Copper is an essential trace element, yet excess copper can lead to membrane damage, protein oxidation, and DNA cleavage. To balance the need for copper with the necessity to prevent accumulation to toxic levels, cells have evolved sophisticated mechanisms to regulate copper acquisition, distribution, and storage. In Saccharomyces cerevisiae, transcriptional responses to copper deficiency are mediated by the copper-responsive transcription factor Mac1. Although Mac1 activates the transcription of genes involved in high affinity copper uptake during periods of deficiency, little is known about the mechanisms by which Mac1 senses or responds to reduced copper availability. Here we show that the copper-dependent enzyme Sod1 (Cu,Zn-superoxide dismutase) and its intracellular copper chaperone Ccs1 function in the activation of Mac1 in response to an external copper deficiency. Genetic ablation of either CCS1 or SOD1 results in a severe defect in the ability of yeast cells to activate the transcription of Mac1 target genes. The catalytic activity of Sod1 is essential for Mac1 activation and promotes a regulated increase in binding of Mac1 to copper response elements in the promoter regions of genomic Mac1 target genes. Although there is precedent for additional roles of Sod1 beyond protection of the cell from oxygen radicals, the involvement of this protein in copper-responsive transcriptional regulation has not previously been observed. Given the presence of both Sod1 and copper-responsive transcription factors in higher eukaryotes, these studies may yield important insights into how copper deficiency is sensed and appropriate cellular responses are coordinated.

Unicellular organisms are constantly exposed to a plethora of changing environments and thus have developed sophisticated uptake, distribution, and storage systems that function to assimilate essential nutrients from the environment. Copper is included among these essential nutrients, and once inside cells, it is incorporated as a catalytic or structural cofactor into a variety of proteins (1, 2). The redox potential that makes copper an important cofactor also allows the ion to undergo Fenton chemistry to produce the potent hydroxyl radical (OH·) (3).

Organisms have evolved sophisticated homeostatic systems to maintain appropriate intracellular copper levels that are below levels that could lead to cellular damage (4, 5).

In Saccharomyces cerevisiae, copper in the extracellular environment is reduced by cell surface reductases, Fre1 and Fre2, and is transported across the plasma membrane by the high affinity copper transporter Ctr1 or the functionally redundant Ctr3 protein (6–8). Inside cells, the Cox17 chaperone facilitates the delivery of copper to the cytochrome c oxidase complex in the mitochondria, and this function is required for aerobic respiration (9–11). Interestingly, recent data have demonstrated that Cox17 localized exclusively to the mitochondria is sufficient for delivery of copper to cytochrome c oxidase (12). This suggests that either an as yet unidentified chaperone or a small molecule carrier is responsible for trafficking of copper from the plasma membrane to Cox17 in the mitochondria. The Atx1 chaperone delivers copper to the Golgi, where it is pumped into the lumen of the secretory compartment by the P-type ATPase Ccc2 (13–15). Ccs1, the copper chaperone for superoxide dismutase (16), is responsible for delivery of copper to Sod1 (Cu,Zn-superoxide dismutase), an enzyme that protects cells against oxidative stress via the disproportionation of superoxide to produce hydrogen peroxide (17, 18).

In S. cerevisiae, the regulation of copper acquisition has been shown to be controlled at the level of transcription by Mac1 (19). Mac1 is activated in response to copper deprivation, leading to transcription of the genes involved in high affinity copper uptake, such as CTR1, CTR3, and FRE1 (20–22). Mac1 is a modular protein consisting of a copper responsive trans-activation domain (TAD) (1) and DNA binding domain (DBD) (23–26). Previous experiments using a fusion protein containing the Gal4 DBD and the Mac1 TAD demonstrated that the TAD is responsive to changes in bioavailable copper levels (23). The carboxyl-terminal Mac1 TAD contains two cysteine- and histidine-rich domains, REP-1 (C1) and REP-II (C2), that each binds four Cu2+ ions in a tetranuclear copper cluster (24). Mutations in the C1 domain lead to constitutively active Mac1up proteins, whereas analogous mutations in the C2 domain decrease the trans-activation of Mac1 (19–21, 23, 25, 27). Copper deprivation also results in increased DNA binding of Mac1 to copper-responsive element (CuRE) regions upstream of its target genes (20–22, 28, 29), and there is evidence that a constitutively active...
Mac1\textsuperscript{up1} protein binds fewer copper ions per molecule than the wild type protein (24). Moreover, Mac1\textsuperscript{up1} is constitutively bound to the promoter of its target genes (20). These observations suggest that loss of copper ions from Mac1 may be important for its activation. Studies using yeast two-hybrid analysis also indicate that copper starvation results in release of an intramolecular interaction between the Mac1 DBD and TAD (24).

Although it has previously been demonstrated that Mac1 protein fragments bind copper ions and that this binding is important for its regulation (24), it is unclear how copper binding may be regulated. Previous studies have shown that Mac1 is a nuclear resident protein, suggesting that copper is either assembled co-translationally or is delivered to the nucleus in order to regulate Mac1 (19, 24). However, there is virtually no free copper in the cell, and it has been demonstrated that copper is associated almost exclusively with either chaperones or the copper-containing proteins that are targets of these chaperones (30).

We began with the hypothesis that one of the three known copper chaperone proteins, Atx1, Cox17, or Ccs1, might be responsible for copper delivery to or removal from Mac1. Here we find that both Ccs1 and its target, Sod1, are necessary for robust activation of Mac1 in response to low copper conditions. We found that the requirement for Ccs1 during Mac1 activation is due to its role in delivery of copper to Sod1 and that the disproportionate of superoxide is necessary for Mac1 activation. However, the role of Sod1 in Mac1 activation appears to be more complex than simply a global protection against oxidative stress, since both genetic and chemical suppression of oxidative stress in sod1 Δ cells failed to restore Mac1 activity to wild type levels. Moreover, we demonstrated that Sod1 and the Ccs1 copper chaperone partially localize to the yeast nucleus and that deletion of SOD1 reduces the ability of Mac1 to bind to CuRE elements in the genome upstream of the CTR1 gene in response to low copper bioavailability. Taken together, these results suggest that in Saccharomyces cerevisiae the Cu,Zn-superoxide dismutase enzyme plays a role in the sensing or responding to copper deficiency to activate gene transcription.

**Experimental Procedures**

**Yeast Strains and Plasmids**—All isogenic S. cerevisiae deletion strains were created by replacement of the endogenous locus with a floxed kanamycin resistance cassette and subsequent removal of this cassette (31). The sod1 Δ pmr1 Δ double mutant was created by deletion of PMR1 in an sod1 Δ strain, and the ccs1 Δ sod1 Δ double mutant was created by deletion of SOD1 in a ccs1 Δ strain. The SOD1-GFP and the MAC1-TAP strains were obtained from the GFP- and TAP-tagged collection (32, 33). The MAC1-TAP sod1 Δ strain was created by deletion of SOD1 in the MAC1-TAP background. The MAC1\textsuperscript{up1} strain and its wild type parental strain have been previously described (19), and the MAC1\textsuperscript{up1} sod1 Δ isogenic variant was created by deletion of SOD1 in this strain. The Y190 yeast strain was used in the yeast one/two-hybrid experiments, and the Y190 sod1 Δ strain was created by deletion of the SOD1 gene in the Y190 background (34).

The GAL1–10-LacZ reporter plasmid was a generous gift from Dr. Alan Hinnebusch. The Caenorhabditis elegans SOD1 plasmid was previously described by Jensen and Culotta (35). The ySOD1 plasmid was created by cloning a PCR fragment containing the SOD1 gene and its endogenous promoter and terminator as an XbaI/XhoI fragment into the pRS416 vector. The SOD1 plasmid was created by subcloning the SOD1 sequence from ySOD1 as an XbaI/XhoI fragment into the pRS415 vector. The SOD1\textsuperscript{R143D} and the SOD1\textsuperscript{G85R} alleles encoding catalytically inactive SOD1 mutants were created by site-directed mutagenesis of SOD1 using overlap PCR and then cloned as BamHI/XhoI fragments into pRS415 (36, 37). A DNA fragment with the coding sequence for the first 105 amino acids of SCO2 as an in-frame amino-terminal fusion with the SOD1 gene under the control of the SOD1 promoter was created using overlap PCR and cloned by gap repair into pRS415 to create the SCO2-SOD1 plasmid. The pGB4D1-Trp MAC1 1–159, pVT102-Leu VP16, pVT102-leu MAC1 240–417, and pVT102-leu MAC1\textsuperscript{up1} 240–417 plasmids were a generous gift from Dr. Dennis Wing (24). For the yeast one-hybrid experiment, a PCR product containing codons 42–417 of the MAC1 gene was cloned by gap repair as an in-frame fusion with the GAL4 DNA binding domain of the pGBK7 plasmid backbone (Clontech).

**β-Galactosidase Activity Assay for Mac1 Function**—Cells were transformed with the previously described Mac1 reporter plasmid pCM64CTR3-LacZ or pRSCTR3-LacZ (20) and grown to mid-log phase in synthetic complete (SC) selective media with or without 10 μM or 100 μM bathocuproinedisulfonic acid (BCS). β-Galactosidase assays were performed as described by Liu et al. (38).

**RNA Blot Analysis**—RNA was extracted from cells grown to mid-log phase using a modified hot phenol method (39). CTR1 or ACT1 gene fragments were radiolabeled with [\textsuperscript{32P}]dCTP to be used as probes. Quantification of the RNA blot was performed using ImageQuant TL version 2003.02 software (Amer sham Biosciences) and processed using Adobe Photoshop version 7.0 (Adobe Systems).

**Immunoblotting**—Protein extracts were prepared either using a glass bead/Triton X-100 method (6) or by alkali extraction (40). Mitochondria were isolated using the Yeast Mitochondria Isolation Kit (Sigma) and then resuspended in buffer containing 2% Triton X-100, 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.5 mM EDTA and solubilized on ice for 30 min. SDS-PAGE was performed, and samples were probed with anti-Sod1 antibody (a generous gift from Dr. Thomas O’Halloran), anti-TAP antibody (Open Biosystems), anti-Pgk1 antibody (Invitrogen), or anti-Por1 antibody (Molecular Probes).

**Functional Assays for Sod1 and Mac1**—For phenotypic analysis, wild type and mutants were spotted on SC plates, SC–lysine plates, SC –methionine –lysine plates, or media containing ethanol (2%) and glycero (3%) as the sole carbon sources (YPEG). To test superoxide dismutase catalytic activity, protein extracts were obtained using the glass bead/Triton X-100 method, and samples were subjected to nondenaturing gel electrophoresis followed by nitro blue tetrazolium staining (41). Mac1-TAP protein function was tested by spotting 10-fold serial dilutions of cells on YPD, YPEG, and YPEG with 100 μM CuSO\textsubscript{4}.  

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*Fluorescence Microscopy*—A BY4742-derived yeast strain with a functional genomic fusion of GFP at the carboxyl terminus of the *SOD1* or *CCS1* open reading frame was used for localization of Sod1 and Ccs1, respectively (33).

*ChIP PCR Analysis*—Chromatin immunoprecipitation was carried out as previously described (42). Cells were grown for 3 h to mid-log phase in YPD medium, 100 µM CuSO₄ or 500 µM BCS was added, and the incubation continued for an additional 15 min before cross-linking with formaldehyde. After cell lysis by vortexing with glass beads and ultrasonication to shear DNA, 250 µg of protein was immunoprecipitated with IgG-Sepharose beads (GE Healthcare). The precipitated DNA was used for PCR with primers for either the *CTR1* promoter region or the *CMD1* promoter region. The primers used to amplify the *CTR1* promoter region were 5’-TAA GGA TCG AAA CTG CAC CTC AAC-3’ and 5’-ACA TAC AAG ACC CTC TCG AGA TGA CA-3’. The primers used to amplify the *CMD1* promoter region were 5’-CGCTTCCTCTCAATTCCCAAAGT-3’ and 5’-GTG ATG TAG GAC ACT CTC CAA GG-3’. PCRs were performed using serial dilutions of the output DNA to be sure that the reaction was in the linear range, and the ChIP experiment was repeated three times with similar results. The data presented are representative of three independent experiments. Digital images of ChIP results were quantitated using ImageQuant software and processed using Adobe Photoshop.

**RESULTS**

The Ccs1 Copper Chaperone Is Required for Robust Activation of Mac1—In *S. cerevisiae*, expression of the high affinity copper uptake system is regulated by the copper-responsive transcription factor Mac1. Mac1 has been demonstrated to be a nuclear resident protein, and protein fragments have been shown to directly bind copper atoms, suggesting that the copper status of Mac1 could be important to its regulation. The mechanism by which copper is incorporated into this protein remains unknown, and it is unclear how copper might enter or leave the nucleus.

We began by testing whether one of the three known copper chaperones, Atx1, Ccs1, or Cox17, is involved in the regulation of Mac1. A *CTR3-LacZ* reporter plasmid that contains two copies of the CuRE from the *CTR3* promoter upstream of the *LacZ* gene was used to quantitate Mac1 activity. We found that the yeasts lacking *CCS1* display a severe defect in the activation of Mac1 in response to decreased copper availability induced by supplementation of the growth medium with the copper-specific chelator BCS. Cells harboring an *atx1Δ* allele do not show defects in activation of the Mac1 reporter, and, as expected, *mac1Δ* cells are completely defective in *CTR3-LacZ* activity in response to copper deficiency (Fig. 1A). A *cox17Δ* mutant also displays a defect in activation of the Mac1 reporter. However, these same cells show a significant reduction in activation of a reporter gene for the unfolded protein response (supplemental Fig. 1). This shows suggest that the *COX17*-dependent defect in activation of the Mac1 reporter is due to a more general loss of transcriptional regulation. RNA blotting analysis of the *CTR1* transcript confirmed that *ccs1Δ* mutants show decreased induction of this Mac1 target gene in response to copper deprivation as compared with wildtype cells. As expected, *mac1Δ* cells show nearly undetectable levels of *CTR1* mRNA (Fig. 1, B and C). Similar results were observed with the transcript of a second Mac1 target, *FRE1*, indicating that Ccs1 plays a more general, rather than a *CTR1*-specific, role in Mac1 activation.

Cu,Zn-superoxide Dismutase Functions in the Activation of Mac1—The copper chaperone Ccs1 delivers copper to the Sod1 enzyme in a series of steps that are critical for Cu,Zn-superoxide dismutase activation in yeast (16, 30, 43–45). Two possibilities could explain the diminished ability of *ccs1Δ* mutants to activate Mac1 in response to low copper. First, it is possible that Ccs1 functions directly in the activation of Mac1. Second, it is possible that the defect of *ccs1Δ* cells is an indirect effect due to an inability to deliver copper to, and thus activate,
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Sod1. To distinguish between these two possibilities, cells harboring a wild type CCS1 gene but lacking SOD1 were tested for the ability to activate the CTR3-LacZ reporter plasmid in response to copper deprivation. sod1Δ mutants display a Mac1 regulation phenotype that phenocopies ccs1Δ cells, consistent with the observation that yeast Sod1 is largely dependent on Ccs1 for its activation (Fig. 2A) (46). As expected, mac1Δ cells are completely defective in activation of CTR1 expression under conditions of copper deficiency. This reduced ability to activate Mac1 is also evident at the level of Mac1 target mRNA, as shown by RNA blotting analysis of the CTR1 transcript (Fig. 2, B and C). The poor activation of Mac1 in an sod1Δ strain is not due to a general defect in gene transcription, since the induction of the galactose-inducible reporter plasmid is unaffected in sod1Δ cells (Fig. 2D). However, deletion of SOD1 in a strain expressing the constitutively active MAC1up1 allele does not affect the regulation of Mac1 protein (Fig. 2E). These results suggest that the Sod1 protein is also required for physiological Mac1 activation in the same pathway as Ccs1, yet this requirement can be bypassed by a constitutively active variant of the Mac1 protein.

Activation of Mac1 Requires Catalytically Active Sod1—Since deletion of either CCS1 or SOD1 leads to a similar defect in Mac1 target gene activation in response to copper deficiency, it is possible that either one or both of these proteins may be required for the response to copper deficiency in S. cerevisiae. Previous experiments have demonstrated that the C. elegans Sod1 is copper-metallated and activated independently of Ccs1, and indeed there is no CCS1 homologue encoded in the C. elegans genome (35). We exploited these observations to ascertain whether a catalytically active Cu,Zn-superoxide dismutase is sufficient to rescue the defect in Mac1 activation in the absence of Ccs1. The C. elegans Sod1 protein was expressed in ccs1Δ sod1Δ cells, and Mac1 activation was measured. As shown in Fig. 3A, activation of the CTR3-LacZ reporter plasmid is restored by expression of C. elegans Sod1, even in the absence of Ccs1. Superoxide dismutase in-gel assays recapitulate previous findings that this C. elegans Cu,Zn-superoxide dismutase is activated in a Ccs1-independent manner when expressed in the ccs1Δ sod1Δ double mutant (Fig. 3B) (35). These results demonstrate that Sod1 is required for activation of Mac1, and Ccs1 is required only for its ability to deliver copper to and activate Sod1.

\[ \text{CTR3-LacZ reporter plasmid were grown to mid-log phase in complete media or media with 10 µM or 100 µM BCS and β-galactosidase assays were performed. ccs1Δ cells and sod1Δ display similar defects in the ability to activate the Mac1 reporter in response to limiting copper. Samples were analyzed in triplicate and data are representative of at least three independent experiments. B, the induction of Mac1 target mRNA upon copper depletion is decreased in sod1Δ cells. RNA blotting analysis for the CTR1 transcript also indicates that sod1Δ mutants show decreased activation of Mac1 in response to copper deprivation. C, quantification of mRNA blots from B. D, sod1Δ cells WT and sod1Δ cells transformed with a galactose-inducible reporter plasmid were grown for 0, 1, or 2 h in media containing galactose as the sole carbon source. β-Galactosidase activity assays demonstrate that β-galactosidase is transcribed/translated at similar levels to WT sod1Δ mutants. E, WT, MAC1Δ, and isogenic MAC1Δ sod1Δ cells transformed with the CTR3-LacZ reporter plasmid were grown to mid-log phase in synthetic complete media supplemented with 1 µM CuSO4 or 100 µM BCS. MAC1Δ and MAC1Δ sod1Δ cells show similar levels of Mac1 reporter activity which is higher than WT control cells.} \]
To further explore the requirement for Sod1 in Mac1 activation, we expressed either wild type yeast Sod1 or two mutants with largely compromised catalytic activity. We expressed either the SOD1R143D allele that disrupts an invariant residue in the electrostatic loop that guides superoxide to the catalytic site of Sod1 or the SOD1G85R allele that has been identified in humans as a mutation that leads to familial amyotrophic lateral sclerosis (47, 48). Previous studies demonstrate that protein expressed from the SOD1R143D allele exhibits an ~100-fold decrease in catalytic activity (49) and that the protein expressed from the SOD1G85R allele binds copper ions yet exhibits severely diminished superoxide dismutase activity (50). As shown in Fig. 4A, although the plasmid-borne wild type yeast Sod1 is able to fully complement for loss of SOD1 in Mac1 activation, the catalytically compromised Sod1, expressed from either the SOD1R143D allele or the SOD1G85R allele, is largely defective in supporting Mac1 activation in response to copper deprivation.

**Reduction in Cytosolic Oxidative Stress Does Not Rescue Mac1 Activation**—Many studies have established that a primary role of Sod1 is to protect cells from oxidative stress (51–54). Since Sod1 catalytic activity is required for activation of Mac1, and Mac1 is a cysteine-rich protein that could be susceptible to oxidation by superoxide, the inability to robustly activate Mac1 in sod1Δ cells could be the result of increased oxidative stress. Extragenic suppressor mutants have been identified that suppress phenotypes associated with the deletion of SOD1, including the ability to synthesize methionine and lysine and a growth defect on nonfermentable carbon sources. One suppressor is due to a mutation in the PMR1 gene, which encodes a...
protein that transports manganese into the Golgi, resulting in the hyperaccumulation of manganese in the cytosol (55). Due to the ability of manganese to scavenge superoxide radicals, the increase in cytosolic manganese can partially suppress sod1Δ phenotypes (56). Deletion of PMR1 in an sod1Δ background restores the ability of S. cerevisiae to grow on synthetic media lacking methionine and lysine (−methionine −lysine) (Fig. 5A). However, the sod1Δ pmr1Δ cells are deficient in Mac1 activation in response to copper deficiency (Fig. 5B). Furthermore, the addition of manganese ions to the growth medium, previously demonstrated to rescue aerobic growth defects associated with deletion of SOD1 (55), has no effect on induction of the Mac1-responsive reporter plasmid (Fig. 5B).

It has previously been shown that either wild type human Cu,Zn-superoxide dismutase (hSod1) or hSod1 targeted to the mitochondrial matrix (hSod1matrix), can rescue the defects associated with increased oxidative stress in sod1Δ yeast cells (57). Indeed, the inability of sod1Δ yeast cells to grow on non-fermentable carbon sources, such as ethanol and glycerol, is restored by expression of either cytosolic or matrix- localized human Sod1 (Fig. 5C). However, neither wild type nor mitochondrial human Sod1 restored the ability of sod1Δ mutants to activate Mac1 during copper deprivation (Fig. 5D). Furthermore, deletion of the gene encoding the mitochondrial matrix manganese superoxide dismutase, SOD2, does not alter the ability to activate Mac1 in response to copper deprivation.4

Ccs1 and Sod1 Proteins Partially Localize to the Yeast Nucleus—The deletion of PMR1 or the expression of human Sod1 protein are able to restore growth defects associated with increases in cytosolic and mitochondrial oxidative stress, yet neither is able to restore wild type Mac1 activity in response to copper deficiency. Since Mac1 is a nuclear protein, it is possible that Sod1 may be required in the nucleus to promote Mac1 activation. It was previously reported that both Ccs1 and Sod1 are required for Mac1 activation during copper deprivation (Fig. 5E). Further- more, deletion of the gene encoding the mitochondrial matrix manganese superoxide dismutase, SOD2, does not alter the ability to activate Mac1 in response to copper deprivation.4

To test whether the localization of Sod1 is important for its role in activation of nuclear localized Mac1, we tethered the yeast Sod1 protein to the mitochondrial inner membrane space by creating an in-frame fusion of the Sod1 protein to the transmembrane domain of the mitochondrial resident protein Sco2 (12). The intact fusion protein was expressed at levels similar to wild type Sod1 protein, as demonstrated by immunoblot analysis of Triton-solubilized whole cell extracts, and this protein is able to restore the ability of sod1Δ cells to grow on non-fermentable carbon sources or to grow in the absence of lysine (Figs. 6, B and C). Immuno blot analysis of fractionated mitochondria indicated that the Sco2-Sod1 protein is localized exclusively to the mitochondria (Fig. 6D). Although the Sco2-Sod1 fusion protein is capable of suppressing oxygen-depend-
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A

Ccs1-GFP

DAPI

Overlay

DIC

Sod1-GFP

DAPI

Overlay

DIC

B

WT

sod1Δ

Vector

SOD1

SCO2-SOD1

Sod1

Pgk1

C

WT

sod1Δ

sod1Δ + SOD1

sod1Δ + SCO2-SOD1

Sod1

Por1

D

WT

sod1Δ

Vector

pSOD1

pSCO2-SOD1

Sod1

Por1

E

300

200

100

50

0

Control

10 μM BCS

100 μM BCS

WT

sod1Δ

sod1Δ + SOD1

sod1Δ + SCO2-SOD1

WT cells co-transformed with a CTR3-LacZ reporter and an empty vector and sod1Δ cells co-transformed with CTR3-LacZ reporter and an empty vector, a plasmid encoding the yeast SOD1 gene (SOD1), or a plasmid encoding the tethered yeast SCO2-SOD1 fusion gene (SCO2-SOD1) were grown to mid-log phase in complete media or media with 10 μM or 100 μM BCS, and β-galactosidase assays were performed.

FIGURE 6. Ccs1 and Sod1 proteins partially localize to the yeast nucleus. A, after staining with the DNA dye 4',6-diamidino-2-phenylindole (DAPI), Ccs1-GFP and SOD1-GFP cells were examined by fluorescence microscopy to visualize the whole cell (differential interference contrast; DIC), the nucleus (DAPI), or either the Ccs1-GFP or the Sod1-GFP fusion protein. Nuclear localization of Ccs1-GFP and Sod1-GFP is evident from the overlay image of the 4',6-diamidino-2-phenylindole and GFP images. B, immunoblot analysis using an antibody against yeast Sod1 (Sod1) demonstrates that Triton-solubilized whole cell protein extracts from WT cells and sod1Δ cells expressing either the SOD1 gene or the SCO2-SOD1 gene from a low copy centromeric plasmid contain similar amounts of Sod1 protein. Immunoblot analysis of 3-phosphoglycerate kinase (Pgk1) shows that loading of each protein sample is equivalent. C, serial dilution on plates containing ethanol and glycerol (YPEG) or plates lacking lysine (–lys) indicates that sod1Δ cells expressing either the yeast SOD1 gene (SOD1) or the mitochondrial tethered yeast SOD1 (SCO2-SOD1) gene are able to grow on the nonfermentable carbon sources or in the absence of lysine. D, yeast cells were separated to separate the mitochondria (M) from the soluble protein extract (S), and proteins were solubilized with 2% Triton. Immunoblot analysis using an antibody against yeast Sod1 (Sod1) demonstrates that a significant portion of Sod1 protein from either WT cells or sod1Δ cells expressing wild type Sod1 is found in the soluble fraction, whereas some Sod1 protein is also localized to the mitochondria. In yeast expressing SCO2-Sod1, the Sod1 fusion protein is localized exclusively to the mitochondria. Immunoblot analysis of the mitochondrial membrane protein porin (Porin) demonstrates that fractionation results in distinct separation of the soluble and mitochondrial portions of the cell. E, WT cells co-transformed with a CTR3-LacZ reporter and an empty vector and sod1Δ cells co-transformed with CTR3-LacZ reporter and an empty vector, a plasmid encoding the yeast SOD1 gene (SOD1), or a plasmid encoding the tethered yeast SCO2-SOD1 fusion gene (SCO2-SOD1) were grown to mid-log phase in complete media or media with 10 μM or 100 μM BCS, and β-galactosidase assays were performed.

oxide levels by Sod1 or by Sod1 mimetics is not sufficient to restore Mac1 activity. Furthermore, these experiments suggest that localization of Sod1 is important for its function in the regulation of Mac1.

Sod1 Is Required for Copper Deficiency-induced DNA Binding by Mac1—Previous studies indicate that Mac1 is regulated by copper deficiency at several different levels, including intramolecular interactions, trans-activation, and the binding of Mac1 to promoter CuRE elements. We used yeast two-hybrid analysis to test whether in response to copper deficiency Sod1 facilitates the loss of the previously observed intramolecular interaction between the DBD and the TAD of Mac1. These experiments showed that there are no significant differences in the intramolecular interaction of Mac1 in a wild type strain as compared with an isogenic sod1Δ strain (supplemental Fig. 2A). We tested whether the trans-activation activity of Mac1 is dependent on Sod1 using a yeast one-hybrid analysis of a Gal4 DBD-Mac1 TAD fusion protein. These experiments showed no difference in activation in response to copper deprivation in either the wild type strain or the isogenic sod1Δ strain, suggesting that Sod1 does not influence trans-activation by Mac1 (supplemental Fig. 2B). ChIP PCR using a functional genomic TAP-tagged version of MAC1 was used to test whether Sod1 affects the DNA binding of Mac1-TAP to CuRE sequences in the CTR1 promoter in response to copper deprivation. The Mac1-TAP fusion protein is expressed at similar levels in both wild type and sod1Δ cells, as demonstrated by immunoblotting (Fig. 7A). Interestingly, the Mac1-TAP fusion protein is functional as demonstrated by the ability of this strain to grow on nonfermentable carbon sources, conditions under which the copper-dependent mitochondrial cytochrome oxidase activity is required (Fig. 7B). As shown in Fig. 7, C and D, Mac1-TAP binding to the CTR1 promoter region is enhanced nearly 4-fold after treatment with the copper chelator BCS. Although binding of Mac1 to the CTR1 promoter appears to have a slight basal elevation in sod1Δ cells, the copper deficiency-induced enhancement of Mac1 binding to the CTR1 promoter is severely compromised in an sod1Δ mutant. Taken together, these results suggest that localization of Sod1 is important for its function in the regulation of Mac1.
Sod1 activity positively influences the ability of Mac1 to bind to the promoter of its target genes in response to copper deficiency.

**DISCUSSION**

Given the importance of copper for aerobic life, all organisms must adjust to changes in intracellular and extracellular levels to ensure an adequate, but not toxic, supply. In *S. cerevisiae*, the expression of genes encoding copper acquisition proteins that include the Ctr1 and Ctr3 high affinity Cu$^{2+}$ importers and the Cu$^{2+}$-Fre1 metalloreductase is induced under conditions of copper deficiency by the Mac1 transcription factor. Previous studies have established that two homologous repeats in Mac1, REP-I and REP-II, contain a critical array of cysteine and histidine residues that are required for regulatory responses to copper. Moreover, studies have demonstrated that Mac1 is bound to CuRE elements in the promoter regions of its target genes in vivo in response to low extracellular copper availability, and the occupation of the CuRE elements is reduced under conditions of copper adequacy (20, 29). Furthermore, studies suggest that Mac1 is subject to changes in intramolecular interactions as a function of copper availability and that the Mac1 trans-activation domain is regulated by copper. Finally, reports suggest that Mac1 is a phosphoprotein, but the precise role of this post-translational event in Mac1 function is not understood (60). Taken together, we currently know little about the mechanisms by which Mac1 senses and or responds to changes in copper availability to activate transcription of genes involved in copper acquisition.

Previous studies suggest that Mac1 binds copper in vivo with a stoichiometry of eight copper ions to one Mac1 protein (24) and that a polypeptide fragment corresponding to the REP-I cysteine-rich minimal regulatory domain binds four Cu$^{2+}$ ions in a tetracopper cluster (61). A Mac1 genetic variant, *MAC1*<sup>up1</sup>, harbors a histidine to glutamine replacement and both constitutively binds CuRE elements in vivo and constitutively activates target gene transcription. The observation that Mac1<sup>up1</sup> has been shown to co-purify with only four copper atoms suggests a model whereby Mac1 may be regulated by reversible metallation in vivo. To address this hypothesis, we tested whether Mac1-mediated target gene activation in response to decreased bioavailable extracellular copper levels is dependent on any of the currently known soluble copper chaperones.

Although our initial studies identified the Ccs1 copper chaperone for Sod1 as being important for Mac1-mediated activation of both *CTR3*-lacZ reporter gene and endogenous *CTR1* gene expression, our subsequent experiments demonstrate that this requirement for Ccs1 in Mac1 activation is indirect, and Ccs1 is simply required for the activation of Sod1. This conclusion is based on the observation that a *C. elegans* Sod1 protein that is copper-loaded in a Ccs1-independent manner can restore full activation of Mac1 in the absence of the yeast *CCS1* and *SOD1* gene. Moreover, the observation that wild type, but not catalytically inactive, yeast Sod1 proteins can rescue the Mac1 activation defect of a strain lacking Sod1 suggests that catalytic activity is necessary for Mac1 activation under conditions of copper deficiency. Because the catalytically compromised Sod1 mutants have previously been shown to bind copper, this defect in Mac1 activation is unlikely to be due to the generation of an altered intracellular copper pool.

Previous studies indicate that Mac1 is regulated at multiple levels in response to changes in the availability of copper. Our experiments demonstrate that Sod1 is required for copper deficiency-induced binding to CuRE elements in the promoter of *CTR1* but not for changes in intramolecular interactions nor for trans-activation when the Mac1 TAD is delivered to the DNA via a surrogate DNA binding domain. How does Sod1 facilitate Mac1 DNA binding in response to copper deficiency? Although Sod1 catalytic activity is essential for this function, the use of pharmacological superoxide scavengers and genetic suppression of *sod1*<sup>Δ</sup> supports the notion that the destruction or sequestration of superoxide anion *per se*, is not the critical step.
Given that the superoxide anion disproportionation reaction carried out by Sod1 also results in the generation of hydrogen peroxide (H₂O₂), it is possible that Sod1-generated H₂O₂ serves as an important signal or reactant for Mac1 activation under conditions of copper deficiency. H₂O₂ is a well established signaling agent that is known to regulate receptor phosphorylation, transcription factor activity, and other regulatory events (62, 63). One well characterized example is OxyR, a bacterial H₂O₂-responsive transcription factor harboring a cysteine that is converted to sulfenic acid as a key step in its activation pathway (64, 65). Additionally, the yeast Yap1 protein is also modified at cysteine residues by H₂O₂ as a central component of its activation mechanism to protect against oxidative stress (66–69). Perhaps more relevant to Mac1, the bacterial Hsp33 protein chaperone has been demonstrated to be activated for substrate binding via H₂O₂-mediated zinc ejection and a concomitant conformational change (70–72). Given that the constitutively active Mac1up protein has been shown to bind fewer copper atoms than wild type Mac1, it is possible that the loss of copper from Mac1 activates its DNA binding function. The localized generation of H₂O₂ by Sod1 could facilitate cysteine oxidation, thereby enhancing the lability of bound copper. Interestingly, our experiments indicate that Mac1up protein functions to activate CTR1 expression independently of Sod1.

Although further investigations will be necessary to decipher the mechanism by which Sod1 activates Mac1, our results suggest that Sod1 and Mac1 are at least partially co-localized. Although yeast Sod1 is known to be present in both the cytosol and mitochondrial intermembrane space, our studies here suggest that Sod1 may also partially localize to the nucleus, as might the Ccs1 copper chaperone that is required for Sod1 activation. Previous studies in mammals have also noted the presence of a subfraction of Cu,Zn-superoxide dismutase, and Ccs1, in the nucleus. Moreover, although tethering Sod1 to the mitochondrial inner membrane was able to reverse oxidative stress phenotypes associated with sod1Δ cells, the Sco2-Sod1 fusion protein did not support wild type Mac1 activation in response to copper deficiency. Although these observations are consistent with a potential nuclear role for Sod1 in Mac1 activation, two-hybrid experiments were negative, and thus it is currently unclear whether there is a direct interaction between Mac1 and Sod1. Moreover, it is not clear why yeast and C. elegans Sod1 support Mac1 activation, but the expression of human Cu,Zn-superoxide dismutase, capable of suppressing oxidative stress phenotypes, cannot support Mac1 activation. Perhaps there are distinct structural differences between human and yeast or worm Sod1 that preclude a functional interaction, or human Sod1 may not exhibit the same localization pattern in yeast as the other Sod1 proteins.

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