A Mitochondrial-Vacuolar Signaling Pathway in Yeast That Affects Iron and Copper Metabolism*

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Mitochondria utilize iron, but the transporters that mediate mitochondrial iron uptake and efflux are largely unknown. Cells with a deletion in the vacuolar iron/manganese transporter Ccc1p are sensitive to high iron. Overexpression of MRS3 or MRS4 suppresses the high iron sensitivity of Δccc1 cells. MRS3 and MRS4 have recently been suggested to encode mitochondrial iron transporters. We demonstrate that deletion of MRS3 and MRS4 severely affects cellular and mitochondrial metal homeostasis, including a reduction in cytosolic and mitochondrial iron acquisition. We show that vacuolar iron transport is increased in Δmrs3Δmrs4 cells, resulting in decreased cytosolic iron and activation of the iron-sensing transcription factor Aft1p. Activation of Aft1p leads to increased expression of the high affinity iron transporter Ftr1p and increased iron uptake. Deletion of CCC1 in Δmrs3Δmrs4 cells restores cellular and mitochondrial iron homeostasis to near normal levels. Δmrs3Δmrs4 cells also show increased resistance to cobalt but decreased resistance to copper and cadmium. These phenotypes are also corrected by deletion of CCC1 in Δmrs3Δmrs4 cells. Decreased copper resistance in Δmrs3Δmrs4 cells results from activation of Aft1p by Ccc1p-mediated iron depletion, as deletion of CCC1 or AFT1 in Δmrs3Δmrs4 cells restores copper resistance. These results suggest that deletion of mitochondrial proteins can alter vacuolar metal homeostasis. The data also indicate that increased expression of the AFT1-regulated gene(s) can disrupt copper homeostasis.

Two important aspects of cellular iron metabolism are localized to mitochondria, heme synthesis and iron-sulfur cluster synthesis. Both synthetic pathways require iron import into mitochondria, but little is known about mitochondrial transporters for either import or export of iron. Recent studies have shown that deletion of both MRS3 and MRS4, members of the mitochondrial carrier family, results in decreased mitochondrial iron content. Double deletion strains (Δmrs3Δmrs4) demonstrate a low iron growth defect and decreased heme and iron-sulfur cluster synthesis (1, 2). Furthermore, Foyer and Roganti (1) deleted MRS3 and MRS4 in a strain lacking Yfh1p, the yeast frataxin homologue. Loss of Yfh1p leads to defects in iron-sulfur cluster synthesis (3–5) and excessive mitochondrial iron accumulation (6). Δyfh1 cells are protected from mitochondrial iron toxicity when MRS3 and MRS4 are deleted (1). Deletion of these genes does not completely abrogate mitochondrial iron uptake, leading to the suggestion that they encode mitochondrial iron transporters whose function only becomes apparent under low iron conditions (2).

Other characteristics of Δmrs3Δmrs4 cells are inconsistent with a role for these genes simply as mitochondrial iron transporters, as broad effects on transition metal metabolism have been observed. In particular, Δmrs3Δmrs4 cells show increased cellular iron acquisition due to up-regulation of the iron regulon (1, 2). Disruption of the MRS3/MRS4 homologue in Cryptococcus neoformans also leads to increased expression of iron transport genes (7). Furthermore, Δmrs3Δmrs4 cells show significant alterations in transition metal metabolism; Δmrs3Δmrs4 cells are more cobalt-resistant and more copper- and cadmium-sensitive than wild type cells (1).

We have examined how deletion of MRS3 and MRS4 affects cellular metal metabolism. We report that deletion of these genes leads to activation of the vacuolar iron transporter Ccc1p and the subsequent depletion of cytosolic iron. The decrease in cytosolic iron leads to activation of Aft1p and transcription of the iron regulon. Aft1p is an iron-sensing transcription factor that translocates to the nucleus when cells are iron-depleted (8, 9). Aft1p is responsible for the transcription of approximately 20 genes, among which are those that encode the high affinity iron transport system and increased iron uptake. Overexpression of MRS3 or MRS4 suppresses the high iron toxicity in Δccc1 cells. These results demonstrate that MRS3 and MRS4 have roles beyond mitochondrial iron metabolism and suggest that alteration of mitochondrial activity can affect vacuolar function.

MATERIALS AND METHODS

Yeast Strains, Growth Media, and Plasmids—Yeast strains used in this study are shown in Table I. Cells were grown in CM, a synthetic medium that includes yeast nitrogen base, amino acids, and glucose. When required, iron, copper, zinc, cadmium, and cobalt were supplemented at the concentrations indicated in each experiment. Iron-limited conditions were achieved by the addition of the impermeable iron chelator bathophenanthroline disulfonate. Where specified, low iron conditions were achieved by the addition of the impermeable iron chelator bathophenanthroline disulfonate. Where specified, low iron medium was supplemented with 0.2% Tween, 30 μg/ml ergosterol, and 56 μg/ml methionine. Construction of pMET3CCC1- and AFT1-controlled plasmids has been described previously (10). Construction of a low copy vector containing CCC1 was performed by cloning the PCR product of the CCC1 gene, including 500 bp of the 5′ promoter region, into YCPPlac3. Construction of plasmids containing MMT1 and MMT2 have been described previously (11) (although the genes in that publication were referred to as MFT1 and MFT2).

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Table I

Yeast strains

| Strain         | Genotype                        |
|---------------|---------------------------------|
| BY4741 wild type | MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 |
| BY4742 wild type | MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 |
| Δmrs3          | MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δmrs3::kanMX4 |
| Δmrs4          | MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 Δmrs4::kanMX4 |
| Δmrs3Δmrs4-1   | MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δmrs3Δmrs4::kanMX4 |
| Δmrs3Δmrs4-2   | MAT a his3Δ1 leu2Δ0 ura3Δ0Δmrs3Δmrs4::kanMX4 |
| Δccc1          | MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δccc1::HIS3 |
| Δmrs3Δmrs4Δccc1 | MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δmrs3Δmrs4::kanMX4Δccc1::HIS3 |
| ΔΔccc1         | MAT a his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0ΔΔccc1::HIS3 |
| Δmrs3Δmrs4ΔΔccc1 | MAT a his3Δ1 leu2Δ0 Δmrs3Δmrs4::kanMX4ΔΔccc1::HIS3 |

Fig. 1. Overexpression of MRS3 or MRS4 suppresses iron sensitivity in a Δccc1 strain. A, cells (wild type, Δccc1, or Δccc1 transformed with a high copy plasmid expressing either MRS3, MRS4, or MMT1 and MMT2) were plated on media containing different concentrations of ferrous ammonium sulfate and incubated aerobically or (B) anaerobically (C). Cells (wild type, Δccc1, Δccc1 cells transformed with either a MRS3- or MRS4-containing plasmid) were plated on different concentrations of ferrous ammonium sulfate and grown aerobically.

Identification of Suppressors of the High Iron Toxicity in Δccc1 Cells—A Saccharomyces cerevisiae genomic library was used to identify high copy suppressors of iron toxicity in Δccc1 cells. The library was obtained from Dr. David Stillman (University of Utah) and has been described previously (12). The library was transformed into Δccc1 cells. Plasmids were recovered and sequenced from cells capable of growing on 5 mM iron. Identified plasmids were subcloned to identify the complementing gene. 

Transposon Insertion Library Screen—An mTr-3×HAT/lacZ-mutagenized genomic library was a gift from Dr. M. Snyder (Yale University). Three pools of libraries were digested with NotI and transformed into Δmrs3Δmrs4Δccc1 Δccc1 rho0 cells. Colonies were replicated on high copper plates (2 mM CuSO4). Candidates that grew on this toxic concentration of copper were isolated and transformed with YIp5. Genomic DNA recovered from these candidates was digested with NotI, ligated overnight, and transformed into Escherichia coli cells by electroporation. Rescued plasmids were amplified and sequenced, and the disrupted genes were identified as described previously (13). 

Microarray Analysis—RNA was extracted from wild type, Δmrs3Δmrs4Δccc1, and Δmrs3Δmrs4Δccc1 cells grown in CM media. Total RNA was isolated using standard techniques (14). Purification of mRNA was performed using the Poly(A) Tract mRNA isolation system (Promega). Fabrication of DNA microarray, synthesis of fluorescent-labeled cDNA, hybridization of the microarrays, and subsequent scanning were performed in the Huntsman Cancer Institute Microarray Core Facility.

Miscellaneous Procedures—Iron transport activity and iron content assays were performed as described (15). A FET3-LacZ construct was constructed and assayed as described previously (15). CTR1-LacZ and CUP1-LacZ constructs were obtained from Dr. Dennis Winge (University of Utah). Aconitase activity was assayed as described (3). Vacuoles were isolated as described previously (15).

Fig. 2. Overexpression of MRS3/MRS4 decreases cellular iron content. A, cells (wild type, Δccc1, or Δccc1 transformed with a high copy plasmid expressing either MRS3, MRS4, MMT1, or MMT2) were incubated overnight in CM. Cells were washed, and the iron content was determined by atomic absorption spectroscopy. B, increased cellular iron through forced expression of FET4 overcomes MRS4-mediated protection. Cells (Δccc1) were transformed with a MRS3 or MRS4 plasmid and with a plasmid containing a GAL4-regulated FET4. Cells were plated on galactose media containing different amounts of ferrous ammonium sulfate.
RESULTS

Overexpression of MRS3 or MRS4 Suppresses Iron Sensitivity of Δccc1 by Reducing Cellular Iron Accumulation—Cells with a deletion in CCC1, which encodes a vacuolar iron and manganese transporter, are more sensitive to growth on high iron medium than wild type cells, presumably because of an inability to remove iron from the cytosol (15). We screened for genes that when overexpressed could suppress the high iron sensitivity of Δccc1 cells. We identified two homologous genes, MRS3 and MRS4, capable of permitting Δccc1 cells to grow on high iron (Fig. 1A). These genes are members of the family of mitochondrial carriers and have been identified as putative mitochondrial iron transporters (1, 2). We considered the possibility that MRS3/MRS4 prevented iron toxicity by reducing iron-induced oxidant damage. If this hypothesis is correct, then we might expect that anaerobiosis would reduce iron sensitivity. Incubation of cells under anaerobic conditions decreased the iron sensitivity of Δccc1 cells, as a higher concentration of iron was required to see a phenotype. Under anaerobic conditions, overexpression of either MRS3 or MRS4 in Δccc1 cells could no longer suppress the iron toxicity seen at the higher iron concentration (Fig. 1B). Although oxygen is required for protection by MRS3 or MRS4, respiration is not required, as overexpression of MRS3 or MRS4 could protect Δccc1 cells that are rho0 from iron toxicity (Fig. 1C).

Overexpression of MRS3 or MRS4 could protect cells from iron toxicity by sequestering iron into mitochondria, as these genes are thought to encode mitochondrial iron transporters. We discovered, however, that overexpression of either gene in either Δccc1 (data not shown) or wild type cells resulted in a lower cellular iron content as assayed by atomic absorption spectroscopy (Fig. 2A). Previously we had identified MMT1 and MMT2 as mitochondrial iron transporters (11). These two genes encode members of the cation diffusion facilitator family localized to mitochondria, and overexpression of either of these transporters led to increased mitochondrial iron. In contrast to overexpression of MRS3 or MRS4, overexpression of MMT1 and/or MMT2 in Δccc1 cells increased iron toxicity (Fig. 1A) and, when overexpressed in wild type cells, increased cell-associated iron (Fig. 2A).

Deletion of plasma membrane iron transport systems, either the high affinity (FET3) or low affinity (FET4) transport systems, both of which import iron into cells, did not rescue the iron sensitivity of Δccc1 cells (data not shown). Increasing cellular iron levels by the ectopic expression of FET4 through the use of a GAL4-driven plasmid, overcame the protection provided by overexpressed MRS3 or MRS4 (Fig. 2B). These results suggest that overexpression of MRS3 and MRS4 protects Δccc1 cells by reducing cellular iron accumulation and that the protective effect requires oxygen metabolism but not respiration.

Deletion of MRS3 and MRS4 Increases Vacuolar Iron Accumulation and Induces Expression of the Iron Regulon—Microarray analysis of Δmrs3Δmrs4 cells showed increased transcription of many Aft1p-regulated genes, confirming previous work (1, 2) (Table II). These data were further supported by

### Table II

| Gene | Δmrs3Δmrs4/wild type | Δmrs3Δmrs4Δccc1/wild type |
|------|----------------------|---------------------------|
| FIT2 | 9.532 ± 1.068        | 1.193 ± 0.507             |
| FIT3 | 9.413 ± 1.175        | 0.765 ± 0.421             |
| ARN3 | 5.335 ± 0.409        | 1.458 ± 0.547             |
| FET3 | 4.699 ± 0.425        | 0.978 ± 0.495             |
| FET4 | 4.509 ± 0.285        | 0.705 ± 0.649             |
| FRE1 | 4.082 ± 0.332        | 1.374 ± 0.233             |
| FTR1 | 3.462 ± 0.301        | 0.834 ± 0.701             |
| ARN2 | 3.184 ± 0.766        | 0.026 ± 0.005             |
| VMR1 | 2.736 ± 0.537        | 0.833 ± 0.045             |
| FRE3 | 2.664 ± 0.579        | 0.547 ± 0.587             |
| COT1 | 2.459 ± 0.500        | 1.441 ± 0.191             |
| ARN1 | 2.192 ± 0.541        | 0.072 ± 0.012             |
| CCC2 | 2.145 ± 0.403        | 0.095 ± 0.013             |
| CTR1 | 1.640 ± 0.355        | 0.912 ± 0.218             |

Fig. 3. Deletion of CCC1 restores iron homeostasis in Δmrs3Δmrs4 cells. A, cells (wild type, Δmrs3Δmrs4, Δccc1, Δmrs3Δmrs4Δccc1) transformed with aFET3LacZ-containing plasmid. Cells were transformed, and β-galactosidase activity was assayed by measuring 59Fe uptake. Ascorbate (1.0 mM) was added to bypass the ferrireductase. C, cellular iron content was determined by atomic absorption spectroscopy, as was vacuolar iron content (D) (note the difference in scale between C and D).
Northern analysis, which showed that Δmrs3Δmrs4 cells have increased levels of FET3 mRNA (data not shown) as well as increased expression of a reporter construct driven by the FET3 promoter (Fig. 3A). The increased expression of the iron regulon resulted in increased iron transport (Fig. 3B) and increased cellular iron (Fig. 3C).

To determine how deletion of MRS3 and MRS4 leads to increased cellular iron accumulation, we first examined the subcellular location of the accumulated iron. We predicted that if cellular iron couldn’t enter the mitochondria due to deletion of the putative iron transporters Mrs3p and Mrs4p, then it might be stored in an organelle other than the mitochondria. Storage of iron would make it unavailable to the iron-sensing transcription factor Aft1p. This could occur through either a direct reduction in cytosolic iron or by affecting the synthesis of iron-sulfur clusters in mitochondria, as the level of iron-sulfur clusters appears to regulate transcription of the iron-regulon (16). Our previous studies demonstrated that yeast store iron in the vacuole and that Ccc1p is a vacuolar protein that transports iron from the cytosol to the vacuole (15). Vacuoles isolated from Δmrs3Δmrs4 cells showed an increase in iron content compared with vacuoles from wild type cells (Fig. 3D). Deletion of CCC1 in a Δmrs3Δmrs4 strain (Δmrs3Δmrs4Δccc1) reduced all of the increased parameters of cellular iron homeostasis, including FET3-LacZ reporter activity (Fig. 3A), cell surface iron transport (Fig. 3B), whole cell iron accumulation (Fig. 3C), and vacuolar iron accumulation (Fig. 3D). Transcript analysis showed that deletion of CCC1 in Δmrs3Δmrs4 cells affects not only transcription of FET3 but other members of the iron regulon as well (Table II). Although all of the measures of iron accumulation are reduced, we note that the extent of the reduction varied for the different measures.

These results suggest that increased transcription of the iron regulon in Δmrs3Δmrs4 cells resulted in part from the increased activity of Ccc1p. We have shown that when CCC1 is overexpressed, vacuolar iron is increased, resulting in a decrease in cytosolic iron (10, 15). Overexpression of CCC1 leads to decreased mitochondrial aconitase activity (3), suggesting that reduced cytosolic iron affects mitochondrial iron uptake and the synthesis of iron-sulfur clusters. Based on these observations, we hypothesized that the increased mitochondrial iron accumulation seen in Δmrs3Δmrs4 cells grown in iron-replete media (1) resulted from an effect on Ccc1p activity. To test this hypothesis, we examined aconitase activity in a Δmrs3Δmrs4Δccc1 strain. We confirmed a previous report (1) showing that Δmrs3Δmrs4 cells have reduced aconitase activity (Fig. 4A). Decreased aconitase activity resulted from an effect of iron deprivation on iron-sulfur cluster synthesis in mitochondria. In a Δmrs3Δmrs4Δccc1 strain there was a return of aconitase activity to near normal levels. Incubation of Δmrs3Δmrs4Δccc1 cells in high iron medium does not lead to the recovery of aconitase activity, whereas incubation of Δmrs3Δmrs4Δccc1 cells in high iron medium leads to supernormal aconitase activity (Fig. 4B). These results indicate that activated Ccc1p can deplete cytosolic iron even when cells are incubated in high iron medium and that loss of Ccc1p permits mitochondria to acquire iron even in the absence of Mrs3p and Mrs4p.

Increased Ccc1p activity lowers cytosolic iron by sequestering iron in the vacuole. One consequence of decreased cytosolic iron is activation of the iron regulon, leading to increased iron transport across the plasma membrane as well as increased iron transport from the vacuole to the cytosol. Both the high affinity cell surface iron transporter system (Fet3p/Ftr1p) and the vacuolar iron export systems (Fet5p/Fth1p and Smf3p) are regulated by the iron-sensitive transcription factor Aft1p (17). Thus, the increased activity of Ccc1p in depleting cytosolic iron should be partially offset by the increased export of iron from
Deletion of AFT1 in a Δmrs3Δmrs4 strain should, therefore, lead to profound iron deprivation. This prediction was confirmed, as Δmrs3Δmrs4Δaft1 cells require high iron for growth (Fig. 5A) and show a profound decrease in mitochondrial aconitase activity (Fig. 5B).

Deletion of CCC1 Restores Transition Metal Sensitivity in Δmrs3Δmrs4 Cells—A number of plate phenotypes have been reported for Δmrs3Δmrs4 cells (1). We confirmed that the Δmrs3Δmrs4 strain shows increased sensitivity to Cd2+ and Cu2+ and decreased sensitivity to Co2+ (Fig. 6). All of these metal sensitivities showed a greater (copper, Co2+) or lesser (Cd2+) reversion to the wild type phenotype when CCC1 was deleted. Furthermore, transformation of Δmrs3Δmrs4Δccc1 cells with a low copy CCC1 plasmid reverted the phenotype toward that of the Δmrs3Δmrs4 strain. These results indicate that disruption of transition metal homeostasis in the Δmrs3Δmrs4 strain is due to Ccc1p.

We explored the basis of the increased copper sensitivity shown by the Δmrs3Δmrs4 strain. Measurement of cell-associated copper in Δmrs3Δmrs4 cells by atomic absorption spectroscopy showed a 2-fold increase in copper concentration (data not shown). Normally, increased cytosolic copper activates the copper-sensing transcription factor Ace1p, resulting in the synthesis of the metallothionein genes CUP1–1 and CUP1–2 (for review see Ref. 18). Metallothionein binds cytosolic copper and prevents copper toxicity. Examination of Ace1p activity in Δmrs3Δmrs4 cells transformed with a CUP1-LacZ reporter construct showed that Δmrs3Δmrs4 cells have lower than normal levels of β-galactosidase activity, suggesting that cytosolic copper levels are low (Fig. 7A). This result was confirmed using cells transformed with a reporter construct driven by the promoter of the high affinity copper transporter CTR1. When cytosolic copper is low, transcription of CTR1 is increased by the Mac1p copper-sensing transcription factor (19, 20). Δmrs3Δmrs4 cells show increased expression of a CTR1-LacZ reporter construct, confirming reduced cytosolic copper levels (Fig. 7B). Deletion of CCC1 in Δmrs3Δmrs4 cells returns copper homeostasis to near normal levels. Overexpression of CCC1 in wild type cells increases the expression of CTR1 reporter constructs, confirming Ccc1p alters copper homeostasis (Fig. 7C).

Changes in Copper Metabolism Result from Ccc1p Activation of Aft1p—One interpretation of our results is that Ccc1p is a copper transporter, and decreased cytosolic copper results from an increase in vacuolar copper. Although we observed an increase in vacuolar copper in Δmrs3Δmrs4 cells, increased copper was found in other membrane fractions such as mitochondria and Golgi (data not shown). We took a genetic approach to define the mechanism by which CCC1 alters copper homeostasis. We transformed Δmrs3Δmrs4 cells with a transposon library, selecting for mutations that permit growth on high copper. One of the mutants that grew on high copper contained a transposon, which destroyed the open reading frame of AFT1. Deletion of AFT1 in a Δmrs3Δmrs4 strain resulted in increased copper resistance (Fig. 8A). Deletion of AFT1 also abrogated the Co2+ resistance in wild type and Δmrs3Δmrs4 cells, confirming a recent report that Co2+ resistance was due to the expression of Aft1p-regulated genes (21). Transformation of wild type cells with an AFT1-1′′ allele results in increased expression of the CTR1-LacZ reporter construct (Fig. 8B) and increased toxicity to high copper (Fig. 8C). To determine the
relationship between AFT1, CCC1, and copper homeostasis, we overexpressed CCC1 in a Δaft1 strain. As expected, overexpression of CCC1 increases CTR1 reporter activity in wild type cells. Expression of CCC1 in Δaft1 cells reduces the expression of the CTR1 reporter construct to near normal levels (Fig. 9A). This result suggests that the Ccc1p effect on copper homeostasis requires the expression of an Aft1p-regulated gene. To test this hypothesis we expressed a constitutive AFT1-1up allele in both wild type and Δccc1 strains. As expected, the AFT1-1up allele led to an increase in CTR1 reporter activity. Deletion of CCC1 in AFT1-1up cells did not affect the expression of CTR1-lacZ activity (Fig. 9B). Overexpression of CCC1 had no more effect than did the presence of the AFT1-1up allele (Fig. 9C). Collectively, these results suggest that Ccc1p is not a vacuolar copper transporter and that changes in copper metabolism result from Ccc1p-induced activation of Aft1p.

Deletion of CCC1 Does Not Restore the Low Iron Growth Defect of Δmrs3Δmrs4 Cells—Although deletion of CCC1 in Δmrs3Δmrs4 cells restores copper, cadmium, and cobalt sensitivities to near wild type levels, it does not permit cells to grow on low iron medium; the low iron defect seen in Δmrs3Δmrs4 cells is independent of vacuolar iron accumulation. The low iron defect due to the absence of MRS3 and MRS4 results from impaired mitochondrial activity, specifically reduced heme biosynthesis. The addition of Tween, methionine, and ergosterol to low iron media permits the growth of Δmrs3Δmrs4Δccc1 cells (Fig. 10). These additives supply metabolites that are the products of heme-based enzymes and which are missing in heme-deficient cells (22). Bypassing the heme requirement permits Δmrs3Δmrs4 cells to grow on low iron medium. Based on these results, we conclude that in low iron medium the absence of MRS3 and MRS4 leads to a reduction in mitochondrial heme production or export.

**DISCUSSION**

Mutants have been identified that affect mitochondrial metal metabolism, but the specific biochemical function of the mutant or normal allele is unclear. This is particularly true for members of the mitochondrial carrier family. Many members of this family mediate anion transport across the mitochondrial membrane. The family includes citrate transporters, ATP/ADP exchangers, and succinate/fumarate exchangers (for review, see Ref. 23). Deletion of these genes leads to marked changes in mitochondrial metal homeostasis. For example, deletion of the mitochondrial carrier YHM1 leads to mitochondrial iron accumulation (24), yet Yhm1p appears to be a GTP/GDP exchanger (25). It is not clear how defects in mitochondrial GTP levels would lead to increased mitochondrial iron accumulation. Deletion of two highly homologous members of the mitochondrial carrier family, MRS3 and MRS4, was shown to affect mitochondrial and cellular metal homeostasis. Foury and Rogati (1) show that deletion of these genes limited mitochondrial iron accumulation, leading to the hypothesis that these genes encode mitochondrial iron transporters. This hypothesis was supported by Muhlenhoff *et al.* (2), who assayed mitochondrial heme and Fe-S synthesis in both Δmrs3Δmrs4 cells and in cells

![Deletion of AFT1 affects copper and cobalt homeostasis in Δmrs3Δmrs4 cells.](https://example.com/deletion_aft1_copper_cobalt_homeostasis.png)
that overexpressed MRS3 or MRS4. Their data are consistent with Mrs3p and Mrs4p functioning as mitochondrial iron transporters whose role only becomes apparent when cellular iron is limiting. However, protection of transporters whose role only becomes apparent when cellular iron levels, is not easy to reconcile with Mrs3p and Mrs4p functioning as mitochondrial iron transporters. Other phenotypes shown by Δmrs3Δmrs4 cells, such as changes in copper and cobalt sensitivity and increased iron transport, are not easily ascribed to changes in mitochondrial iron transport, particularly when cells are incubated in iron-replete media.

Plants and fungi, as opposed to other eucaryotes, store iron in the vacuole, as they do not synthesize cytosolic ferritin. Ccc1p was identified in two separate yeast genetic screens as a vacuolar iron and manganese transporter. Increased expression of CCC1 lowered the cytosolic concentration of both metals (15, 26). We suggest that deletion of MRS3/MRS4 affects Ccc1p activity leading to cytosolic iron depletion and that cytosolic iron depletion is responsible for most of the phenotypes seen in Δmrs3Δmrs4 cells (see model Fig. 11). There are two major lines of evidence which suggest that deletion of MRS3/MRS4 increases Ccc1p activity. The first line of evidence is that overexpression of CCC1 mimics many of the phenotypes seen in Δmrs3Δmrs4 cells. We discovered Ccc1p as a high copy suppressor able to prevent excessive mitochondrial iron accumulation in Δyfh1 cells (10). A prerequisite for mitochondrial iron accumulation in Δyfh1 cells is high cytosolic iron (27, 28). Increased expression of CCC1 results in increased vacuolar iron storage, lowering cytosolic iron. Because cytosolic iron is the immediate precursor pool for mitochondrial iron acquisition, decreased cytosolic iron leads to decreased mitochondrial iron and prevents iron accumulation in Δyfh1 mitochondria (10). Overexpression of CCC1 leads to increased cell surface iron transport and decreased mitochondrial aconitase activity (3, 10).

The second line of evidence that supports an effect of MRS3/MRS4 deletion on Ccc1p activity is that deletion of CCC1 in Δmrs3Δmrs4 cells restores iron and metal homeostasis to near normal values. We note that deletion of CCC1 does not restore all of the measures of iron homeostasis in Δmrs3Δmrs4 cells to normal, suggesting that deletion of MRS3/MRS4 may affect other (vacuolar/cell surface) transporters in addition to Ccc1p. We do not know what metabolite(s) is generated in Δmrs3Δmrs4 cells or how it affects Ccc1p. We do know that neither CCC1 mRNA nor protein levels are increased. We think that a specific metabolite alters the activity of Ccc1p because we have been unable to alter its activity by conditions that lead to increased cellular iron (e.g., high media iron) nor does increased cellular iron lead to the phenotypes seen in Δmrs3Δmrs4 cells.
Further evidence which suggests that Mrs3p and Mrs4p have effects that cannot simply be related to mitochondrial iron accumulation is that overexpression of Mmt1p and Mmt2p, other mitochondrial metal transporters, does not protect cells against iron toxicity or nor do they activate Ccp1p. Indeed, Muhlenhoff et al. (2) show that overexpression of MRS3 or MRS4 stimulated iron-sulfur cluster synthesis and that this stimulation could not be accounted for by increased iron transport. Their explanation for this stimulatory activity was that Mrs3p or Mrs4p may transport molecules other than ferrous iron.

Our data show that many of the phenotypes in Δmrs3Δmrs4 cells result from downstream consequences of reduced cytosolic iron, which activates the iron-sensitive transcription factor Aft1p. Increased cobalt resistance and decreased copper resistance both result from activation of Aft1p. Both metal sensitivities are returned to normal when either mrs3 or mrs4 deletions are required to see a change in phenotype. Our data suggest that these target genes lead to reduced cytosolic copper and increased membrane-associated copper. It is this combination that prevents copper sequestration by metallothioneins, resulting in increased toxicity. Our studies suggest that iron can affect the expression of genes regulated by copper, as deletion of CCCI in cells with an active Aft1p results in decreased cytosolic copper. Recently, Gross et al. (29) show that high copper induced the iron regulon and that this effect required the copper-sensing transcription factor Ace1p. We speculate that when Aft1p is activated, particularly in the presence of iron, a protein encoded by an Aft1p-regulated gene transports copper into membrane compartments, leading to copper-deprived cytosol.

Our data are not discrepant with a role for Mrs3p and Mrs4p in mitochondrial iron transport. Deletion of CCCI does not suppress the inability of Δmrs3Δmrs4 cells to grow on low iron, consistent with the possibility that Mrs3p and Mrs4p may be mitochondrial iron transporters active under low iron conditions as previously suggested (1, 2). The addition of Tween, ergosterol, and methionine, products of heme containing enzymes, suppresses the low iron growth defect resulting from deletion of MRS3 and MRS4. We think that in Δmrs3Δmrs4 cells activation of Ccp1p lowers cytosolic iron, exacerbating the mitochondrial defect. We cannot rule out the possibility that deletion of MRS3 and MRS4 affects vacuolar transporters other than Ccp1p, and activation of these transporters exacerbates the low iron growth defect of Δmrs3Δmrs4 cells.

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