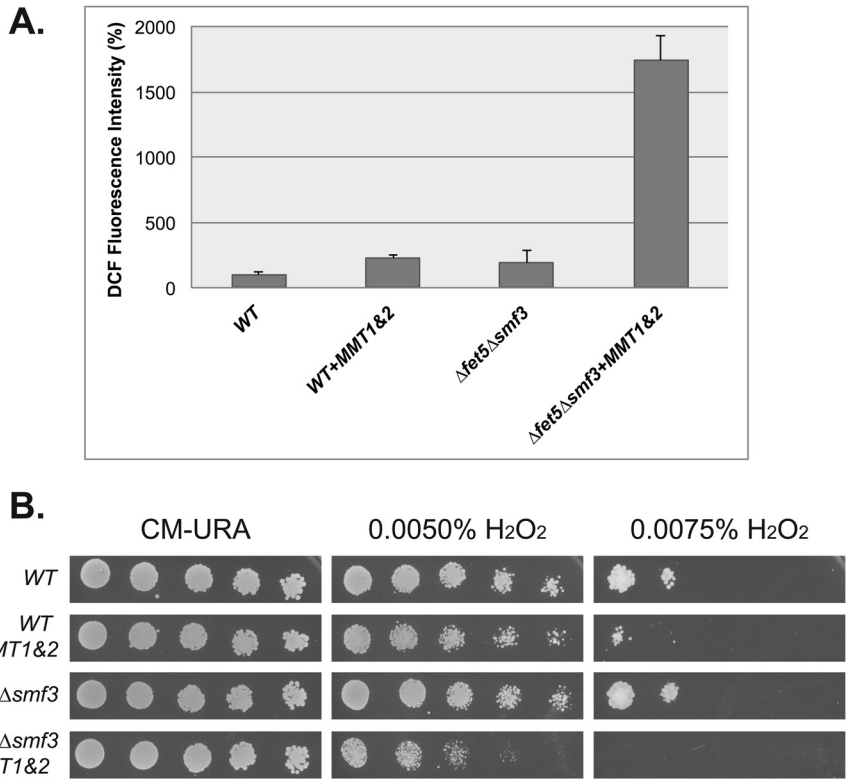

FIGURE 6. **Effect of *MMT1&2* overexpression on cellular oxidants and oxidant sensitivity in cells deleted for the vacuolar iron exporters *FET5* and *SMF3*.** *A*, wild type cells and Δ*fet5*Δ*smf3* cells were transformed with either a control plasmid (pTf63) or a *MMT1&2*-expressing plasmid. Cells were grown overnight and 0.00125% H_2O_2 was added for 15 min followed by addition of 10 μM 2′,7′-dichlorodihydrofluorescein diacetate for 1 h and then fluorescence intensity was determined. The data are expressed as the mean percent DCF fluorescence compared with wild type cells normalized to 100% ± S.D. of two independent transformants and is a representative experiment ($n = 3$). *B*, serial dilutions of cells as described in *A* were plated on CM-URA medium with the specified concentrations of H_2O_2.

overexpression of *MMT1* or *MMT2* led to increased cellular iron (3) and induction of the iron regulon (see Fig. 3). Recently, we employed an assay for cytosolic iron that measured the activity of the bacterial iron-dependent enzyme, c-GDO, expressed in yeast (19). We used this assay to determine if changes in mitochondrial iron transporters affected cytosolic iron levels. Overexpression of *MRS3* in either wild type or Δ*ccc1* cells results in decreased c-GDO activity (Fig. 7*A*). This result is consistent with decreased levels of cellular iron assayed by ICP-OES (3). The decrease in cellular iron may be attributed to reduced levels of *FET3/FTR1*, which occurs upon overexpression of *MRS3* (data not shown). Deletion of *MRS3/MRS4* also led to lower levels of c-GDO (Fig. 7*B*), yet these cells have increased expression of Fet3/Ftr1 and increased cellular iron as assayed by ICP-OES (3). Deletion of *CCC1* in wild type cells resulted in increased c-GDO. Deletion of *CCC1* in Δ*mrs3*Δ*mrs4* cells reduced the level of c-GDO compared with Δ*ccc1* cells, but the level of GDO activity was still higher than that seen in Δ*mrs3*Δ*mrs4* cells. The loss of *CCC1* led to decreased cellular iron but higher cytosolic iron compared with wild type cells. Deletion of *CCC1* in Δ*mrs3*Δ*mrs4* cells, however, did not completely restore cytosolic iron levels to that of Δ*ccc1* cells. This finding is consistent with our previous results showing that deletion of *CCC1* did not completely suppress the effects seen in Δ*mrs3*Δ*mrs4* cells (3). These results suggest that Ccc1 is not the sole responder to changes due to *MRS3/MRS4*.

Similar analyses were performed in cells overexpressing *MMT1*. Overexpression of *MMT1* in wild type or Δ*ccc1* cells led to increased c-GDO activity, which was not reduced by deletion of *CCC1* (Fig. 8*A*). Indeed, the level of c-GDO was higher in Δ*ccc1* cells than in wild type cells and even higher in Δ*ccc1*-overexpressing *MMT1* cells than in wild type-overexpressing *MMT1* cells, suggesting that cytosolic iron levels increased. Although the level of c-GDO was increased by deletion of *CCC1*, the level of total cellular iron, as measured by ICP-OES was decreased (~50%) by deletion of *CCC1* (Fig. 8*B*). Cells overexpressing *MMT1* showed a large increase in cellular iron, which is consistent with increased expression of the low iron regulon, as shown above (Fig. 3*B*). Deletion of *CCC1* in *MMT1*-overexpressing cells suppressed the increased iron accumulation. Our interpretation of this result is that Mmt1 exported mitochondrial iron, increasing cytosolic iron but most of that iron accumulated in the vacuole in the presence of *CCC1*.

Mmt1 and Mmt2 Can Export Iron from Mitochondria—If iron is being exported from mitochondria, then deletion of *MMT1/2* should affect cytosolic iron by retaining iron in mitochondria. This is observed by the c-GDO assay in both wild type cells and in Δ*ccc1* cells grown in CM medium (Fig. 9*A*). The effects of loss of mitochondrial iron export should be particularly apparent under conditions in which both cellular iron acquisition and vacuolar iron storage are prevented. To accom-

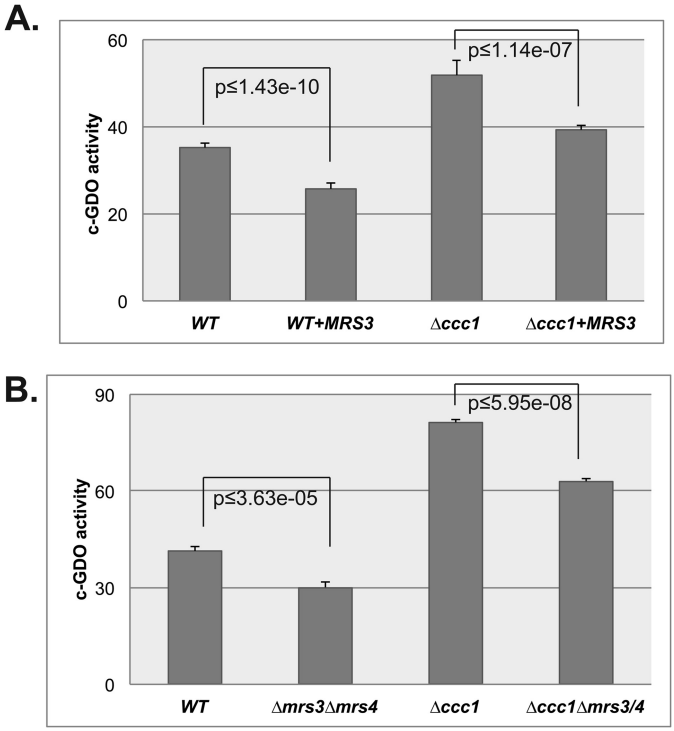

FIGURE 7. **Expression of *MRS3* affects cytosolic iron.** *A*, wild type and Δ*ccc1* cells were transformed with either a control vector or a *MRS3*-expressing vector in which *MRS3* is regulated by the *MET3* promoter. Cells were also transformed with a plasmid expressing c-GDO-FLAG under the control of the *ADH1* promoter. Cells were grown in methionine free medium overnight, and the activity of c-GDO as well as the cell protein was determined. The data are expressed as the mean specific activity ± the S.D. of four individual transformants. *B*, cells (wild type, Δ*mrs3*Δ*mrs4*, Δ*ccc1*, Δ*ccc1*Δ*mrs3mrs4*) were transformed with a c-GDO-FLAG expressing plasmid. Cells were grown overnight, and the activity of c-GDO as well as cell protein was determined. The data are expressed as the mean specific activity ± S.D. of two independent transformants and is a representative experiment ($n = 3$).

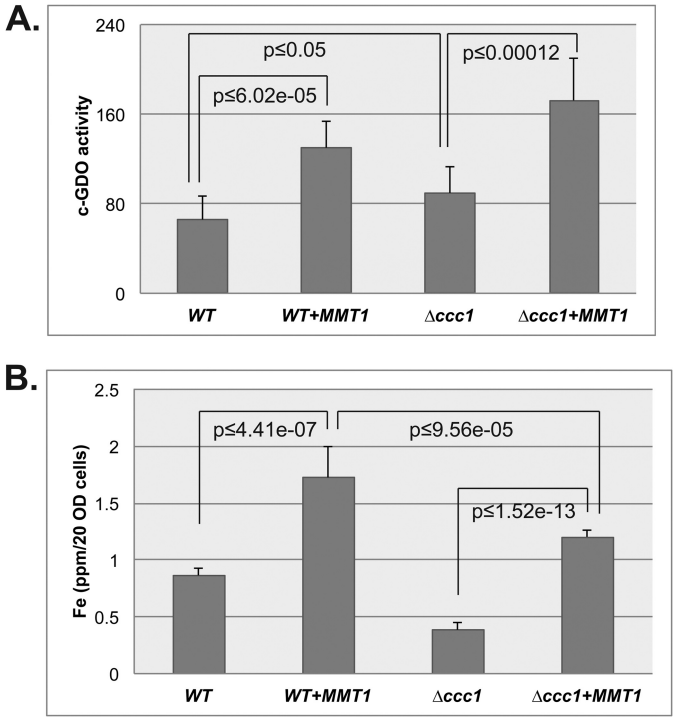

FIGURE 8. **Expression of *MMT1* affects cytosolic and cellular iron levels.** *A*, wild type and Δ*ccc1* cells were transformed with either a control vector or a *MET3* regulated *MMT1-FLAG* vector. Cells were also transformed with a plasmid expressing c-GDO-FLAG under the control of the *ADH1* promoter. Cells were incubated overnight in methionine-free medium and harvested, and cell protein and cytosolic GDO activity were determined. The data are expressed as the mean specific activity ± S.D. of four individual transformants. *B*, cells treated as described in *A* were harvested and assayed for cellular iron by ICP-OES. The data are expressed as the mean iron (ppm/20 OD cells) ± S.D. of four individual transformants.

plish this, we examined the effect of deletion of *MMT1/2* on the activity of c-GDO in cells deleted for *CCC1*. In the absence of vacuolar iron storage, iron can accumulate in the mitochondria over time, which should magnify the effects of deleting *MMT1/2*. Consequently, cells were grown in iron overnight and then placed in medium lacking iron (through the addition of the iron chelator BPS). When Δ*ccc1* cells were placed in low iron medium there was a time dependent decrease in c-GDO activity reflecting decreased cytosolic iron (Fig. 9*B*). There was a greater rate of loss of c-GDO activity in Δ*ccc1*Δ*mmt1*Δ*mmt2*, suggesting that lack of these transporters affected export of iron from mitochondria to cytosol.

An analogous protocol was used to examine the effect of overexpression of *MMT1*. For this experiment, Δ*ccc1* cells were used to eliminate the vacuole as an iron storage organelle and to obviate effects due to Ccc1-mediated vacuolar iron transport. Cells were transformed with a c-GDO-expressing plasmid, which permitted us to monitor cytosolic iron and c-GDO levels. Cells were also transformed with a plasmid expressing a methionine-regulated *MMT1* with a carboxyl FLAG epitope. Cells were grown overnight in iron-containing medium in the presence of 10× methionine (to repress expression of *MMT1*). The cells were then shifted to methionine-free medium (to permit expression of *MMT1*) that was made low iron through the addition of BPS. Under these conditions, *MMT1* is only overexpressed once cells are placed in low iron (Fig. 10, *A* and *B*). The expression of *MMT1*, which becomes noticeable at 2 h, resulted in higher c-GDO activity than in cells that did not overexpress *MMT1*. This result supports the hypothesis that *MMT1*-induced mitochondrial iron export results in an increase in cytosolic iron.

DISCUSSION

The concentration of iron in biological fluids is tightly regulated. Cytosolic iron levels are regulated by transporters that import iron into the cytosol either across the plasma membrane or from the vacuole. In plants and yeast, iron is stored in the vacuole. In yeast, vacuolar iron levels are regulated by transporters that export iron from the vacuole (Fet5/Fth1, Smf3) or import iron into the vacuole (Ccc1). Mitochondrial iron transporters are necessary for mitochondrial iron consuming processes such as Fe-S cluster and heme synthesis. The finding that mitochondrial iron storage can protect Δ*ccc1* cells from iron toxicity led us to speculate that there may well be transporters that could efflux mitochondrial iron (13). Here, we provide data suggesting that Mmt1 and Mmt2, members of the CDF family, maybe mitochondrial iron exporters.

There are two lines of evidence that suggest that Mmt1/Mmt2 can export iron from mitochondria. The first is based on the fact that the same phenotypes seen when *MMT1&2* are

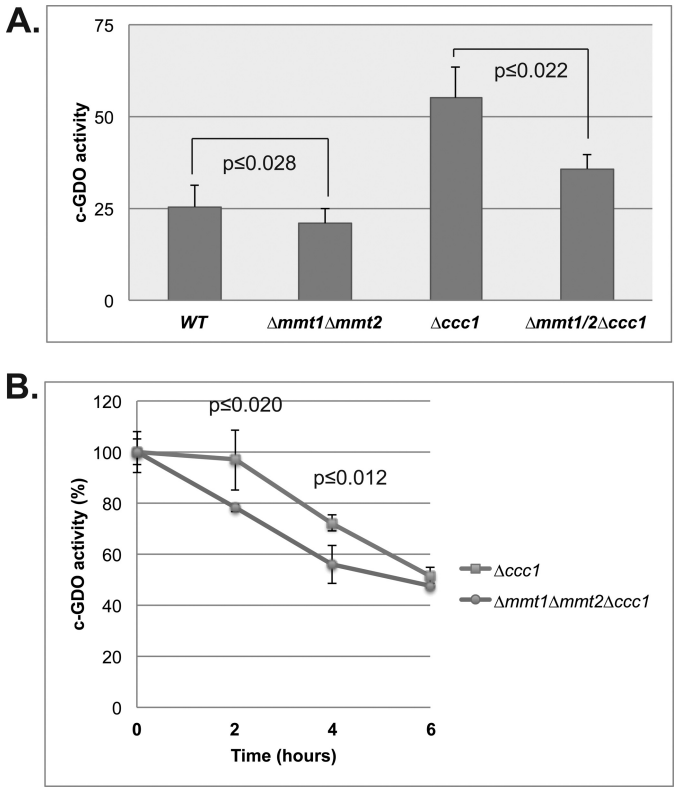

FIGURE 9. **Deletion of *MMT1/MMT2* affects cytosolic iron levels.** *A*, cells (wild type, Δ*mmt1*Δ*mmt2*, Δ*ccc1*, Δ*mmt1*Δ*mmt2*Δ*ccc1*) were transformed with a c-GDO-FLAG expressing plasmid. Cells grown overnight in CM medium were harvested and assayed for cell protein and c-GDO activity. The data are expressed as the mean specific activity ± S.D. of four individual transformants. *B*, cells (Δ*ccc1*, Δ*mmt1*Δ*mmt2*Δ*ccc1*) were transformed with a c-GDO-expressing plasmid. Cells grown overnight in 200 μM iron were washed and incubated in medium made iron-deficient through the addition of the iron chelator BPS. At the specified times, cells were harvested and assayed for cell protein and c-GDO activity. The data are normalized to the zero time value (100%) for each group. The data are presented as the mean ± standard deviation of two independent transformants within a single experiment. This experiment presented is representative of three independent experiments.

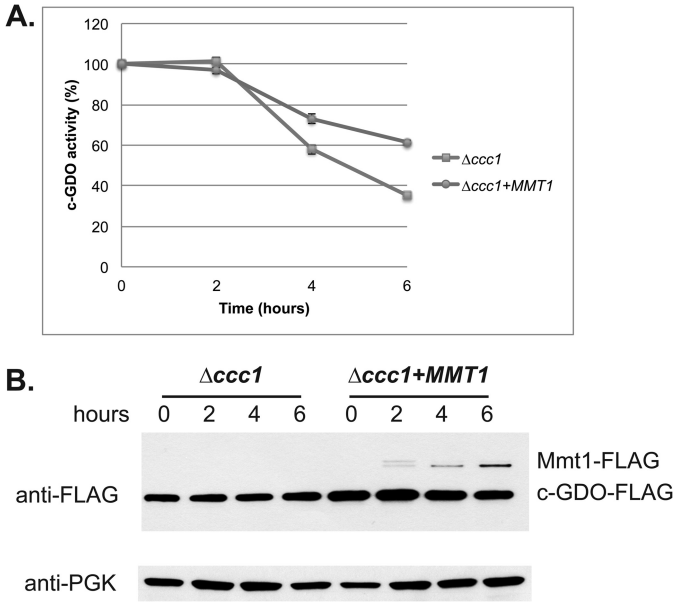

FIGURE 10. **Overexpression of *MMT1* affects cytosolic iron independent of *CCC1*.** *A*, cells (Δ*ccc1*) were transformed with either a control plasmid or a plasmid containing *MMT1-FLAG* under the control of the *MET3* promoter. Cells were grown to mid log phase in the presence of 200 μM iron and high methionine (10× Met) medium to repress expression of *MMT1*. Cells were washed and then placed in iron-depleted BPS-containing medium with no added methionine to permit expression of *MMT1*. At the specified times, cells were harvested, and c-GDO activity was determined. The data are normalized to the zero time value (100%) for each group. The data are presented as the mean ± S.D. of two independent transformants within a single experiment ($n = 2$). *B*, samples from one of the transformants from *A* were analyzed by Western blot for c-GDO-FLAG and Mmt1-FLAG. Antibodies to PGK were used as a loading control.

overexpressed are seen when *MRS3* and *MRS4* are deleted. Mrs3/Mrs4 and their homologues in higher eukaryotes (mitoferrins) are mitochondrial iron importers (1–4, 7). Extensive biochemical and phenotypic alterations are seen upon deletion of these mitochondrial iron importers. Many of those alterations are suppressed by deletion of the vacuolar iron importer *CCC1*. Similar findings have been made in *C. albicans*, in which deletion of the only mitochondrial iron importer results in changes in transition metal metabolism, which are suppressed by deletion of *CCC1* (22). These results show that reduction in mitochondrial iron import can lead to changes, which affect vacuolar iron uptake and cytosolic iron levels.

The second line of evidence indicating that Mmt1/Mmt2 may export mitochondrial iron is based on the use of GDO to assay cytosolic iron. Attempts to utilize a mitochondrial tagged GDO were unsuccessful due to low levels of mitochondrial GDO activity. Data obtained using c-GDO suggest that alterations in the levels of the mitochondrial iron importers Mrs3 and Mrs4 affect cytosolic iron in a manner opposite that of the putative iron exporters Mmt1 and Mmt2. In general, deletion of *MMT1/2* results in a decrease in cytosolic iron, whereas overexpression of *MMT1&2* led to an increase in cytosolic iron. Overexpression of Mrs3 resulted in increased mitochondrial iron, which then led to decreased expression of the low iron regulon. Similarly, deletion of *MMT1/2*, by decreasing mitochondrial iron export led to increased mitochondrial iron and decreased expression of the iron regulon. Deletion of *MRS3/MRS4* resulted in reduced mitochondrial iron by reducing mitochondrial iron import and activating Ccc1, which lowered cytosolic iron. Overexpression of *MMT1&2* reduced mitochondrial iron by exporting iron and activating Ccc1, albeit to a lower extent then did deletion of *MRS3/4*.

There are differences in cells with deletions of *MRS3/4 versus* overexpression of *MMT1&2* such as the level of cytosolic iron and the level of oxidants. We believe that the differences are quantitative not qualitative. Deletion of *MRS3/4* resulted in a large increase in endogenous oxidants and a marked decrease in cytosolic iron. Both alterations are dependent on the presence of Ccc1. In cells overexpressing *MMT1&2*, the increase in endogenous oxidants was modest at best. Overexpression of *MMT1&2*, however, affected oxidant production in response to H_2O_2, deletion of *FRA1* or when vacuolar iron export was reduced by deletion of *FET5/SMF3*. Similarly, deletion of *MRS3/4* had dramatic effects on reducing cytosolic iron levels, although cellular iron levels were increased. In *MMT1*-expressing cells, both cytosolic iron and cellular iron are increased even in a Δ*ccc1* strain. We interpret these results to suggest that *MMT1*-mediated mitochondrial iron export results in a modest increase in oxidants that affect Ccc1 activity, which results in increased iron entry into the vacuole and thus increased in cellular

iron. Deletion of *CCC1* in *MMT1*-overexpressing cells resulted in a decrease in cellular iron, but because of mitochondrial iron export, cytosolic iron was higher than seen in Δ*ccc1* cells.

The finding that changes in mitochondrial iron levels can affect the vacuole, and that vacuolar activities can affect the mitochondria are consistent with recent studies in yeast that show that changes in vacuolar activities can affect mitochondrial function. In yeast, decreased vacuolar pH affects mitochondrial function leading to altered mitochondrial membrane potential and morphology (25). Recently, Diab and Kane (24) showed that decreased vacuolar pH resulted in chronic oxidative stress, decreased activity of mitochondrial aconitase and induction of the iron regulon leading to increased cellular iron. Their data indicated that induction of the iron regulon resulted from a decrease in cytosolic pH, which is a consequence of decreased vacuolar pH. We do not think that deletion of *CCC1* affects vacuolar pH, as the vacuolar transition metal transporters Zrc1 or Cot1 can protect Δ*ccc1* cells from zinc or cobalt toxicity (26). These transporters rely on the activity of the vacuolar H^+ATPase for metal import, suggesting that vacuolar H^+ATPase activity is not severely affected. Our data, however, suggest that changes in vacuolar iron import or export can result in increased cellular oxidants.

CDF family members are found in all kingdoms. CDF transporters in prokaryotes most often play a role in metal resistance by exporting metals from cells (for review see (27)). There are numerous CDF transporters in eukaryotes, found in diverse organelles including vacuole (lysosomes), plasma membrane, endoplasmic reticulum, and Golgi. Most studies show that these transporters export metals from the cytosol either into organelles or across the plasma membrane. Znt2, a member of the CDF family, is a zinc transporter localized to mitochondria in human cells and is thought to increase mitochondrial zinc levels at the expense of cytosolic zinc (28, 29). Mmt1 and Mmt2 are the only identified mitochondrial CDF members in fungi. MTP6 is a homologue of *MMT1/MMT2* present in higher plants but not in algae (30). Expression of MTP6 in yeast results in phenotypes similar to overexpression of *MMT1/MMT2*: induction of the iron regulon and increased cobalt resistance (data not shown). This result suggests that MTP6 is a functional homologue of *MMT1/MMT2*. Vertebrates do not appear to have mitochondrial CDF members. These observations lead to the question of whether mitochondrial iron export is limited to fungi and plants. Alternatively, there might be non-CDF transporters in mitochondria that can mediate the same function. A recent study by Ichikawa *et al.* (17) suggested that the mitochondrial ATP transporter ABCB8 might provide a similar function. Increased expression of ABCB8 resulted in decreased mitochondrial iron while reduction in ABCB8, either through gene deletion or RNAi, resulted in increased mitochondrial iron. These results, in concert with the results presented in this communication, suggest that the pool of mitochondrial iron may vary and be subject to mitochondrial iron exporters.

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