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Mobilization of Intracellular Copper Stores by the Ctr2 Vacuolar Copper Transporter*⑤

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Copper plays an essential role in processes including signaling to the transcription and protein trafficking machinery, oxidative phosphorylation, iron mobilization, neuropeptide maturation, and normal development. Whereas much is known about intracellular mobilization of ions such as calcium, little information is available on how eukaryotic cells mobilize intracellular copper stores. We describe a mechanism by which the *Saccharomyces cerevisiae* Ctr2 protein provides bioavailable copper via mobilization of intracellular copper stores. Whereas Ctr2 exhibits structural similarity to the Ctr1 plasma membrane copper importer, microscopic and biochemical fractionation studies localize Ctr2 to the vacuole membrane. We demonstrate that Ctr2 mobilizes vacuolar copper stores in a manner dependent on amino acid residues conserved between the Ctr1 and Ctr2 copper transport family and that *ctr2Δ* mutants hyper-accumulate vacuolar copper. Furthermore, a Ctr2 mutant that is mislocalized to the plasma membrane stimulates extracellular copper uptake, supporting a direct role for Ctr2 in copper transport across membranes. These studies identify a novel mechanism for copper mobilization and suggest that organisms cope with copper deprivation via the use of intracellular vesicular stores.

Metal ions such as calcium, copper, iron, and zinc play numerous and diverse roles in cellular physiology. Much is known about the role of calcium import from extracellular sources and mobilization from intracellular compartments in signaling processes including nutrient sensing (1), stress adaptation (2–4), cell-cycle control (5, 6), and protein processing in the endoplasmic reticulum (7). The mechanism of transcriptional regulation by calcium signaling via the calmodulin/calcineurin pathway identified in mammalian cells is conserved in yeast, suggesting that these signaling mechanisms are common to most organisms (8–10). The need to maintain adequate levels of intracellular calcium, yet keep the cytosolic concentrations of this second messenger under tight control, demands that much of the cellular calcium be sequestered until use. In yeast cells the vacuole possesses more than 95% of the total cellular calcium (11–13).

The yeast vacuole is an important and dynamic organelle that is most similar to lysosomes in mammalian cells. Vacuole function has been shown to be critical for maintenance of intracellular pH, and the vacuole also acts as a storage compartment for amino acids, metal ions, and other molecules (14). Previous studies suggest that, like calcium, the yeast vacuole is a significant storage compartment for iron and zinc (15–18). Specific mechanisms for transport of vacuolar calcium (19–21), iron (22–25), and zinc (26–28) have been identified and demonstrated to play an important role in the homeostatic regulation of these metals. However, whereas vacuole biogenesis mutants have been demonstrated to be sensitive to copper toxicity (16, 29), little is known about the mechanisms by which vacuoles participate in copper homeostasis to augment copper-dependent signaling or copper-dependent enzyme activities.

Eukaryotes from yeast to humans utilize Ctr1[1] proteins for the high affinity transport of Cu(I) across the plasma membrane (30–36). The Ctr1 high affinity copper transporters are characterized by the presence of three transmembrane domains and a series of methionine residues in both the extracellular amino terminus and the second transmembrane domain that are critical for copper transport activity (37). Intracellular copper is routed to copper-dependent enzymes in the cytosol such as Cu,Zn-superoxide dismutase, in mitochondria (cytochrome oxidase), and in the secretory pathway (for example, the Fet3 or ceruloplasmin ferroxidase in yeast and mammals, respectively) through the action of specific copper chaperone proteins (38–41). Recent experiments have demonstrated that *S. cerevisiae* cells lacking the high affinity plasma membrane transporters are capable of routing copper to both Fet3 and Cu,Zn-superoxide dismutase (42). This activity was shown to be dependent at least in part on the Fet4 plasma membrane broad-specificity copper, iron, zinc transporter, and the Ctr2 protein (42). Ctr2 was first identified based on its strong homology to Copt1, a plant copper transporter that is a member of the Ctr1 family, and suggested to be a low affinity copper importer (32). Two reports have localized an epitope-tagged form of Ctr2 and a Ctr2-GFP fusion protein to the vacuolar membrane and punctate spots, or the endoplasmic reticulum, respectively (42, 43). However, the subcellular location of Ctr2 has not been firmly established, nor has its role in copper homeostasis been clearly defined.

Here we demonstrate that *S. cerevisiae* Ctr2 is localized to the vacuole membrane where it functions to mobilize vacuolar copper stores to cytosolic copper chaperones. We show direct evidence that Ctr2 function affects vacuolar copper levels and that a *CTR2* mutant that suppresses the requirement for the

⑤ The on-line version of this article (available at http://www.jbc.org) contains Figs. S1 and S2.

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[1] The abbreviations used are: Ctr, copper transporter; GFP, green fluorescent protein; ICP-MS, inductively coupled plasma mass spectrometry; PIPES, 1,4-piperazinediethanesulfonic acid.

high affinity plasma membrane Cu(I) transporters is mislocalized to the plasma membrane and facilitates the kinetics of copper uptake into cells. Taken together, these studies identify a mechanism whereby cells mobilize intracellular copper stores to render this metal available for cellular signaling and a number of copper-dependent enzymes whose activity is essential for normal growth and development.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The *ctr1Δctr3Δctr2Δ* strain was constructed by integrating the *HIS3* gene at the *CTR2* locus in the MPY17 strain (44). Wild-type *CTR2* and *CTR2* mutant alleles were amplified from genomic DNA by PCR. Site-directed mutagenesis was performed by the overlap extension method (45). For the *CTR2-GFP* fusion, a NotI restriction enzyme site was introduced in-frame just upstream of the stop codon and the GFP open reading frame was inserted. Yeast strains were transformed with plasmids using standard techniques and grown in minimal selective media at 30 °C with agitation.

Functional Complementation of ctr2 Mutants—*ctr1Δctr3Δctr2Δ* cells were transformed with wild-type, mutant *CTR2*, or empty plasmids and grown to exponential phase in selective media with agitation. 10-Fold serial dilutions were spotted on selective media, ethanol (2%) and glycerol (3%) media (YPEG), and YPEG containing 10, 15, 20, 25, and 100 μM copper containing 1.5% agar and incubated for 3–7 days at 30 °C.

Fluorescence Microscopy—The *ctr1Δctr3Δctr2Δ* strain was transformed with p416GPD-*CTR2-GFP* and grown in selective media to exponential phase at 30 °C. FM4-64 (Molecular Probes) was added to a final concentration of 40 μM and cells were incubated at 30 °C for 15 min. Cells were then collected and resuspended in fresh media for further incubation at 30 °C without agitation for 30–60 min. Cells were visualized with a Zeiss Axioskop upright, wide field fluorescence microscope equipped with a filter wheel. Indirect immunofluorescence was performed as previously described (46). Polyclonal antibody against Ctr2 was generated by Bethyl Laboratories, Inc. from a peptide sequence (CVHKRQLSQRVLLPNRSLTK) in the intracellular loop region of Ctr2 and affinity purified.

Vacuole Isolation and ICP-MS—Vacuoles were isolated from spheroplasted yeast cells by previously described methods (47). Briefly, spheroplasts were resuspended in buffer containing PIPES and 15% Ficoll. DEAE-dextran was added to permeabilize the plasma membrane. Lysates were then loaded into Beckman SW41 ultraclear tubes and overlaid with the same buffer containing 8 and 4% Ficoll, and then tubes were filled completely with buffer. Gradients were centrifuged at 30,000 rpm for 1.5 h at 4 °C. Samples were then collected from each of the three interfaces of the gradients (0/4, 4/8, and 8/15%) and total protein concentrations were determined using the BCA method. 50 μg of total protein was used for ICP-MS analysis. For ICP-MS analysis, cells were incubated with 20 μM copper for 1 h prior to harvesting the cells.

Immunoblotting—Whole cell extracts made by the alkali extraction method (48) or samples from the vacuole isolation were separated by 10 or 15% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-GFP (Chemicon), anti-Pma1 (Santa Cruz), anti-alkaline phosphatase, anti-3-phosphoglycerate kinase, or anti-carboxypeptidase Y (all from Molecular Probes).

Identification of CTR2 Mutants—A PCR-based random mutagenesis strategy was used to generate *CTR2* mutant alleles (49). The *CTR2* mutant alleles were cloned into the constitutive GPD promoter-driven yeast expression vector. Approximately 1×10^4 expression plasmids containing *CTR2* alleles were generated. This *CTR2* expression library was transformed into *ctr1Δctr3Δ* cells, and the yeast cells were selected on YPEG media. Plasmids containing Ctr2 mutant alleles were isolated from yeast cells growing on YPEG media and sequenced.

Copper Accumulation and ⁶⁴Cu Uptake—Whole cells were used to measure copper accumulation and ^{64}Cu uptake. Radioactive ^{64}Cu was purchased from the Mallinckrodt Institute of Radiology at Washington University, St. Louis, MO. Copper accumulation was measured by ICP-MS, and ^{64}Cu was quantified in a Packard Cobra II γ-counter as described before (37).

RESULTS

Ctr2 Contributes Copper to Mitochondrial Respiration—All characterized members of the Ctr1 family of high affinity plasma membrane copper transporters possess specific conserved structural features (50), which include three putative transmembrane domains, the second of which contains a Met-X_3-Met motif required for function (Fig. 1*A*). Additionally, many Ctr1 family members identified to date possess a methionine-rich amino terminus and a cysteine/histidine-rich carboxyl terminus. Along with these structural features, all Ctr1 family members possess a methionine residue, shown to be essential for function, ~20 amino acids upstream of the first transmembrane domain (37). Interestingly, Ctr2 has two methionine residues separated by one amino acid in this position. Whereas *S. cerevisiae* Ctr2 shares only ~23% amino acid identity overall with Ctr1, the majority of sequence conservation occurs within the hydrophobic transmembrane domains and the ~20 amino acid residues from the conserved methionine to the first transmembrane domain. Fig. 1*A* summarizes the structural features that are conserved in *S. cerevisiae* Ctr1, Ctr2, and potential Ctr2 homologs from *Schizosaccharomyces pombe*, mouse, and humans.

Work by Culotta and colleagues demonstrated that *ctr2Δ* mutants, in the context of a *ctr1Δctr3Δ* double mutant background, exhibited decreased activity of both Cu,Zn-superoxide dismutase and the Fet3 multicopper ferroxidase that functions in high affinity iron uptake (42). To further explore the physiological role of Ctr2 in copper homeostasis we ascertained whether *ctr1Δctr3Δctr2Δ* strains displayed a more pronounced defect in delivery of copper to mitochondrial cytochrome oxidase than the parental *CTR2* strain, as measured by growth on the non-fermentable carbon sources glycerol and ethanol. As shown in Fig. 1*B*, a *ctr1Δctr3Δ* strain cannot grow on ethanol-glycerol media (YPEG), as it possesses a defective mitochondrial respiratory chain because of the inability of cytochrome *c* oxidase to obtain its copper cofactor. The addition of exogenous copper to the growth medium at concentrations of 15 to 20 μM or higher rescues this phenotype, indicating the mitochondrial defect is because of a lack of copper and not general mitochondrial dysfunction. Deletion of *CTR2* in the *ctr1Δctr3Δ* background resulted in a more severe respiratory deficiency, with *ctr1Δctr3Δctr2Δ* cells unable to grow even when supplemented to 20 μM copper, but retaining growth in the presence of 100 μM copper (Fig. 1*B*). Furthermore, overexpression of the wild type *CTR2* gene or a *CTR2-GFP* fusion allele restored growth on YPEG containing 10 μM copper. Taken together, these results suggest that inactivation of *CTR2* causes an exacerbated copper deficiency and that increased levels of Ctr2, or a Ctr2-GFP fusion protein, are beneficial for cell growth under conditions of copper deprivation.

Ctr2 Is a Vacuolar Membrane Protein—Despite the primary structural and topological similarities between Ctr2 and the Ctr1 family and their involvement in copper-dependent physiological processes, Ctr2 cannot complement the growth defect associated with *ctr1Δctr3Δ* mutants (Fig. 1*B*) (32, 42). Although Ctr2 was initially proposed to function as a low affinity copper importer (32), a partially functional epitope-tagged *CTR2* allele was localized to intracellular membranes that were postulated to be largely vacuolar (42). Moreover, a genome-wide GFP fusion analysis placed Ctr2-GFP in the endoplasmic reticulum (43). To establish Ctr2 subcellular location, the functional Ctr2-GFP fusion protein was localized both by fluorescence microscopy and by vacuolar purification on discontinuous Ficoll density gradients. As shown in Fig. 2A, *ctr2Δ* cells bearing the *CTR2-GFP* expression plasmid displayed co-localization of Ctr2-GFP with the perimeter of the vacuole, as ascertained by DIC imaging and by overlapping fluorescence with the red fluorescent lipophilic dye FM4-64, which is endocytosed to the vacuole membrane. We also observed vacuolar localization of Ctr2-GFP for a chromosomal *CTR2-GFP* fusion allele (data not shown), and this localization did not change in the presence or
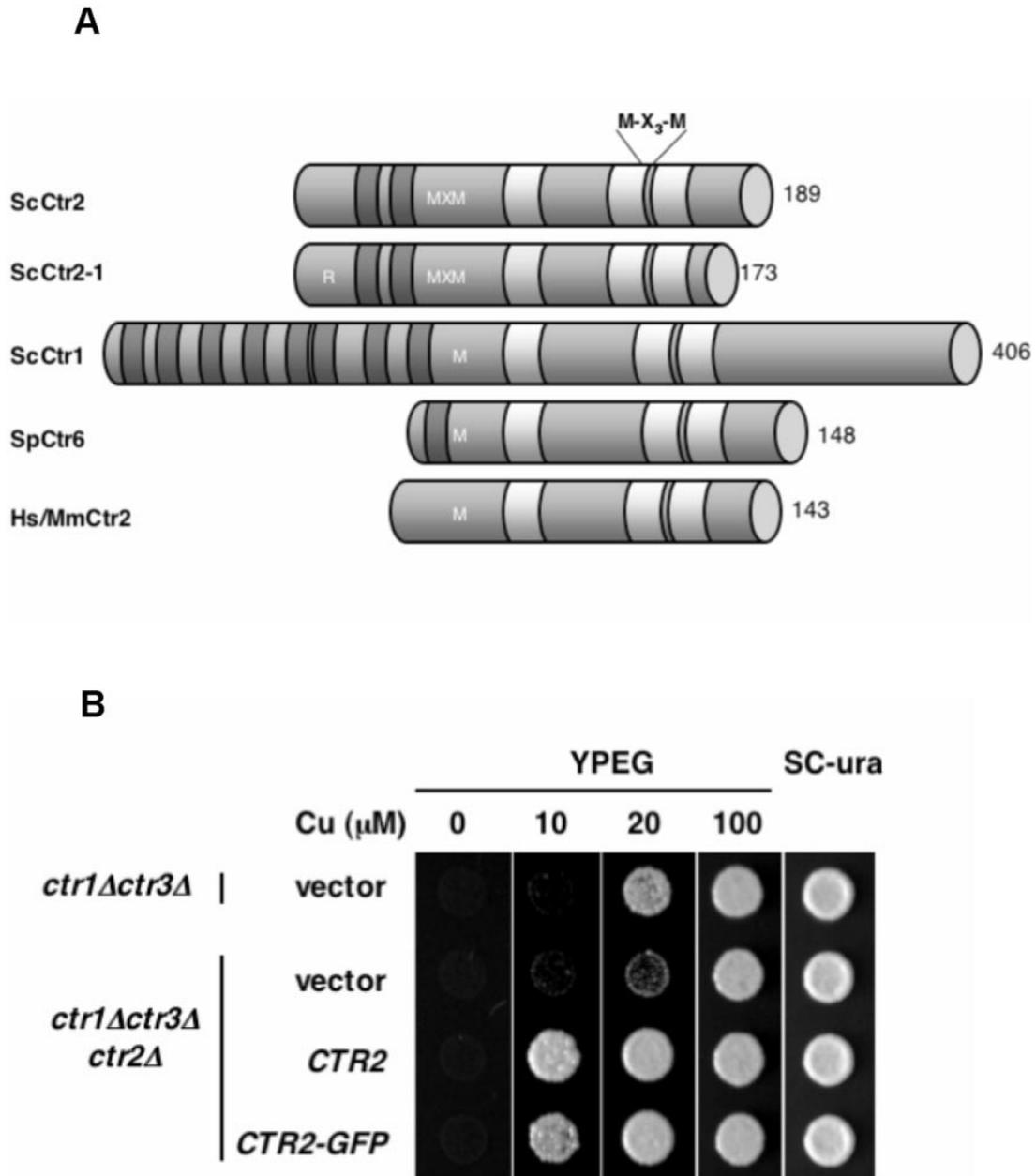
FIG. 1. **Ctr2 shares structural and functional features with the Ctr family of copper transporters.** *A*, alignment of Ctr proteins. *S. cerevisiae* Ctr2 (*ScCtr2*) and a mutant allele (*ScCtr2*-1) share conserved structural features with *S. cerevisiae* Ctr1 (ScCtr1 and putative functional homologs from other organisms (*S. pombe* (Sp) Ctr6 and human (*Hs*) and mouse (*Mm*) Ctr2). These features include three transmembrane domains (*light gray*), a conserved methionine residue(s) (*M* or *MXM*) approximately 20 amino acids upstream of the first transmembrane domain, and a Met-X_3-Met motif in the second transmembrane domain. Most Ctr family members also possess amino termini rich in methionine residues (*dark gray*). *Numbers* to the *right* of each protein indicate the length in amino acids. *B*, Ctr2 contributes copper to mitochondrial respiration. *ctr1Δctr3Δ* cells or *ctr1Δctr3Δctr2Δ* cells transformed with vector, *CTR2*, or *CTR2-GFP* under the control of the GPD promoter were spotted onto selective media (*Sc-ura*) and ethanol-glycerol media (*YPEG*) with and without copper. Deletion of *CTR2* in the *ctr1Δctr3Δ* background results in a more severe growth defect that can be rescued by overexpression of wild-type *CTR2* and the *CTR2-GFP* fusion.

absence of copper or the plasma membrane copper transporters *CTR1* and *CTR3* (data not shown).

Based on the microscopic localization of Ctr2-GFP, we independently confirmed the vacuolar localization of Ctr2-GFP by vacuole purification and immunoblotting experiments. The dynamic nature of yeast vacuoles allows them to be isolated intact from whole yeast cells; centrifugation of permeabilized yeast spheroplasts on a discontinuous density gradient causes the vacuoles to expand and float to the top, whereas the other organelles and cellular debris fractionate elsewhere in the gradient. A sample from each interface of the density gradient was collected and separated by SDS-PAGE, followed by immunoblotting to detect Ctr2-GFP. As shown in Fig. 2*B*, Ctr2-GFP co-fractionates with the integral vacuolar membrane protein alkaline phosphatase as well as carboxypeptidase Y, a protein contained in the lumen of the vacuole. The GFP fusion does not affect localization of Ctr2, as a non-tagged plasmid-born copy of Ctr2 shows an identical fractionation pattern (Fig. 6*A*). 3-Phosphoglycerate kinase is a cytosolic protein that does not appear in the same fraction as Ctr2-GFP, alkaline phosphatase, and carboxypeptidase Y, indicating the vacuoles have indeed been purified away from other cellular components. Marker proteins in the mitochondria (Cox2) and trans-Golgi apparatus (Kex2) also do not appear in the fraction with the vacuoles (data not shown). Whereas there is slight contamination of the vacuole fraction with the plasma membrane ATPase Pma1, there is no
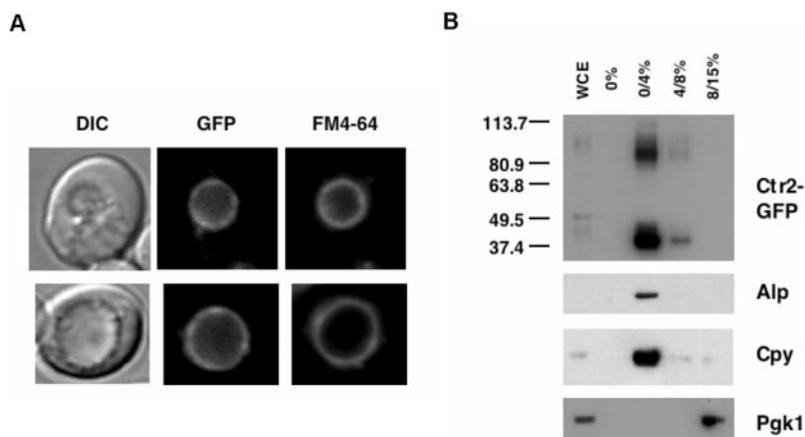
FIG. 2. **Ctr2 is localized to the vacuole membrane.** *A*, the *ctr1Δctr3Δctr2Δ* strain was transformed with p416GPD-*CTR2-GFP* and grown to exponential phase. Cells were visualized by fluorescence microscopy to see the whole cell (*DIC*), Ctr2-GFP fluorescence (*GFP*), or the fluorescent vacuole membrane marker FM4-64 (*FM4-64*). The vacuole is seen as an indentation in the DIC panels on the *left*. *B*, the same cells as in *A* were spheroplasted, permeabilized, and fractionated on a discontinuous Ficoll density gradient. Samples from whole cell extracts (*WCE*), the top layer (0%), and each interface of the gradient (0/4, 4/8, and 8/15% Ficoll) were collected and separated by SDS-PAGE. Ctr2-GFP was detected by immunoblotting with anti-GFP antibody. Antibodies against alkaline phosphatase (*Alp*) and carboxypeptidase Y (*Cpy*) were used as vacuole markers, and 3-phosphoglycerate kinase (*Pgk*) as a cytosolic control.

enrichment of the plasma membrane in the vacuole samples over the other fraction where Pma1 is observed (Fig. 6*A*). Given the vast abundance of Pma1 in the plasma membrane, the actual plasma membrane contamination present in the vacuole samples is likely very low.[2] Together with the results of the fluorescence microscopy, these biochemical data firmly support a vacuolar localization for Ctr2.

Ctr2 Mobilizes Vacuolar Copper Stores—Genetic evidence, subcellular localization studies, and the homology of Ctr2 to the Ctr1 family of plasma membrane copper transporters are consistent with the hypothesis that Ctr2 functions in copper homeostasis by transporting copper across the vacuolar membrane. To date, however, little information is available on the mobilization of copper from the lumen of internal organelles to the cytosolic copper chaperones. If Ctr2 functions in the export of vacuolar copper stores to the cytosol, we reasoned that vacuolar copper levels should vary with changes in the levels of expression or functional state of Ctr2 protein. To test this hypothesis we quantified vacuolar-associated copper by inductively coupled plasma mass spectrometry (ICP-MS) in an otherwise wild-type *ctr2Δ* strain transformed with either an empty vector or *CTR2* under the control of the constitutive *GPD* promoter. One hour prior to harvesting cells for the vacuole purification, cultures were supplemented with 20 μM copper, and vacuoles were isolated as described under "Experimental Procedures." The data in Fig. 3, which represent three independent experiments, demonstrate that cells lacking *CTR2* possess vacuolar copper levels that are ~4-fold higher than cells constitutively expressing *CTR2*. This same trend was also observed when cells were supplemented with 100 μM copper for 30 min prior to harvesting, and in the *ctr1Δctr3Δctr2Δ* strain supplemented with 20 μM copper prior to harvest (data not shown). Taken together, these results demonstrate that loss of *CTR2* results in vacuolar copper accumulation, and suggest that Ctr2 functions during copper deficiency by mobilizing vacuolar copper stores from the vacuolar lumen to the cytosol.

Methionine Residues and Multimerization Are Critical for Ctr2 Function—Much is known about the mechanisms by which copper transporting P-type ATPases pump copper into the lumen of the secretory compartment in both yeast and mammals (51–53). In contrast, little is known about the mechanisms by which cells mobilize stores of intracellular copper.
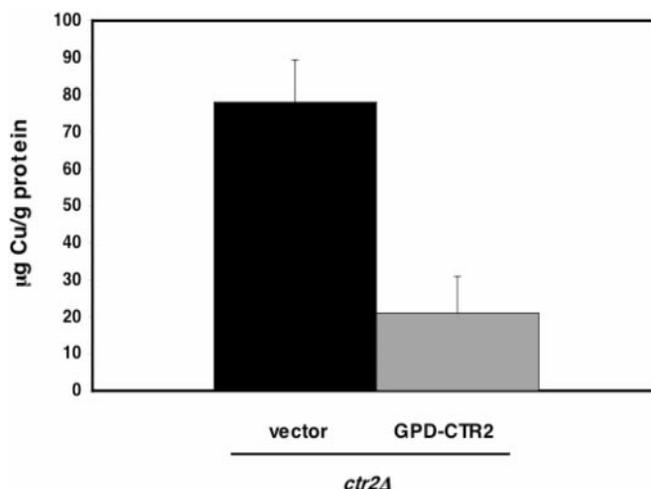

FIG. 3. **Ctr2 mobilizes vacuole copper stores.** A *ctr2Δ* strain was transformed with the p416GPD vector or p416GPD-*CTR2*. Cells were grown in selective media, and 1 h prior to harvesting cells, 20 μM copper was added. Vacuoles were isolated as described and 50 μg of protein from the vacuole samples were analyzed by ICP-MS. Data represent the mean ± S.D. of three experiments.

Given the significant primary structural and topological similarity to Ctr1, and the data pointing to a role for Ctr2 in vacuolar copper mobilization, we carried out structure-function studies to ascertain whether Ctr2 and Ctr1 might utilize a similar mechanism to move copper across distinct subcellular compartments. Both Ctr1 and Ctr2 possess conserved methionine residues in the amino terminus as well as a Met-X_3-Met motif within transmembrane domain two, which are essential for the function of Ctr1 in high affinity copper import from the plasma membrane (Figs. 1*A* and 4*A*). To determine whether these conserved methionine residues are required for Ctr2 function, mutant alleles of *CTR2* were tested for complementation of the respiratory-deficient phenotype of *ctr2Δ* cells in the context of *ctr1Δctr3Δ*. The Ctr2 amino terminus contains two methionine residues, Met-59 and Met-61, that are 22 and 20 residues upstream of the first transmembrane domain, respectively. *CTR2* mutant alleles with either single alanine mutations (M59A or M61A) or a double mutation (M59A,M61A) were transformed into the *ctr1Δctr3Δctr2Δ* triple mutant strain and grown on YPEG media. As seen in Fig. 4*B*, both single mutant alleles (M59A and M61A) still comple-

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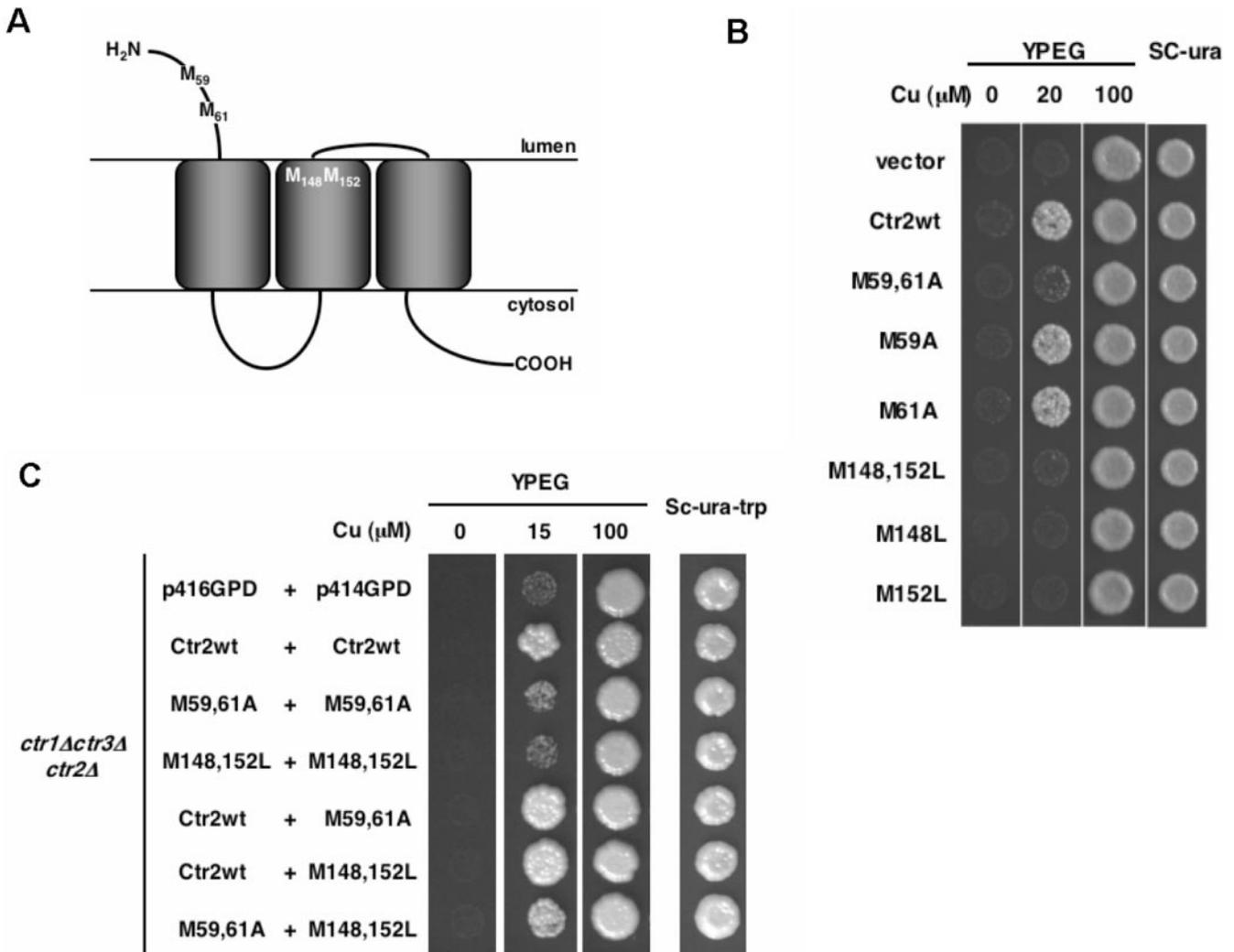[2] A. Chang, personal communication.

FIG. 4. **Genetic evidence suggests Ctr2 needs conserved methionine residues and homomultimerization for function.** *A*, the predicted topology of Ctr2 indicates the positions of the conserved methionines in the amino terminus (Met[59]-*X*-Met[61]) and second transmembrane domain (Met[148]-*X*-*X*-*X*-Met[152]) that are mutated in the following experiments. *B*, the *ctr1Δctr3Δctr2Δ* strain expressing mutant alleles of *CTR2* was tested for function using the respiratory growth phenotype shown in Fig. 1*B*. Mutation of either of the conserved methionines (Met[59] and Met[61]) in the amino terminus of Ctr2 complements the phenotype (M59A and M61A), but mutation of both residues to alanine (M59A,M61A) does not. For the conserved methionine residues in the transmembrane domain (Met[148] and Met[152]), individual mutations (M148L and M152L) and the double mutation (M148L,M152L) all result in a loss of Ctr2 function. *C*, co-expression of two different non-functional *CTR2* alleles provides genetic evidence for homomultimerization. Ctr2 methionine mutants (M59A,M61A and M148L,M152L) were subcloned into the p414GPD vector. The *ctr1Δctr3Δctr2Δ* strain was co-transformed with p416GPD and p414GPD vectors alone, or wild-type Ctr2 (*Ctr2wt*), M59A,M61A, M148L,M152L, Ctr2wt + M59A,M61A, Ctr2wt + M148L,M152L, or M59A,M61A + M148L,M152L. Cells were grown in SC-ura-trp media and tested for growth on ethanol-glycerol media (*YPEG*). Cells were incubated for 3 days at 30 °C on SC-ura-trp and YPEG + 100 μM copper, and for 7 days on YPEG and YPEG + 15 μM copper plates.

mented the growth-defect phenotype. However, substitution of both methionine residues with alanine (M59A,M61A) resulted in a non-functional Ctr2 protein that could not rescue the respiratory deficiency (Fig. 4*B*). Biochemical fractionation data indicated that this non-functional Ctr2 protein retained proper expression and localization (see Fig. 6*A*). Analogous to Ctr1 (37), changing both methionine residue substitutions in Ctr2 to cysteine or histidine residues (M59C,M61C and M59H,M61H), other potential copper ligands, restored growth with the addition of 20 μM copper (data not shown), suggesting these methionine residues are not only critical for function, but may potentially coordinate copper ions at some point during the copper mobilization process.

Mutations were also generated, singly (M148L or M152L) and in combination (M148L,M152L), in the Met-*X*$_3$-Met motif in the second transmembrane domain of Ctr2. Each of the mutant alleles, when expressed in the *ctr1Δctr3Δctr2Δ* strain and seeded on a non-fermentable carbon source supplemented

with 20 μM copper, resulted in a growth defect (Fig. 4*B*). This was not because of a difference in localization between wild-type and mutant Ctr2, as confirmed by indirect immunofluorescence with the M148L,M152L mutant (data not shown). Furthermore, the decrease in vacuolar copper levels observed in cells expressing wild-type *CTR2* was not observed when the M148L,M152L mutant was expressed, consistent with its inability to transport copper (data not shown). The functional conservation of these methionine residues between Ctr1 and Ctr2 supports the idea that, despite their localization to the plasma membrane and vacuolar membrane, respectively, these proteins transport copper across membranes via similar mechanisms.

Based on both biochemical and genetic experiments, a current model for Ctr1 function in copper transport across the plasma membrane is consistent with Ctr1 forming a homomultimer, with evidence for homotrimers (31, 37). Given the role of Ctr2 in vacuolar copper mobilization, the presence of conserved
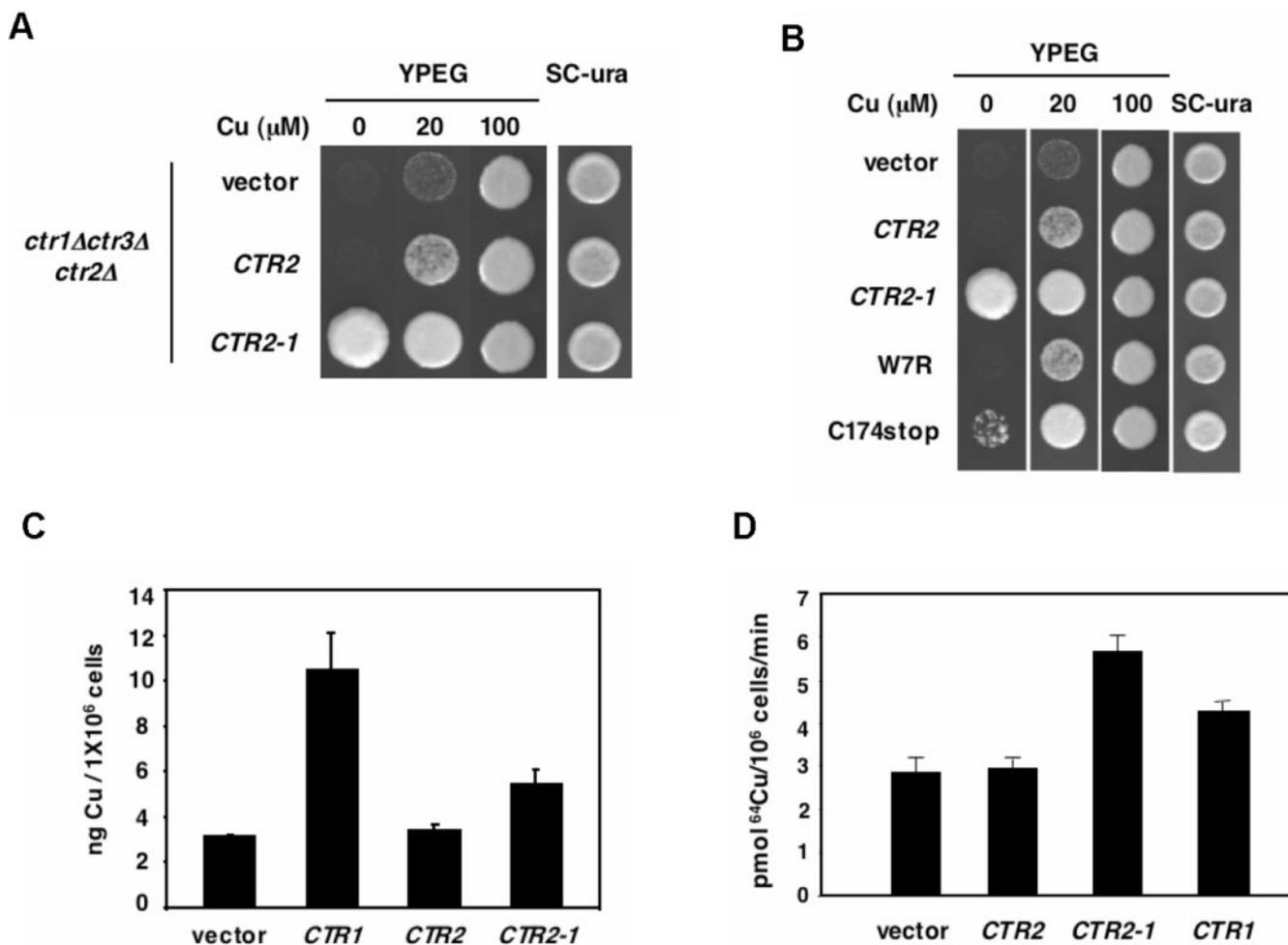
FIG. 5. **Identification of a mutant allele of *CTR2* that stimulates copper accumulation and ^{64}Cu uptake.** *A*, PCR-based random mutagenesis was used to construct a *CTR2* mutant library. *CTR2* mutant alleles were transformed into MPY17 (*ctr1Δctr3Δ*) and selected for stimulation of copper uptake by growth on YPEG. *CTR2-1* is one of four independent mutant alleles found that suppressed the *ctr1Δctr3Δ* phenotype (see Supplemental Materials). *B*, *CTR2-1* contains two base substitutions resulting in an amino acid substitution from tryptophan to arginine at position seven, and a truncation of the final 16 amino acids (see Fig. 1*A*). Mutant alleles were cloned that contain either the amino terminus mutation (W7R) or the carboxyl terminus truncation (C174stop), and these constructs were tested for their ability to suppress the *ctr1Δctr3Δ* respiratory defect. *C*, *ctr1Δctr3Δctr2Δ* cells expressing vector, wild-type *CTR2*, *CTR2-1*, or *CTR1* were analyzed for cell-associated copper by ICP-MS. *D*, ^{64}Cu uptake was measured in the cells used for *C*.

and functionally important methionine residues, and a similar predicted topology with three putative transmembrane domains, we ascertained whether Ctr2 may also form obligatory homomultimers. As shown in Fig. 2*B*, the Ctr2-GFP fusion protein fractionates by SDS-PAGE as both unit length and larger species consistent with higher-order multimers. A similar observation was made for endogenous Ctr2 using polyclonal anti-Ctr2 antiserum, which detects polypeptide species consistent with a monomer, dimer, and trimer (Fig. 6*A*). To test whether this apparent homomultimerization occurs *in vivo* and is important for Ctr2 function, we transformed the *ctr1Δctr3Δctr2Δ* yeast strain with plasmids independently expressing the non-functional M59A,M61A or M148L,M152L alleles of *CTR2*, or both alleles, and tested for respiratory proficiency. As shown in Fig. 4*C*, whereas independent expression of either the M59A,M61A or M148L,M152L alleles did not restore growth in this experiment, co-expression of the M59A,M61A and M148L,M152L alleles rescued the copper-dependent respiratory deficiency observed in the *ctr2Δ* strain. In addition, both of these Ctr2 mutations are recessive, as *ctr1Δctr3Δctr2Δ* cells co-expressing either M59A,M61A or M148L,M152L with wild-type Ctr2 grow on copper-supplemented ethanol-glycerol (Fig. 4*C*). Therefore, genetic evidence suggests that, similar to Ctr1 in transporting copper across the plasma membrane, Ctr2

assembles as a homomultimer in the mobilization of copper across the vacuolar membrane.

CTR2–1 Suppresses the ctr1Δctr3Δ Mutant Phenotype by Stimulating Copper Uptake at the Plasma Membrane—Whereas Ctr2 shares structural features with the Ctr1 family of high affinity plasma membrane copper transporters, and defects in Ctr2 cause hyperaccumulation of vacuolar copper, it is important to understand whether Ctr2 enhances the kinetics of copper movement across membranes. To address this issue, we selected for *CTR2* variants capable of by-passing the requirement for the Ctr1 and Ctr3 high affinity plasma membrane copper transporters for copper acquisition, perhaps through their mislocalization to the plasma membrane. The *CTR2* gene was randomly mutagenized by error-prone PCR, a library of mutants introduced on plasmids into a *ctr1Δctr3Δ* strain, and transformants identified that are capable of growth on YPEG in the absence of added copper. By plasmid rescue and DNA sequencing, four independent *CTR2* mutant alleles were found to suppress the loss of the high affinity plasma membrane transporters (see Supplementary Materials). One such suppressor allele, designated *CTR2-1*, confers robust growth of the *ctr1Δctr3Δ* strain on YPEG without copper supplementation and was chosen for further investigation (Fig. 5). *CTR2-1* harbors two T → A base substitutions, resulting in

a Trp → Arg amino acid substitution at position seven and the generation of a stop codon at position 174, resulting in a truncated form of the Ctr2 protein that lacks the carboxyl-terminal 16 amino acid residues (Fig. 1*A*). Each of the other three *CTR2* suppressor alleles is truncated at or near this same codon, with distinct amino acid substitutions at other positions within the *CTR2* open reading frame (see Supplementary Materials). Interestingly, dissection of the two mutations in *CTR2-1* demonstrates that while the carboxyl-terminal truncation is sufficient for some of the *ctr1Δctr3Δ* suppression activity, the two mutations function together for maximal suppression (Fig. 5*B*). Further analysis will be required to understand the basis for the interactions between the two mutations underlying robust suppression of *ctr1Δctr3Δ* by *CTR2-1*.

CTR2 encodes a vacuolar membrane protein that functions to mobilize copper from the vacuolar lumen to cytosolic copper chaperones. To understand how the Ctr2-1 protein suppresses the YPEG growth phenotype of the *ctr1Δctr3Δ* strain, a plasmid bearing wild-type *CTR2*, the *CTR2-1* allele, *CTR1*, or no insert was transformed into a *ctr1Δctr3Δctr2Δ* strain and total cell copper accumulation and the kinetics of ^{64}Cu uptake were measured. Copper accumulation measurements by ICP-MS demonstrated that expression of *CTR1* increased the accumulation of copper ∼4-fold over that observed in cells transformed with an empty vector. Interestingly, cells transformed with the *CTR2-1* allele exhibited an almost 2-fold increase in total cellular copper accumulation over vector-transformed cells, demonstrating that the Ctr2-1 protein functions to enhance copper accumulation (Fig. 5*C*). Furthermore, as shown in Fig. 5*D*, the rate of ^{64}Cu uptake in cells expressing the *CTR2-1* allele was ∼2-fold higher than either cells transformed with vector alone or with a plasmid expressing wild-type *CTR2* (Fig. 5*D*). The increase was nearly abolished when experiments were performed at 0 °C, suggesting that the increased uptake was not because of general diffusion or nonspecific mechanisms (data not shown). Note that overexpression of wild-type *CTR2* stimulates neither copper accumulation (Fig. 5*C*) nor the rate of ^{64}Cu uptake (Fig. 5*D*). Taken together these results strongly suggest that *CTR2-1* by-passes the requirement for the *CTR1* and *CTR3* high affinity copper uptake proteins by enhancing copper accumulation through facilitating copper uptake from the exterior of the cell.

The ability of *CTR2-1* to stimulate copper accumulation and uptake implies a plasma membrane localization for the Ctr2-1 protein. Therefore, we investigated the subcellular localization of Ctr2-1 by both biochemical fractionation and indirect immunofluorescence microscopy. Spheroplast lysates were fractionated on a Ficoll discontinuous step gradient followed by immunoblotting using the affinity purified polyclonal antiserum directed against the presumptive Ctr2 cytosolic loop domain. The data in Fig. 6*A* demonstrate that whereas Ctr2 and the M59A,M61A mutant co-purify with vacuolar fractions, Ctr2-1 co-fractionated with both the vacuolar fraction (0/4% Ficoll) and the plasma membrane fraction (4/8% Ficoll), as shown by the presence of the Pma1 plasma membrane ATPase in the latter fraction. Note that truncation of the carboxyl-terminal 16 residues of Ctr2-1 leads to a faster electrophoretic mobility as compared with that of wild-type Ctr2, with both proteins displaying species whose migration is consistent with monomers, dimers, and homotrimers. Furthermore, as shown by indirect immunofluorescence microscopy using the affinity purified anti-Ctr2 antiserum, wild-type Ctr2 is found only on vacuolar membranes (Fig. 6*B*). However, whereas much of the Ctr2-1 protein localizes to the vacuolar membrane, a significant signal is seen on the plasma membrane. Taken together with the copper accumulation and uptake data for *CTR2-1*, we conclude

that the ability of *CTR2-1* to suppress the *ctr1Δctr3Δ* mutant growth defect on YPEG medium is attributable to partial localization to the plasma membrane. Importantly, the generation of a mislocalized Ctr2-1 protein has allowed a clear demonstration that this protein stimulates the kinetics of copper transport across membranes. Furthermore, these data support the hypothesis that wild-type Ctr2 protein functions in copper mobilization by facilitating the movement of copper from vacuolar luminal stores across the vacuole membrane and ultimately to copper chaperones.

DISCUSSION

The ability to acquire metals from the environment, and mobilize them from intracellular stores for signaling or as co-factors to drive biochemical reactions, are important features of metal homeostasis. Whereas much progress has been made in understanding the mechanisms whereby mammalian and yeast cells compartmentalize and mobilize calcium, zinc, iron, and other metals, little information is available on how copper stores are generated and utilized. Data presented here strongly suggest that Ctr2 is a vacuolar membrane copper transporter that functions to mobilize vacuolar copper stores. Like the Ctr1 and Ctr3 high affinity copper transporters at the plasma membrane, Ctr2 provides copper to all three of the copper chaperone pathways.

Whereas earlier studies suggested that Ctr2 is a low affinity copper transporter at the plasma membrane because of its inability to suppress a *ctr1Δctr3Δ* mutant when overexpressed without added copper (32), work presented here and in other studies (42, 43) demonstrates that Ctr2 resides on intracellular membranes. Both fluorescence microscopy with a functional Ctr2-GFP fusion and the use of polyclonal anti-Ctr2 antibody, as well as biochemical fractionation data firmly establish a vacuolar membrane localization for Ctr2. This localization does not depend on the presence of Ctr1 and Ctr3 or the amount of copper within a cell (data not shown), and indirect immunofluorescence of a Ctr2 fusion protein in temperature-sensitive yeast strains defective for receptor-mediated or fluid-phase endocytosis suggests Ctr2 is not endocytosed from the plasma membrane, but instead traffics directly to the vacuole from the secretory pathway (42).

Despite the difference in subcellular location, the mechanisms by which Ctr2 transports copper across the vacuole membrane may nevertheless be similar to that of the Ctr1 family of plasma membrane transporters. Both biochemical cross-linking experiments and *in vivo* trans-complementation experiments strongly suggest that Ctr1 functions as a homomultimer, most likely a homotrimer (37). Our data demonstrate that the methionine residues located in the amino terminus and in the second transmembrane domain that are important for Ctr1 function are conserved in Ctr2 and required for its function. Indeed, co-expression of two distinct non-functional alleles of *CTR2* to reconstitute function in the delivery of copper to the mitochondrial oxidative phosphorylation machinery provides further genetic evidence in support of Ctr2 homomultimerization. The use of a common mechanism for copper transport across two distinct membranes is analogous to the copper transporters ATP7A and ATP7B in mammalian cells. Whereas ATP7A pumps copper into the lumen of the secretory apparatus for incorporation into proteins such as tyrosinase or peptidylglycine α-amidating monooxygenase, in response to high intracellular copper concentrations ATP7A traffics to the plasma membrane where it serves as a plasma membrane copper efflux pump (53). In liver cells, ATP7A is not expressed, and ATP7B delivers copper to ceruloplasmin or effluxes copper into the bile via a mechanism of transport similar to ATP7A (53). Whereas it is not yet clear whether Ctr1 and Ctr2 utilize
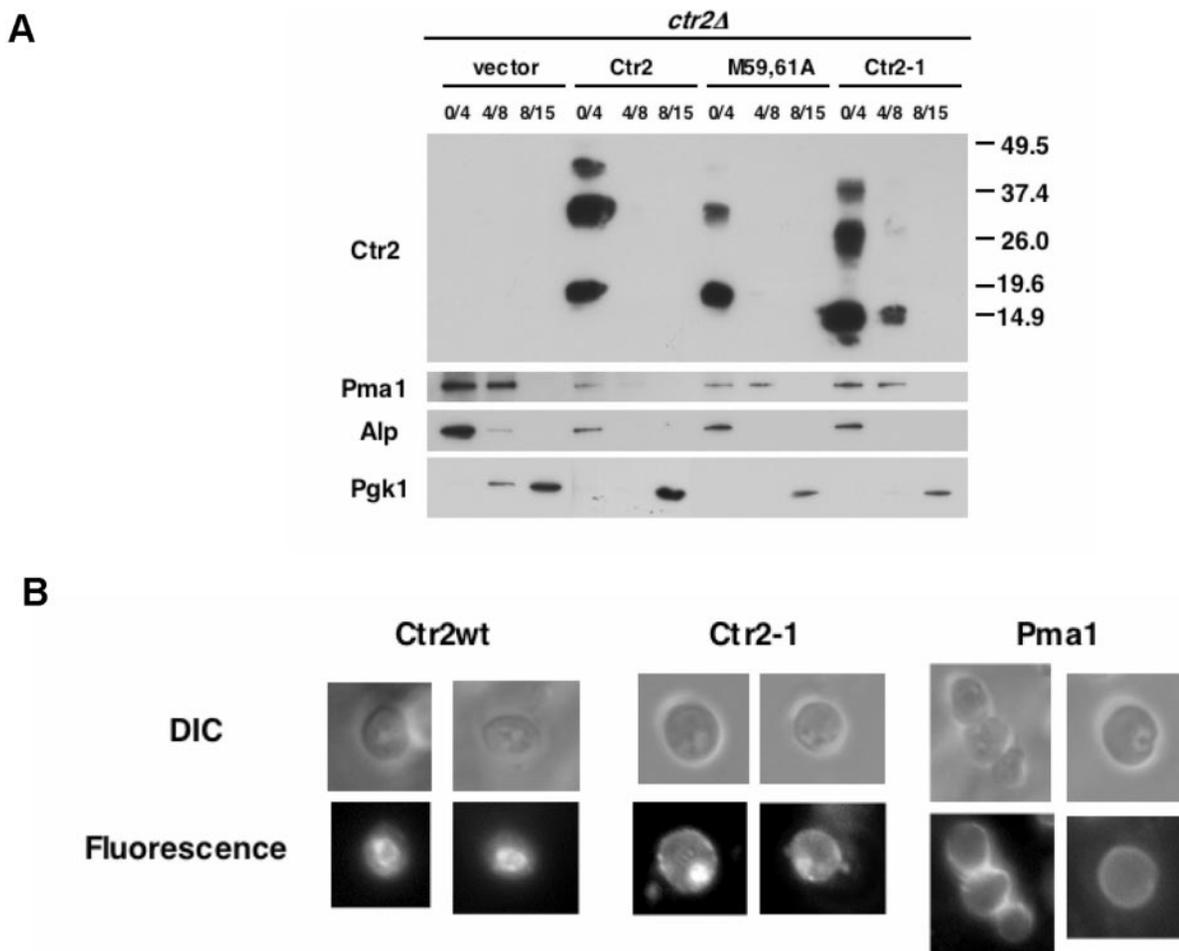
FIG. 6. **Ctr2-1 is partially mislocalized to the plasma membrane.** *A*, *ctr2Δ* cells expressing vector, wild-type Ctr2, Ctr2 M59A,M61A, or Ctr2-1 were grown to exponential phase, and vacuoles were isolated and analyzed by immunoblotting as described in the legend to Fig. 2*B*, except Ctr2 was detected by an anti-Ctr2 antibody. Markers against the plasma membrane (*Pma1*), vacuole (*Alp*), and cytosol (*Pgk*) were used. *B*, *ctr1Δctr3Δctr2Δ* cells expressing vector or *CTR2-1* were grown to exponential phase and then fixed by the addition of 4% formaldehyde to the growth media, spheroplasted, and adhered to polylysine-coated slides. Cells were permeabilized with 0.1% SDS and then incubated with primary antibody, followed by fluorescein isothiocyanate-conjugated secondary antibody. Cells were viewed by fluorescence microscopy.

a common copper transport mechanism, if this is the case it would also suggest that Ctr2 would transport Cu(I) rather than the oxidized Cu(II). The importance of the conserved methionine residues in Ctr2, predicted to function as Cu(I) ligands for Ctr1 (37), suggests that Ctr2 may transport Cu(I) and would further suggest a requirement for a vacuolar metalloreductase to function in concert with Ctr2. Current studies are underway to further explore this hypothesis.

The yeast vacuole plays an important role in overall cellular homeostasis for amino acids, phosphate, pH, and many other ions and metabolites. Previous reports have demonstrated that vacuoles represent an important organelle for the homeostatic regulation of zinc and iron levels (15–18). Furthermore, mutants in genes essential for normal vacuolar biogenesis have been demonstrated to exhibit slow growth phenotypes on medium containing elevated copper concentrations (16, 29), but the precise role the vacuole plays in copper homeostasis is not clear. One step toward a better understanding of the role of the vacuole in copper homeostasis comes from work done in the fission yeast *S. pombe* (54). Labbé and colleagues (54) found that deletion of the *ctr6+* gene resulted in a significant decrease in the activity of Cu,Zn-superoxide dismutase, and overexpression of *ctr6+* made the cells sensitive to high copper concentrations. Interestingly, the copper sensitivity was not because of an increase in copper uptake, in fact, ^{64}Cu measurements indicated a decrease in uptake when *ctr6+* is overexpressed. The authors localized Ctr6 to the vacuole membrane and suggested, based on the above observations, that this gene product functions to mobilize vacuolar copper stores, which in turn results in a reduction in the expression of the plasma membrane uptake machinery through the down-regulation of the copper-sensing transcription factor Cuf1. However, it is not clear whether *ctr6+* function affects vacuolar copper levels and whether Ctr6 directly transports copper.

In this work we have purified vacuoles and directly measured vacuolar-associated copper. Our ICP-MS results provide direct evidence for mobilization of vacuolar copper stores by Ctr2, as there is approximately a 4-fold decrease in vacuolar-associated copper levels when *CTR2* is overexpressed ($\sim$21 μg of copper/g of vacuole protein) compared with a *ctr2Δ* mutant ($\sim$78 μg of copper/g of vacuole protein). Quantification of vacuolar iron and zinc under these conditions in our vacuole purification suggest the amounts of iron and zinc per g of vacuole-associated protein are higher than the amounts of copper ($\sim$5- and 45-fold, respectively) and do not directly depend on the activity of Ctr2 (data not shown). However, these metal levels will obviously fluctuate depending on the cellular demand for a metal ion at a given time, as well as the extracellular metal concentrations and the expression of vacuolar metal importers and exporters. Whereas both the vacuole importers and exporters for iron and zinc have been identified (22–28), the protein or protein complex responsible for import of copper into the vacuole has not yet been identified.

Because of its partial mislocalization, the mutant *CTR2* allele generated here may provide some insight into the sorting mechanism required for wild-type Ctr2 to traffic to the vacuole, whereas two other proteins relatively similar in structure and function (Ctr1 and Ctr3) traffic to the plasma membrane. Ctr2-1 contains a Trp → Arg substitution at amino acid position 7, as well as a truncation of the carboxyl-terminal 16 amino acid residues. All four of the *CTR2* mutant alleles found to suppress the *ctr1Δctr3Δ* mutant phenotype by selection on YPEG media have truncations of the carboxyl-terminal residues immediately following the third transmembrane domain (see Supplementary Materials). However, each of these mutant alleles also contains at least one other amino acid substitution at some point along the length of the protein. For Ctr2-1, the Trp → Arg substitution is required in concert with the carboxyl-terminal truncation for full suppression of the *ctr1Δctr3Δ* respiratory deficiency. One possible explanation is that the Trp → Arg substitution increases the activity of the protein, and the carboxyl terminus of Ctr2 contains a vacuolar sorting signal that is lost in Ctr2-1, resulting in its plasma membrane localization. It is also possible that there are redundant sorting signals in Ctr2. Studies in mammalian cells on the lysosomal cystine exporter cystinosin have found that mutation of a carboxyl-terminal lysosomal sorting motif leads to partial mislocalization of this protein to the plasma membrane and a subsequent stimulation of extracellular cystine uptake (55). Interestingly, deletion of a portion of the third cytosolic loop of cystinosin along with the mutation in the carboxyl terminus leads to complete mislocalization of this protein (56). Further studies must be done to clarify the effect of each of the mutations in Ctr2-1 and identify the complex sorting signals of the *S. cerevisiae* Ctr copper transport proteins that function at the vacuole and on the plasma membrane.

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Supplementary Figure legends

Figure S1: Alignment of Ctr2 wild type and mutant amino acid sequences. Four independently generated Ctr2 mutants (Ctr2-1, -2, -3, and -4) were found to suppress the *ctr1Δctr3Δ* respiratory defect. The amino acid sequence of wild type Ctr2 is given for reference, and amino acid substitutions are shown in blue. The position of each of the three transmembrane domains (TM1, 2, and 3) is indicated by the horizontal lines. Biochemical and genetic analyses with Ctr2-1 indicate this mutant is partially mislocalized to the plasma membrane where it stimulates ^{64}Cu uptake, providing evidence that Ctr2 directly transports Cu.

Figure S2: Four *CTR2* mutant alleles, but not wild type *CTR2*, can suppress the *ctr1Δctr3Δ* respiratory defect. *ctr1Δctr3Δ* cells expressing an empty vector or plasmid-born *CTR2*, either wild type or mutant, were seeded on the non-fermentable carbon source (YPEG) with or without Cu supplementation. While expression of wild type *CTR2* restores growth in the presence of exogenous Cu, all four mutant alleles (*CTR2-1*, *CTR2-2*, *CTR2-3*, and *CTR2-4*) promote growth on unsupplemented YPEG.

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Ctr2WT    MDDKKTWSTVTLRTFNQLVTSSLIGYSKKMDSMNHKMEGNAGHDHSDMHM
Ctr2-1    MDDKKTRSTVTLRTFNQLVTSSLIGYSKKMDSMNHKMEGNAGHDHSDMHM
Ctr2-2    MDDKKTWSTVTLGTFNQLVTSSLIGYSKKMDSMNHKMEGNAGHDHSDMHM
Ctr2-3    MDDKKTRSTVTLRTFNQLVTSSLIGYSKKMDSMNHKMEGNAGHDHSDMHM
Ctr2-4    MDDKKTWSTVTLRTFNQLVTSSLIGYSKKMDSMNHEMEGNAGHDHSDMHM
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                                        TM1

```
Ctr2WT    GDGDDTCSMNMLFSWSYKNTCVVFEWWHIKTLPGLILSCLAIFGLAYLYE
Ctr2-1    GDGDDTCSMNMLFSWSYKNTCVVFEWWHIKTLPGLILSCLAIFGLAYLYE
Ctr2-2    GDGDDTCSMNMLFSWSYKNTCVVFEWWHIKTLPGLILSCLAIFGLAYLYE
Ctr2-3    GDGDDTCSMNMLFSWSYKNTCVAFEWWHIKTLPGLILSCLAIFGLAYLYE
Ctr2-4    GDGDDTCSMNMLFSWSYKNTCVVFEWWHIKTLPGLILSCLAIFGLAYLYE
```
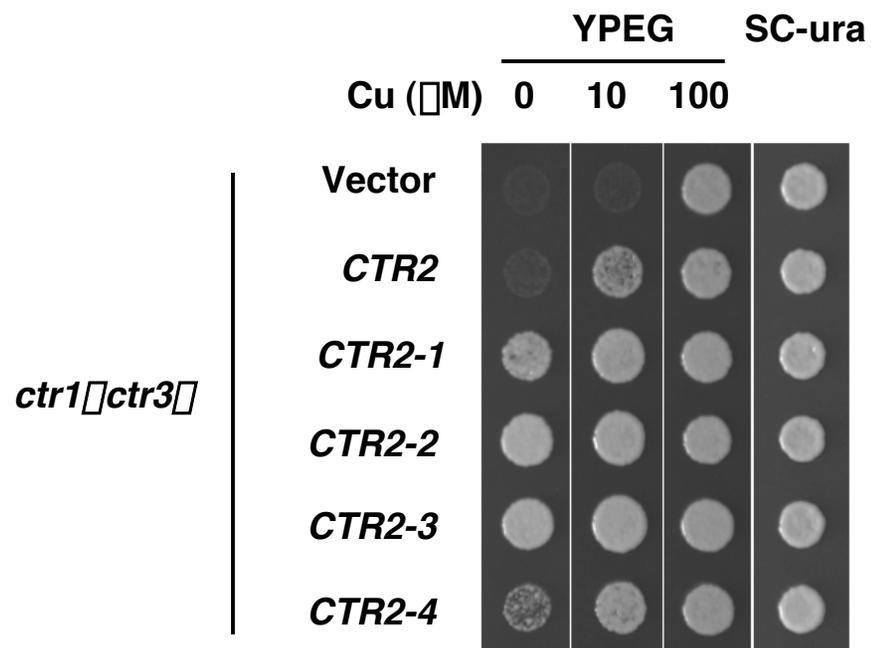
                                        TM2

```
Ctr2WT    YLKYCVHKRQLSQRVLLPNRSLTKINQADKVSNSILYGLQVGFSFMLMLV
Ctr2-1    YLKYCVHKRQLSQRVLLPNRSLTKINQADKVSNSILYGLQVGFSFMLMLV
Ctr2-2    YLKYCVHKRQLSQRVLLPNRSLTKINQADKVSNSILYGLQVGFSFMLMLV
Ctr2-3    YLKYCVHKRQLSQRVLLPNRSLSKINQADKVSNSILYGLQVGFSFMLMLV
Ctr2-4    YFKYCVHKRQLSQRVLLPNRSLAKINQADKVSNSILYGLQVGFSSMLMLV
```

                    TM3

```
Ctr2WT    FMTYNGWLMLAVVCGAIWGNYSWCTSYSPEIDDSSLACH
Ctr2-1    FMTYNGWLMLAVVCGAIWGNYSW
Ctr2-2    FMTYNGWLMFAVVCGAIWGNYSW
Ctr2-3    FMTYNGWLMLAVVCGAIWGNYSW
Ctr2-4    FMTYNGWLMLAVVCGAIWGNYSCVLRIVL
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Rees et al, Supplementary figure S1

Rees et al, Supplementary figure S2