A Role for the Saccharomyces cerevisiae ATX1 Gene in Copper Trafficking and Iron Transport*

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The ATX1 gene of Saccharomyces cerevisiae was originally identified as a multi-copy suppressor of oxidative damage in yeast lacking superoxide dismutase. We now provide evidence that Atx1p helps deliver copper to the copper requiring oxidase Fet3p involved in iron uptake. atx1Δ null mutants are iron-deficient and are defective in the high affinity uptake of iron. These defects due to ATX1 inactivation are rescued by copper treatment, and the same has been reported for strains lacking either the cell surface copper transporter, Ctr1p, or the puta-
tive copper transporter in the secretory pathway, Ccc2p. Atx1p localizes to the cytosol, and our studies indicate that it functions as a carrier for copper that delivers the metal from the cell surface Ctr1p to Ccc2p and then to Fet3p within the secretory pathway. The iron deficiency of atx1Δ mutants is augmented by mutations in END3 blocking endocytosis, suggesting that a parallel pathway for intracellular copper trafficking is mediated by endocytosis. As additional evidence for the role of Atx1p in iron metabolism, we find that the gene is induced by the same iron-sensing trans-activator, Aft1p, that regulates CCC2 and FET3.

The yeast Saccharomyces cerevisiae represents an excellent model system for the study of heavy metal metabolism. In the past few years, several genes involved in the transport and trafficking of copper and iron have been identified in this yeast. CTR1 encodes a high affinity transporter for copper ions (1, 2). Once copper enters the cell via Ctr1p, it can be transported to various distinct cellular locations. First copper can be transported to the mitochondria where the metal is needed to activate cytochrome oxidase. This pathway requires both Ctr1p and a small cystolic protein, Cox17p (2, 3). Secondly, copper is needed to activate cystolic copper-binding proteins such as the copper and zinc requiring superoxide dismutase (Sod1p). Accordingly, ctri mutants are defective in Sod1p activity (2). Ctr1p is also necessary for efficient copper-regulated expression of the Cup1 metallothionein, and thus may be needed for copper transport to the nucleus (2). Additionally, Ctr1p is involved in the delivery of copper ions to the secretory pathway. One protein requiring this mode of copper delivery is Fet3p, a multi-copper oxidase that is needed for high affinity iron uptake (1, 4–6). The copper-loaded Fet3p functions as part of a complex with the iron permease Ftr1p to mediate iron uptake at the plasma membrane (7, 8). Because of this requirement for copper-Fet3p in iron transport, cells lacking Ctr1p are defective in iron uptake (1, 4).

An additional component of the copper delivery pathway to Fet3p is CCC2 (5, 9), the yeast homologue of the human Wilson (10–12) and Menkes (13–15) genes. Like its mammalian counterparts, Ccc2p possesses sequences common to P-type ATPases and also copies of the conserved copper-binding motif MTCXXC (9). These reputed ATPases of yeast and man are believed to function in the translocation of copper across intra- cellular membranes into the secretory pathway. Indeed, the Menkes protein has been localized by Gitlin and co-workers to a compartment in the Golgi (16). Although CCC2 functions in copper delivery, the CCC2 transcript falls under the transcriptional control of the iron-responsive trans-activator Aft1p (17, 18). This is consistent with the role of Ccc2p in copper delivery to Fet3p involved in iron uptake. By contrast, CTR1 is involved in global copper trafficking and is regulated by the copper-responsive activator, Mac1p.1

The mechanism(s) by which Ccc2p and the Menkes/Wilson gene products obtain their copper for translocation into the secretory pathway has been unknown. By virtue of its cytotoxic nature, copper is unlikely to exist free in the cystosol. One hypothesis is that a cytosolic carrier for copper delivers the metal to the ATPase for transport into the secretory pathway. The studies presented here focus on one likely candidate for such a copper carrier: the small copper homeostasis factor, Atx1p.

We originally isolated ATX1 (anti-oxidant) as a multi-copy suppressor of oxygen toxicity in yeast devoid of copper/zinc superoxide dismutase (Sod1p) (19). Cells lacking Sod1p exhibit a number of metabolic defects when grown in air, including aerobic auxotrophies for lysine and methionine (20–23). Overexpression of ATX1 suppresses all the Sod1p-linked defects in a manner dependent upon cellular copper uptake (19). ATX1 encodes an 8.2-kDa polypeptide homologous to the MerP mercury transport proteins of bacteria (19). Close homologues to ATX1 were also identified in Caenorhabditis elegans, and more recently, in humans (33). In each case, the Atx1-like protein contains a single copy of the MTCXXC metal binding motif also found in Cce2p and the Wilson and Menkes gene products (19).

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1 A. Dancis, unpublished data.
In the present study, we provide strong evidence that yeast Atx1p is localized to the cytosol and functions in a copper trafficking pathway (Ctr1p-Atx1p-Ccc2p) mediating delivery of copper to Fet3p.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, Media, and Growth Conditions—The S. cerevisiae strain SL202 was purified by deleting the SOD1 gene of YPH250 (24) using a sod1Δ::LEU2 plasmid (25). Strains SL108 and SL214 were constructed by creating an atx1Δ mutation in A255 (26), SL202, YPH250 (24), RH144-3D, and SM2186, respectively (isogenic wild type and end3Δ mutant strains; kind gifts of S. Michaelis), using either the atx1Δ::HIS3 plasmid described below (for SL214) or the atx1Δ::LEU2 plasmid (remaining strains) described earlier (19). M2P is the AFT1Δ derivative of wild type CM360 (2). Strain 3 is a ccc2Δ mutant strain obtained from T. Dunn (5). Strains EG103 and the corresponding atx1Δ derivative SL104ΔA1 have been described (19, 23).

The pDA1-HIS atx1Δ::HIS3 deletion plasmid was constructed by mobilizing the atx1Δ deletion fragment from plasmid pDA1 (19) through digestion with XhoI and SacI, and then by inserting this fragment into the XhoI and SacI sites of the HIS3 integrating vector, pRS403 (24). p413-A1 represents an ATX1 CEN vector constructed by amplifying ATX1 sequences -397 to +800 by the polymerase chain reaction (PCR), inserting the product into the pCRII vector (Invitrogen), and subcloning the ATX1 fragment into the BamHI and XhoI sites of pRS413 (24).

To insert ATX1 into the overexpression vector pET11d, the ATX1 coding region was amplified by PCR using mutagenic primers to introduce unique restriction sites at both the 5’ (NcoI) and 3’ (BamHI) ends of the gene. The PCR product and pET11d vector were both digested with NcoI and BamHI, the desired fragments were purified from low melt agarose gels and subsequently ligated to produce the pET11d-ATX1 expression vector.

Stocks of yeast strains were maintained on a standard yeast extract-peptone-dextrose (YPD) medium (27). Iron dependence tests were carried out as described previously (2) on the indicated strains grown in enriched medium supplemented with 10 μM bathocuproine disulfonate (+BCS) or 100 μM CuSO4 (+Cu), as specified. Ferrous uptake is expressed in units (picomoles) of iron/10⁶ cells/h. Results represent the averages of three samples, where range was not more than 5%. Strains utilized are as follows: wild type, YPH250; Δatx1, SL215; Δccc2, strain 3; Δsod1, SL202; Δsod1 Δatx1 Δccc2, SL214.

In FIG. 1, the effect of atx1Δ mutations on iron dependence and on ferrous iron uptake. A, the indicated strains were grown for 5 days at 30 °C on minimal medium plates containing 3 mM ferrozine and where indicated (+Fe), the medium was also supplemented with 350 μM ferrous ammonium sulfate. B, measurements of ferrous iron uptake were carried out as described previously (2) on the indicated strains grown in enriched medium supplemented with 10 μM bathocuproine disulfonate (+Bcs) or 100 μM CuSO4 (+Cu), as specified. Ferrous uptake is expressed in units (picomoles) of iron/10⁶ cells/h, as indicated. Measurements of ferrous iron uptake were conducted in triplicate samples essentially as described (1). All yeast transformations were carried out by electroporation (28). Transforms of strains containing sod1Δ mutations required initial cultivation in anaerobic culture jars (23).

Preparation of an Anti-Atx1p Antibody—Atx1p was purified from an E. coli strain carrying a T7 expression vector containing the ATX1 gene. Complete details regarding Atx1p overexpression and purification will be published later. The purified Atx1p was judged to be greater than 95% homogeneous by SDS-PAGE and further analyzed by electrospray ionization-mass spectrometry to confirm the size of the intact protein.

The purified Atx1p was used to produce anti-Atx1p antibodies in rabbits by Cocalico Biologicals, Inc. The antibody was purified using an ImmunoPure IgG (Protein A) purification kit, according to manufacturer’s specifications (Pierce).

Yeast Fractionation and Immunofluorescence Microscopy—For Western blot analysis, yeast cells were initially grown overnight in selecting SD medium to confluence, then diluted in 50 ml of SD medium to an A600 nm of 0.3 and allowed to grow for an additional 6 h. Cells were then broken by glass bead homogenization in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM each of EDTA and EGTA, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. Extracts were subjected to 15% SDS-PAGE and Western analysis using the purified anti-Atx1p IgG at a 1:500 dilution. The secondary antibody consisted of anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) diluted 1:10,000. Detection employed the ECL kit (Amersham), according to manufacturer’s specifications. For resolution of membrane and soluble components, extracts were prepared by osmotic shock as described previously (29), and subjected to centrifugation at 100,000 × g in a Beckman 50 Ti-50 rotor. The pellets were resuspended in lysis buffer to the same volume, and all samples were prepared for SDS-PAGE and Western blot analysis, as above.

For detection of Atx1p by immunofluorescence, the atx1Δ strain

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1 The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole.

2 The effect of atx1Δ mutations on iron dependence and on ferrous iron uptake. A, the indicated strains were grown for 5 days at 30 °C on minimal medium plates containing 3 mM ferrozine and where indicated (+Fe), the medium was also supplemented with 350 μM ferrous ammonium sulfate. B, measurements of ferrous iron uptake were carried out as described previously (2) on the indicated strains grown in enriched medium supplemented with 10 μM bathocuproine disulfonate (+Bcs) or 100 μM CuSO4 (+Cu), as specified. Ferrous uptake is expressed in units (picomoles) of iron/10⁶ cells/h, as indicated. Measurements of ferrous iron uptake were conducted in triplicate samples essentially as described (1). All yeast transformations were carried out by electroporation (28). Transforms of strains containing sod1Δ mutations required initial cultivation in anaerobic culture jars (23).

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SL108 transformed with pRS-A1 (2 µ ATX1) was grown in 10 ml of selecting SD medium to a final A600 of 1.0. Cells were fixed with formaldehyde, permeabilized by zymolyase treatment, and prepared for antibody staining (30). Incubation with rabbit anti-Atx1p (diluted 1:250) proceeded for 2 h. Detection was carried out with a fluorescein isothiocyanate (FITC)-labeling kit (Boehringer Mannheim) and utilized a goat anti-rabbit secondary antibody (diluted 1:500) coupled to FITC. Nucleic acids were stained by DAPI (Sigma), and FITC and DAPI staining was monitored by fluorescence microscopy.

RESULTS

Cells Lacking a Functional ATX1 Gene Are Defective in Iron Uptake—The CTR1 and CCC2 genes of yeast are involved in distinct copper transport steps (1, 5). Mutants at these loci are defective in the high affinity uptake of ferrous iron (1, 5) and require iron supplemented medium for growth. To study whether ATX1 participates in the same pathway of copper trafficking, we tested the effects of an atx1Δ gene deletion on iron dependent growth. An isogenic pair of atx1A and wild type strains was plated onto a minimal medium prepared with the iron-specific chelator ferrozine (31, 32), and growth was monitored. As shown in Fig. 1A, the atx1Δ strain exhibited no growth on this medium, and this defect was reversed by transforming the mutant with a CEN vector harboring wild type ATX1. The growth inhibition of atx1Δ strains on ferrozine was also completely reversed by supplementation with iron concentrations sufficient to reverse the effects of the iron chelator (Fig. 1A).

We also obtained direct measurements of ferrous iron uptake. As shown in Fig. 1B, iron uptake in a wild type strain was reduced by treatment with the BCS copper-specific chelator, confirming the copper dependence of high affinity iron transport (1, 4, 5). Consistent with previous results (5), ferrous iron uptake was undetectable in a strain containing a ccc2 Δ gene deletion and this deficiency was completely rescued by supplementation with copper (Fig. 1B). The atx1Δ strain also exhibited a reduction in iron uptake that was corrected by copper. However, the atx1Δ mutation resulted in an incomplete blockage (65–70% inhibition) of iron uptake, compared with the total inhibition of transport observed with ccc2Δ strains (Fig. 1B).

Nevertheless, the effects of atx1 mutations on the uptake and dependence on iron suggested that Atx1p operates in the same pathway as Ctr1p and Ccc2p to deliver copper to Fet3p. This notion is confirmed in the accompanying paper, where an atx1 mutant strain is shown to be deficient in the production of copper-Fet3p (33).

Cellular Localization of Atx1—To understand further how ATX1 functions in copper metabolism, we examined the intracellular localization of the encoded polypeptide. Atx1p is a small protein that lacks transmembrane domains or other localization signals (19), suggesting that it may be cytosolic.

To monitor Atx1p production, we prepared anti-Atx1p antibodies. Purified Atx1p protein was obtained following expression in E. coli and used to produce polyclonal rabbit antibodies. The efficacy of this anti-Atx1 was then assessed by Western blot. As seen in Fig. 2A, a single major band exhibiting an apparent molecular mass of 7.0 kDa was visualized from a strain harboring ATX1 on a 2 µ plasmid, but was absent in the isogenic atx1Δ strain. Atx1p produced from a CEN vector or the single-copy chromosomal gene could also be visualized (Fig. 2B), but yielded weak signals, suggesting that ATX1 may be normally expressed at low levels in yeast. In all cases, Atx1p migrated slightly faster on SDS gels than would be expected from its predicted molecular mass of 8.2 kDa, and this has also been observed with purified Atx1p produced in E. coli. Overexpression of Atx1p in E. coli resulted in a full-length protein lacking its N-terminal methionine as determined by electrospray ionization-mass spectrometry.4

A cell fractionation experiment was conducted to test whether Atx1p is a soluble cytosolic protein. Extracts were prepared by gentle lysis as described (29) from cells harboring ATX1 on a CEN vector and were subjected to high speed centrifugation to resolve soluble and membrane-associated cellular constituents. As a control for these experiments, we monitored fractionation of the Atx2-HA protein, which is known to localize to vesicles in the secretory pathway (29). As seen in Fig. 2C, Atx1p was totally recovered in the supernatant following a 100,000 × g centrifugation, whereas this same treatment caused precipitation of the Golgi-localized Atx2-HA protein. The Golgi protein was only solubilized upon treatment of extracts with Triton X-100 (Fig. 2C). This fractionation study demonstrated that Atx1p is a soluble protein and is most likely cytosolic.

Indirect immunofluorescence microscopy was used to study Atx1p localization further. Cells harboring the ATX1 2 µ plasmid were fixed and permeabilized, and were probed with the rabbit anti-Atx1 antibody and a secondary anti-rabbit antibody conjugated to FITC. By FITC staining, Atx1p exhibited uni-

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form staining throughout the cytosol and was excluded from the nucleus, as defined by co-staining with DAPI (Fig. 3). Atx1p produced from the CEN vector showed a similar pattern of staining, although the immunofluorescence signal was much weaker in this case (data not shown). Our immunofluorescence experiments together with the cell fractionation study strongly indicated that Atx1p is a cytosolic protein. The complete absence of Atx1p in the nucleus rules against a possible role for the protein as a copper-transcription factor.

The Interacting Roles of Ccc2p, Atx1p, and Endocytosis in Copper Trafficking—The localization of Atx1p to the cytosol suggested that this protein may be involved in the cytosolic trafficking of the metal to Ccc2p and then to Fet3p. As an alternative scenario, Atx1p and Ccc2p could act in parallel, redundant pathways to separately deliver copper to Fet3p. If this latter possibility were true, Ccc2p and Atx1p should cross-compensate for one another. We therefore tested whether multi-copy CCC2 and ATX1 could suppress the effects of an atx1Δ and ccc2Δ null mutation, respectively. As seen in Fig. 4B, multi-copy ATX1 was incapable of suppressing the iron deficiency of a ccc2 mutant, even though the Atx1p protein was clearly overproduced under these conditions (Fig. 2 and Ref. 19). This result indicated that Atx1p does not function in parallel with Ccc2p. However, in contrast to results obtained with multi-copy ATX1, overexpressed CCC2 was capable of suppressing the iron dependence of an atx1Δ mutant (Fig. 4A). The ability of multi-copy CCC2 to compensate for ATX1 (but not the converse) supports the notion that Ccc2p functions downstream of Atx1p. These studies also show that an Atx1p-independent pathway can substitute for Atx1p when Ccc2p is overproduced.

Additional evidence for the Atx1p-independent pathway of copper transport was obtained through iron uptake studies. As seen in Fig. 1B, ferrous iron uptake was reduced in the atx1Δ deletion strain, in comparison to the total elimination of iron transport associated with a deletion of CCC2. This partial effect of atx1 mutations was also seen in a growth test for iron dependence. atx1Δ strains exhibited no growth on ferrozine-containing medium that was not supplemented with additional iron (Fig. 1A). However, the addition of 50 μM ferrous iron to the ferrozine medium supported growth of the atx1Δ strain, but not of the ccc2Δ mutant (Fig. 5). These observations suggested that a “back up” system for copper delivery exists in atx1Δ mutants. Possible candidates for this auxiliary system included Sod1p and the Cup1p and Crs5p metallothioneins, as all three are small soluble copper-binding proteins (34–36). However, we observed that ferrous iron uptake was not eliminated in an atx1Δ strain also containing mutations in SOD1 (Fig. 1B) or in a strain containing triple deletions in ATX1, CUP1, and CRS5 (data not shown). Thus the metallothioneins and Sod1p do not aid in copper delivery to Fet3p.

Endocytosis is another method by which copper could be delivered to intracellular locations. We therefore tested the role of endocytosis as the secondary means of trafficking copper in atx1Δ strains. These studies utilized a temperature-sensitive end3 mutation known to block endocytosis at 37 °C (37). end3 mutants are defective in both receptor-mediated and fluid-phase endocytosis, yet other vesicle-mediated processes are not affected (37). A temperature-sensitive mutation in END3 did not cause growth inhibition on the low iron medium (Fig. 5), consistent with other studies showing that end3 mutants are not defective in high affinity ferrous iron uptake. However, at the non-permissive temperature, this end3 mutation did enhance the iron deficiency of an atx1Δ strain. At 37 °C, but not at 25 °C, an atx1Δ end3 double mutant exhibited the same iron dependence of a ccc2 mutant and showed no growth on ferrozine medium supplemented with only 50 μM ferrous ammonium sulfate (Fig. 5). Growth of the atx1Δ end3 double mutant was stimulated by 350 μM ferrous ammonium sulfate and also by 500 μM CuSO4 (Fig. 5), indicating that iron deficiency resulted from inadequate copper delivery to Ccc2p and Fet3p. Hence, the apparent residual trafficking of copper to Fet3p in atx1 mutants involves endocytosis.

Fig. 4. The effects of multi-copy ATX1 and CCC2 on the iron dependence of ccc2 and atx1 mutants. The indicated strains were grown on ferrozine-containing medium as described in Fig. 1A (A) or on medium containing 1 mM ferrozine supplemented with 50 μM ferrous ammonium sulfate (B); +Fe, medium supplemented with 350 μM ferrous ammonium sulfate. Strains utilized are as described in Fig. 1. 2p CCC2 and Δccc2, 2μ ATX1, strains transformed with plasmids B2 (kind gift, T. Dunn, Ref. 9) and pRS-A1, respectively.

Fig. 5. The effects of combining end3 and atx1 mutations on iron-dependent growth. The indicated strains were plated on minimal medium prepared with 1 mM ferrozine and 50 μM ferrous ammonium sulfate (top), with the same medium supplemented with either 350 μM ferrous ammonium sulfate (middle), or with 50 μM ferrous ammonium sulfate plus 500 μM CuSO4 (bottom). Cells were grown for 3 days at the indicated temperatures. Shown (clockwise) are strains SL216 (Δatx1), SM2186 (end3Δ), SL217 (end3Δ Δatx1), YPH250 (wild type), strain 3 (Δccc2), and RH114–3D (wild type; isogenic to end3Δ and atx1Δ strains).
Regulation of ATX1 Gene Expression—The genes required for high affinity ferrous iron uptake in yeast fall under the control of the iron-sensing trans-activator, Aft1p (17, 18). Included in this list of iron regulated genes are FRE1 (encoding ferric reductase; Ref. 38), FTR1 (iron permease), FET3, and CCC2 (17). In comparison, CTR1, which is involved in global copper homeostasis, is regulated by Mac1p (39), a copper-sensing trans-activator. Upon observation of ATX1 upstream sequences we noted a single Aft1p consensus sequence TG-CACCC at nucleotides −110 to −116 (Fig. 6A). However, possible recognition sequences for the Mac1p trans-activator could not be found. This observation suggested that ATX1 may fall under the Aft1p regulon.

Northern blot analysis was used to test the regulation of ATX1 by copper and iron. For studies on copper, ATX1 expression was monitored in strains treated with the copper chelator bathocuproine sulfonate under conditions known to induce the CTR1 and CTR3 copper transport genes (2, 40). We also tested the effects of null and hyper-active alleles of MAC1 (39) on ATX1 mRNA levels. However, in all cases, ATX1 gene expression remained unaffected by copper depletion or by mutations in MAC1 (data not shown), indicating that ATX1 is not copper-regulated. To examine the possible regulation by iron and Aft1p, ATX1 gene expression was monitored in strains containing either an aft1 null mutation or a hyper-active allele of AFT1 (AFT1-1; Ref. 18). As shown in Fig. 6B, ATX1 mRNA was up-regulated in a strain containing the hyper-active AFT1-1 allele, as was FET3 mRNA known to be a target for Aft1p-regulation (17). Densitometric tracings revealed that the induced level of ATX1 and FET3 mRNA in the AFT1-1 allele compared with the isogenic wild type was 3.0- and 3.6-fold, respectively. Surprisingly however, the basal level of ATX1 gene expression was unaffected in strains containing an aft1Δ null mutation (Fig. 6B) or in wild type strains treated with repressing concentrations of iron that inactivate Aft1p (data not shown; Ref. 17). In comparison, FET3 expression was virtually eliminated in the aft1Δ strain (Fig. 6B). CCC2 mRNA levels are also known to be markedly decreased in an aft1Δ strain (17). Hence unlike CCC2 and FET3, ATX1 is not absolutely dependent on Aft1p, and other trans-acting factors must be involved in ATX1 regulation.

**DISCUSSION**

In bakers’ yeast, multiple pathways of copper trafficking emerge from the plasma membrane copper transporter, Ctr1p. Copper taken up by Ctr1p is delivered to the mitochondria where the metal is needed for electron transport, to a compartment needed for activation of cytosolic copper proteins (such as Sod1p), and to the secretory pathway (1, 2, 4, 5, 7, 8). The studies presented herein describe a small copper carrier, Atx1p, that functions in the Ctr1 pathway that delivers copper to secretory compartments.

Several lines of evidence support a role for Atx1p in the trafficking of copper from Ctr1p to the secretory pathway. Like the copper transporters Ctr1p and Ccc2p, Atx1p is required for activation of Fet3p, a multi-copper oxidase involved in iron transport (1, 4, 5, 8). Strains containing mutations in ATX1 are deficient in ferrous iron uptake and show iron-dependent growth. Furthermore, experiments in the accompanying paper (33) demonstrate that atx1Δ mutants are defective in production of copper-Fet3p, in vivo. Our localization of Atx1p to the cytosol, together with our genetic epistasis experiment, indicates that Atx1p functions as a cytosolic carrier for copper, shuttling the metal from the cell surface Ctr1p to the copper translocating ATPase, Ccc2p, in the secretory pathway. Consistent with its role as a soluble copper carrier, purified Atx1p specifically coordinates copper ions in vitro. It is noteworthy that the bacterial homologue to Atx1p, MerP, likewise functions as a small metal carrier and is believed to help shuttle mercury ions across the periplasmic space to the MerT mercury transporter in the cell membrane (41, 42).

**Ccc2p and Fet3p can also obtain copper ions in an ATX1-
independent manner. *atx1* mutants exhibited partial inhibition of ferrous iron uptake. In comparison, iron uptake was eliminated in *cct2* mutants. Our studies show that the apparent residual copper trafficking in *atx1* mutants involves endocytosis. A blockage in endocytosis together with a mutation in *atx1* conferred a dependence on iron for growth that was indistinguishable from that observed with *cct*Δ mutants. The mechanism by which endocytosis could facilitate intracellular copper transport is currently not known. One possibility is that Ctr1p vesicles are involved. Ctr1p has been shown to be internalized to vesicles by endocytosis (43). A mutation in *ATX1* only the Atx1p-dependent pathway of copper delivery, but also to vesicles by endocytosis (43). A mutation in vesicles are involved. Ctr1p has been shown to be internalized transport is currently not known. One possibility is that Ctr1p blocks not D Aft1 strain and for helpful discussions.

mutants do not exhibit residual copper trafficking in *S. cerevisiae ATX1 and Copper Trafficking* 9220

We demonstrate here that oxygen-sensing trans-activator, Yap1p (44). The regulation of genes involved in iron uptake, including the *ferrous permease, FET3*, and *CCT2* (17). We demonstrate here that *ATX1* can also fall under Aft1p control; however, unlike other Aft1p-regulated genes, *ATX1* does not show an absolute requirement for Aft1p. The bulk of *ATX1* expression is unaffected by a null mutation in *AFT1*, and another trans-activator must be responsible. Our preliminary studies suggest that Aft1p also falls under the control of the oxygen-sensing trans-activator, Yap1p (44). The regulation of *ATX1* by both iron status and oxidative stress would be consistent with the dual roles for copper-Aft1p in protection against oxygen radical toxicity and in the delivery of copper to Fet3p.

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5 S.-J. Lin and V. C. Culotta, unpublished data.
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