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 H2O2.

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 H2O2. 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 Δmrs3Δmr4 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 Mr3 and Mr4 that are members of the mitochondrial carrier facilitator transporter family (1–4). Homologs of these genes, termed mitoferins, 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 iron storage. Studies in CCC1− yeast have suggested that mitochondrial iron transporters 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.
expression 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 H₂O₂, 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 Δmrs3Δmrs4 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 FeSO₄.

**FIGURE 1. Deletion or overexpression of mitochondrial iron transporters affects the sensitivity of Δccc1 cells to high iron.** A, serial dilutions of cells (WT, Δmrs3Δmrs4, Δccc1, Δccc1Δmrs3Δmrs4) were plated on CM containing the specified concentrations of iron (Fe); B, serial dilutions of cells (WT, Δmmt1Δmmt2, Δccc1, Δccc1Δmmt1Δmmt2) 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 Δmrs3Δmrs4 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 Δmmt1Δmmt2 (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.
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-a-tomic 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 Δccc1 cells from iron toxicity by transporting cytosolic iron into mitochondria (3, 13). Deletion of MRS3/4 in Δccc1 cells resulted in the opposite effect and increased iron sensitivity (Fig. 1A). 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 Δccc1 resulted in increased iron toxicity (3). Based on this finding, we then examined the effect of deletion of MMT1/MMT2 on iron toxicity in Δccc1 cells. Deletion of both MMT1 and MMT2 had no effect on iron toxicity in wild type cells but showed modest suppression of toxicity in Δccc1 cells (Fig. 1B). 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. 2A). In contrast, Δmrs3Δmrs4 cells showed increased copper sensitivity while overexpression of MRS3 resulted in decreased copper sensitivity (Fig. 2B). 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. 2C, see also Fig. 3A). 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-
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 \(/\text{H9004 mrs3}/\text{H9004 mrs4}\) 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 \(/\text{H9004 mrs3}/\text{H9004 mrs4}\) cells were due to Aft1-mediated transcription, as deletion of AFT1 suppressed 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 \(/\text{mmt1/2}\) 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. 3A). This result is consistent with the established requirement for Aft1-mediated transcription in cobalt resistance. Deletion of both MMT1 and MMT2 led to a reduction in the expression 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. 3B). 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 \(/\text{mrs3/mrs4}\) cells, but deletion of CCC1

![FIGURE 4. Deletion of CCC1 suppresses many of the effects of MMT1&2 overexpression. A, wild type or \(/\text{ccc1}\) 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 \(/\text{ccc1}\) 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).](image-url)
abrogates the \( \Delta mrs3\Delta mrs4 \) phenotypes (3, 12). Deletion of \( MRS4 \) in \( C. albicans \) also results in altered sensitivity to \( Li^+ \) and \( Cu^{2+} \), which is suppressed by deletion of \( CCC1 \) (22). As the metal phenotypes seen in \( \Delta mrs3\Delta mrs4 \) 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. 4A). Similarly, deletion of \( CCC1 \) reduced the increase in \( FET3\)-lacZ activity seen in \( MMT1\&2 \)-overexpressing cells (Fig. 4B). 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. 4C). 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 \( \Delta ccc1 \) cells results in a decrease in aconitase activity compared with \( \Delta ccc1 \) 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 \( \Delta mrs3\Delta mrs4 \) 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. 5A). \( FRA1 \) encodes a protein, which binds to antioxidant effectors Grx3, Grx4 (23), Tsa1 (9), and Tsa2 (24) and suppresses oxidant damage. Deletion of \( FRA1 \) in \( \Delta mrs3\Delta mrs4 \) 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 \( \Delta fra1 \) cells resulted in a significant increase in DCF fluorescence although much less than deletion of \( FRA1 \) in \( \Delta mrs3\Delta mrs4 \) 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 \( \Delta fra1 \) cells (Fig. 5B, note change in scale). In contrast, \( \Delta fra1 \) cells overexpressing \( MMT1\&2 \) showed a dramatic increase in DCF fluorescence. Deletion of \( CCC1 \) suppressed the increase in DCF fluorescence in overexpressing \( MMT1\&2 \) \( \Delta fra1 \) 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 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. 6A). In contrast, \( \Delta fet5\Delta smf3 \) 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. 6B). In contrast, \( \Delta fet5\Delta smf3 \) 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...
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 \( \Delta \)ccc1 cells results in decreased c-GDO activity (Fig. 7A). 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. 7B), 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 \( \Delta \)mrs3\( \Delta \)mrs4 cells reduced the level of c-GDO compared with \( \Delta \)ccc1 cells, but the level of GDO activity was still higher than that seen in \( \Delta \)mrs3\( \Delta \)mrs4 cells. The loss of CCC1 led to decreased cellular iron but higher cytosolic iron compared with wild type cells. Deletion of CCC1 in \( \Delta \)mrs3\( \Delta \)mrs4 cells, however, did not completely restore cytosolic iron levels to that of \( \Delta \)ccc1 cells. This finding is consistent with previous results showing that deletion of CCC1 did not completely suppress the effects seen in \( \Delta \)mrs3\( \Delta \)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 \( \Delta \)ccc1 cells led to increased c-GDO activity, which was not reduced by deletion of CCC1 (Fig. 8A). Indeed, the level of c-GDO was higher in \( \Delta \)ccc1 cells than in wild type cells and even higher in \( \Delta \)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. 8B). 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. 3B). 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 \( \Delta \)ccc1 cells grown in CM medium (Fig. 9A). 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-
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**FIGURE 7. Expression of MRS3 affects cytosolic iron.** A, wild type and Δccc1 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 ± S.D. of four individual transformants. B, cells (wild type, Δmrs3Δmrs4, Δccc1, Δccc1Δmrs3Δmrs4) 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 Δccc1 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.

To accomplish 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 Δccc1 cells were placed in low iron medium there was a time dependent decrease in c-GDO activity reflecting decreased cytosolic iron (Fig. 9B). There was a greater rate of loss of c-GDO activity in Δccc1Δmmt1Δmmt2, 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, Δccc1 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. In plants and yeast, iron is stored in the vacuole. Iron transporters that export iron from the vacuole (Fet5/Fth1, Smf3) or from mitochondria (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 mitochondrial iron from 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, may be 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
overexpressed are seen when \textit{MRS3} and \textit{MRS4} are deleted. \textit{MRS3}/\textit{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 \textit{CCC1}. Similar findings have been made in \textit{C. albicans}, in which deletion of the only mitochondrial iron importer results in changes in transition metal metabolism, which are suppressed by deletion of \textit{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 \textit{MRS3} and \textit{MRS4} affect cytosolic iron in a manner opposite that of the putative iron exporters Mmt1 and Mmt2. In general, deletion of \textit{MMT1&2} results in a decrease in cytosolic iron, whereas overexpression of \textit{MMT1&2} led to an increase in cytosolic iron.

Overexpression of \textit{Mrs3} resulted in increased mitochondrial iron, which then led to decreased expression of the low iron regulon. Similarly, deletion of \textit{MMT1&2}, by decreasing mitochondrial iron export led to increased mitochondrial iron and decreased expression of the iron regulon. Deletion of \textit{MRS3}/\textit{MRS4} resulted in reduced mitochondrial iron by reducing mitochondrial iron import and activating Ccc1, which lowered cytosolic iron. Overexpression of \textit{Mti1&2} reduced mitochondrial iron by exporting iron and activating Ccc1, albeit to a lower extent than did deletion of \textit{MRS3}/\textit{MRS4}.

There are differences in cells with deletions of \textit{MRS3}/\textit{MRS4} versus overexpression of \textit{Mti1&2} such as the level of cytosolic iron and the level of oxidants. We believe that the differences are quantitative not qualitative. Deletion of \textit{MRS3}/\textit{MRS4} 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 \textit{Mti1&2}, the increase in endogenous oxidants was modest at best. Overexpression of \textit{Mti1&2}, however, affected oxidant production in response to \textit{H2O2}, deletion of \textit{FRA1} or when vacuolar iron export was reduced by deletion of \textit{FETS}/\textit{SMF3}. Similarly, deletion of \textit{MRS3}/\textit{MRS4} had dramatic effects on reducing cytosolic iron levels, although cellular iron levels were increased. In \textit{Mti1}-expressing cells, both cytosolic iron and cellular iron are increased even in a \textit{Dccc1} strain. We interpret these results to suggest that \textit{Mti1}-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 **Delta****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 **Delta****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 **MXT1/MMT2** present in higher plants but not in algae (30). Expression of MTP6 in yeast results in phenotypes similar to overexpression of **MXT1/MMT2**, induction of the iron regulon and increased cobalt resistance (data not shown). This result suggests that MTP6 is a functional homologue of **MXT1/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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