

Transcriptional Activation in Yeast in Response to Copper Deficiency Involves Copper-Zinc Superoxide Dismutase^{*S}

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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)³ 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-I (C1) and REP-II (C2), that each binds four Cu¹⁺ ions in a tetranuclear copper cluster (24). Mutations in the C1 domain lead to constitutively active Mac1^{up} 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

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³ The abbreviations used are: TAD, trans-activation domain; DBD, DNA binding domain; CuRE, copper-responsive element; GFP, green fluorescent protein; BCS, bathocuproinedisulfonic acid; SC, synthetic complete; ChIP, chromatin immunoprecipitation; hSod1, human Sod1; WT, wild type; TAP, tandem affinity purification.

Mac1^{up1} protein binds fewer copper ions per molecule than the wild type protein (24). Moreover, Mac1^{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 disproportionation 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 *sod1Δ* 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 *sod1Δ pmr1Δ* double mutant was created by deletion of *PMR1* in an *sod1Δ* strain, and the *ccs1Δ sod1Δ* double mutant was created by deletion of *SOD1* in a *ccs1Δ* strain. The *SOD1*-GFP and the *MAC1*-TAP strains were obtained from the GFP- and TAP-tagged collections (32, 33). The *MAC1*-TAP *sod1Δ* strain was created by deletion of *SOD1* in the *MAC1*-TAP background. The *MAC1^{up1}* strain and its wild type parental strain have been previously described (19), and the *MAC1^{up1} sod1Δ* 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 *sod1Δ* strain was created by deletion of the *SOD1* gene in the Y190 background (34).

The *GALI-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 *γSOD1* 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 *γSOD1* as an *XbaI/XhoI* fragment into the pRS415 vector. The *SOD1^{R143D}* and the *SOD1^{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^{up1}* 240–417 plasmids were a generous gift from Dr. Dennis Winge (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 pGBKT7 plasmid backbone (Clontech).

***β*-Galactosidase Activity Assay for Mac1 Function**—Cells were transformed with the previously described Mac1 reporter plasmid pCm64*CTR3-LacZ* or pRS*CTR3-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 [³²P]dCTP to be used as probes. Quantification of the RNA blot was performed using ImageQuant TL version 2003.02 software (Amersham 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 glycerol (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 CuSO₄.

Transcription in Response to Copper Deficiency

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 CuSO_4 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'-CGCTTCCTCTCAATTCCCAA-AGT-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 *atx1* Δ allele do not show defects in activation of the Mac1 reporter, and, as expected, *mac1* Δ cells are completely defective in *CTR3-LacZ* activity in response to copper deficiency (Fig. 1A). The *cox17* Δ 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). These results 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 *ccs1* Δ mutants show decreased induction of this Mac1 target gene

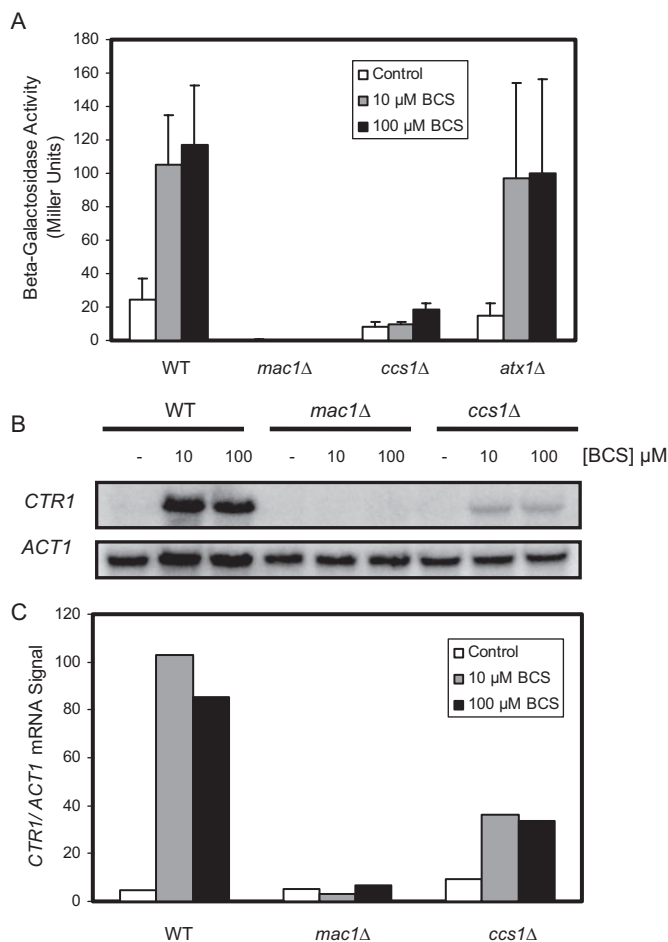


FIGURE 1. The Ccs1 copper chaperone is required for robust activation of Mac1. A, *ccs1* Δ cells show defects in the induction of a Mac1 reporter plasmid upon copper depletion. WT, *mac1* Δ , and *ccs1* Δ cells transformed with the pCm64*CTR3-LacZ* reporter plasmid were grown to mid-log phase in synthetic complete media with no supplementation (Control) or media treated with either 10 or 100 μM copper chelator BCS. β -Galactosidase assays were performed and analyzed in triplicate. The data are representative of at least three independent experiments. B, the induction of Mac1 target mRNA upon copper depletion is decreased in *ccs1* Δ cells. RNA blotting analysis for the *CTR1* transcript was carried out from cells grown under control conditions (–) or in the presence of the indicated concentration of the copper-specific chelator BCS. The *ACT1* transcript is shown as an RNA loading control. C, quantification of mRNA blots from B.

in response to copper deprivation as compared with wild type cells. As expected, *mac1* Δ 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.⁴

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 *ccs1* Δ 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 *ccs1* Δ cells is an indirect effect due to an inability to deliver copper to, and thus activate,

⁴ L. K. Wood and D. J. Thiele, unpublished data.

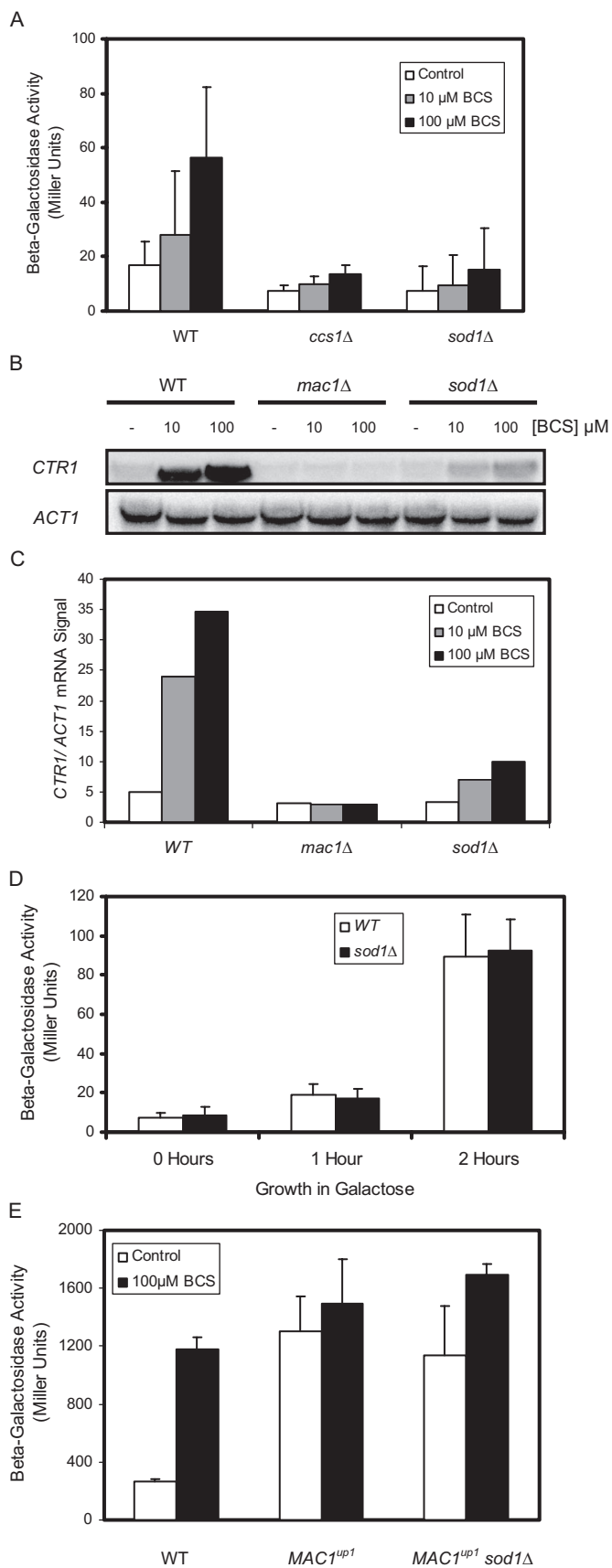


FIGURE 2. Cu,Zn-superoxide dismutase functions in the activation of Mac1. *A*, *sod1* Δ cells exhibit defects in the induction of a Mac1 reporter plasmid upon copper depletion. WT, *ccs1* Δ , and *sod1* Δ cells transformed with the

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. *sod1* Δ mutants display a Mac1 regulation phenotype that phenocopies *ccs1* Δ cells, consistent with the observation that yeast *Sod1* is largely dependent on *Ccs1* for its activation (Fig. 2*A*) (46). As expected, *mac1* Δ 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 *sod1* Δ strain is not due to a general defect in gene transcription, since the induction of the galactose-inducible reporter plasmid is unaffected in *sod1* Δ cells (Fig. 2*D*). However, deletion of *SOD1* in a strain expressing the constitutively active *MAC1*^{up1} allele does not affect the regulation of Mac1 protein (Fig. 2*E*). 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 *ccs1* Δ *sod1* Δ cells, and Mac1 activation was measured. As shown in Fig. 3*A*, 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 *ccs1* Δ *sod1* Δ double mutant (Fig. 3*B*) (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*.

CTR3-LacZ reporter plasmid were grown to mid-log phase in complete media or media with 10 μ M or 100 μ M BCS and β -galactosidase assays were performed. *ccs1* Δ cells and *sod1* Δ 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 *sod1* Δ cells. RNA blotting analysis for the *CTR1* transcript also indicates that *sod1* Δ mutants show decreased activation of Mac1 in response to copper deprivation. *C*, quantification of mRNA blots from *B*. *D*, *sod1* Δ cells WT and *sod1* Δ 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 in *sod1* Δ mutants. *E*, WT, *MAC1*^{up1}, and isogenic *MAC1*^{up1} *sod1* Δ cells transformed with the *CTR3-LacZ* reporter plasmid were grown to mid-log phase in synthetic complete media supplemented with 1 μ M CuSO_4 or 100 μ M BCS. *MAC1*^{up1} and *MAC1*^{up1} *sod1* Δ cells show similar levels of Mac1 reporter activity which is higher than WT control cells.

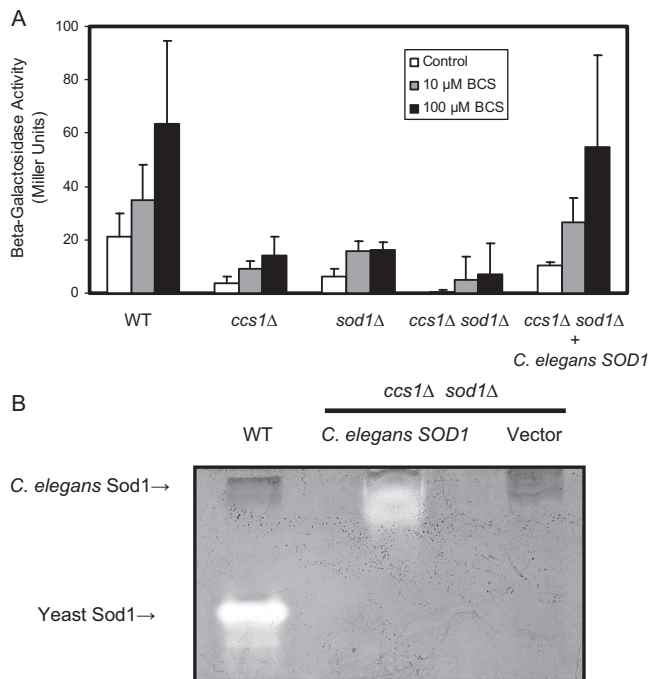


FIGURE 3. *C. elegans* SOD1 rescues Mac1 activation in a Ccs1-independent manner. *A*, the *SOD1* gene from *C. elegans* (*C. elegans SOD1*) rescues the activation of Mac1 protein in *ccs1Δ sod1Δ* mutants. WT, *ccs1Δ*, *sod1Δ*, and *ccs1Δ sod1Δ* cells co-transformed with a *CTR3-LacZ* reporter plasmid and an empty pRS415 vector. *ccs1Δ sod1Δ* double mutants were also co-transformed with a vector carrying the *SOD1* gene from *C. elegans* (*C. elegans SOD1*). Growth to mid-log phase in complete media or media with 10 or 100 μM BCS and subsequent β-galactosidase assays indicates the *C. elegans SOD1* rescues the activation of Mac1 in a *CCS1*-independent manner. *B*, consistent with previous reports that copper is inserted into the *C. elegans* Sod1 protein in a Ccs1-independent manner, superoxide dismutase in-gel activity assays demonstrate that the *C. elegans* Sod1 protein has catalytic activity in the absence of Ccs1, and the *ccs1Δ sod1Δ* double mutant shows no detectable superoxide dismutase activity.

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 *SOD1^{R143D}* allele that disrupts an invariant residue in the electrostatic loop that guides superoxide to the catalytic site of Sod1 or the *SOD1^{G85R}* 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 *SOD1^{R143D}* allele exhibits an ~100-fold decrease in catalytic activity (49) and that the protein expressed from the *SOD1^{G85R}* 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 *SOD1^{R143D}* allele or the *SOD1^{G85R}* allele, is largely defective in supporting Mac1 activation in response to copper deficiency. Immunoblot analysis demonstrates that both the wild type and the mutant Sod1 proteins are expressed at similar levels from low copy episomal plasmids (Fig. 4B). Consistent with previous studies, the products of the *SOD1^{R143D}* and *SOD1^{G85R}* alleles display little detectable superoxide dismutase activity (Fig. 4C). These results suggest that Sod1 catalytic activity is required for Mac1 activation in response to copper

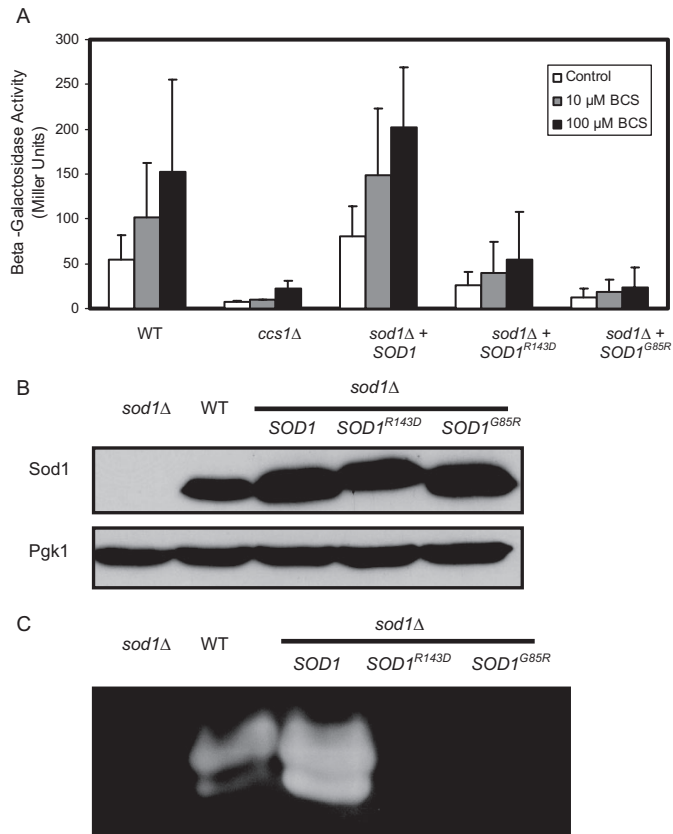


FIGURE 4. Activation of Mac1 requires catalytically-active Sod1. *A*, catalytic activity of Sod1 is necessary for robust activation of Mac1. WT cells were co-transformed with a *CTR3-LacZ* reporter and an empty vector, and *sod1Δ* cells were co-transformed with *CTR3-LacZ* reporter and an empty vector, a plasmid encoding the yeast *SOD1* gene (*SOD1*), or a plasmid encoding the yeast *SOD1* gene with either a mutation that disrupts the proton channel of the Sod1 enzyme (*SOD1^{R143D}*) or a mutation corresponding to a human mutation identified in familial amyotrophic lateral sclerosis (*SOD1^{G85R}*). Cells were grown to mid-log phase in complete media or media with 10 or 100 μM BCS, and β-galactosidase assays were performed. Although the wild type yeast Sod1 can rescue Mac1 activation in *sod1Δ* cells, neither of the catalytically inactive Sod1 mutants' proteins are able to restore Mac1 reporter activity. *B*, immunoblot analysis using an antibody against yeast Sod1 (*Sod1*) demonstrates that catalytically inactive Sod1 proteins are expressed at levels equivalent to wild type Sod1. Immunoblot analysis of 3-phosphoglycerate kinase (*Pgk1*) shows that loading of each protein sample is equivalent. *C*, the *Sod1^{R143D}* and the *Sod1^{G85R}* proteins are catalytically inactive as determined using superoxide dismutase in-gel activity assays. Cells transformed with either an empty vector or the mutant *SOD1^{R143D}* gene or the *SOD1^{G85R}* gene exhibit no detectable superoxide dismutase activity, whereas WT cells and *sod1Δ* expressing the WT *SOD1* gene show considerable superoxide dismutase activity.

deprivation and that the incorporation of copper into Sod1 is not sufficient for its role in the activation of Mac1.

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 (hSod1^{matrix}), 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 partially localize to the nucleus of mammalian cells (45, 58, 59). We carried out fluorescence microscopy of cells expressing a functional Ccs1-GFP fusion protein. As shown in Fig. 6A, Ccs1-GFP partially localizes to the yeast nucleus. Moreover, fluorescence microscopy of a functional Sod1-GFP fusion protein showed that this protein also partially localizes to the nucleus of cells (Fig. 6A). Microscopy was carried out using cells grown in the presence of either 1 μM CuSO₄ or 100 μM BCS, and no difference was seen in the localization of either the Ccs1-GFP or Sod1-GFP proteins.

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 trans-membrane domain of the mitochondrial resident protein Sco2 (12). The intact fusion protein was expressed at levels similar to the 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). Immunoblot 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-

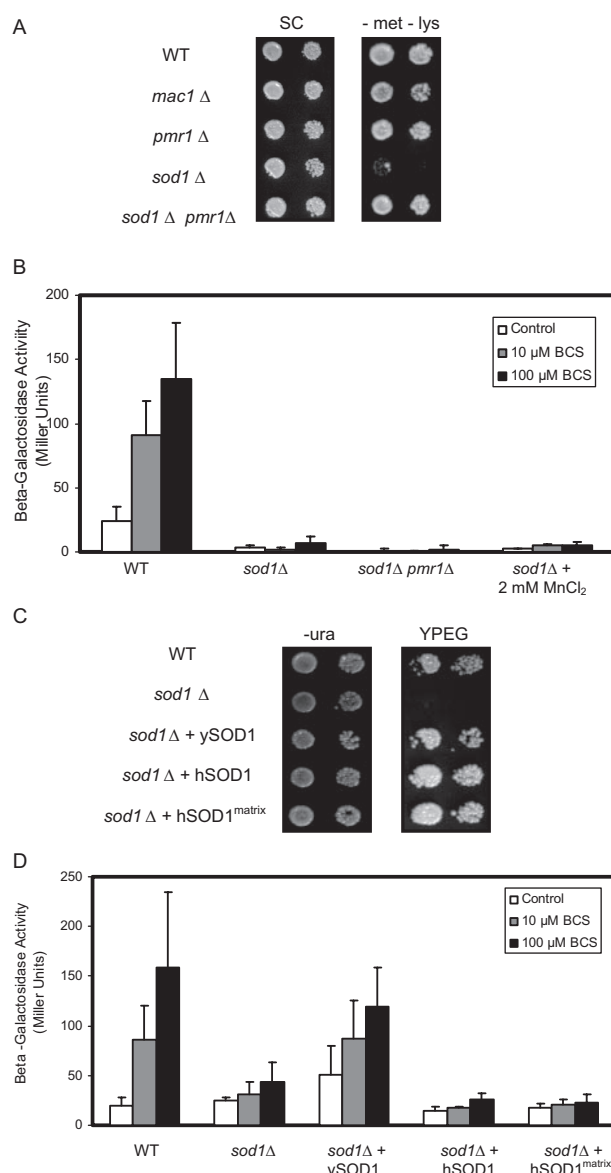


FIGURE 5. Cytosolic reduction in oxidative stress does not rescue Mac1 activation. A, deletion of *PMR1* is able to rescue the sensitivity to oxygen seen in *sod1Δ* cells. Serial dilutions of WT, *mac1Δ*, *pmr1Δ*, *sod1Δ*, and *sod1Δ pmr1Δ* cells were plated on synthetic complete (SC) media or on SC –methionine –lysine media. Deletion of *PMR1* is able to rescue the growth defect of *sod1Δ* cells on SC media without methionine or lysine. B, neither the deletion of *PMR1* nor the addition of exogenous MnCl₂, both of which rescue the oxidative stress phenotypes associated with *SOD1* deletion, is able to complement the defect in Mac1 activation seen in *sod1Δ* mutants. C, yeast or human Sod1 rescues the oxidative stress phenotype associated with *SOD1* deletion. Serial dilutions spotted onto plates containing ethanol and glycerol (YPEG) indicated that *sod1Δ* cells expressing either the yeast *SOD1* gene or the wild type or the mitochondrial matrix tethered human *SOD1* gene are able to grow on the nonfermentable carbon sources. D, the expression of the yeast, but not the human, *SOD1* gene is able to restore Mac1 activity in *sod1Δ* cells. Although the transformation of *sod1Δ* cells with a vector encoding yeast *SOD1* gene restores activity of a Mac1 reporter to wild type levels, transformation with either an empty vector or wild type or the mitochondrial matrix-tethered human *SOD1* does not restore the ability of *sod1Δ* cell to activate the reporter.

ent growth defects when tethered in the mitochondrial inner membrane space, it is unable to restore the ability of *sod1Δ* cells to activate Mac1 in response to low copper (Fig. 6E). Taken together, these results indicate that a global reduction in super-

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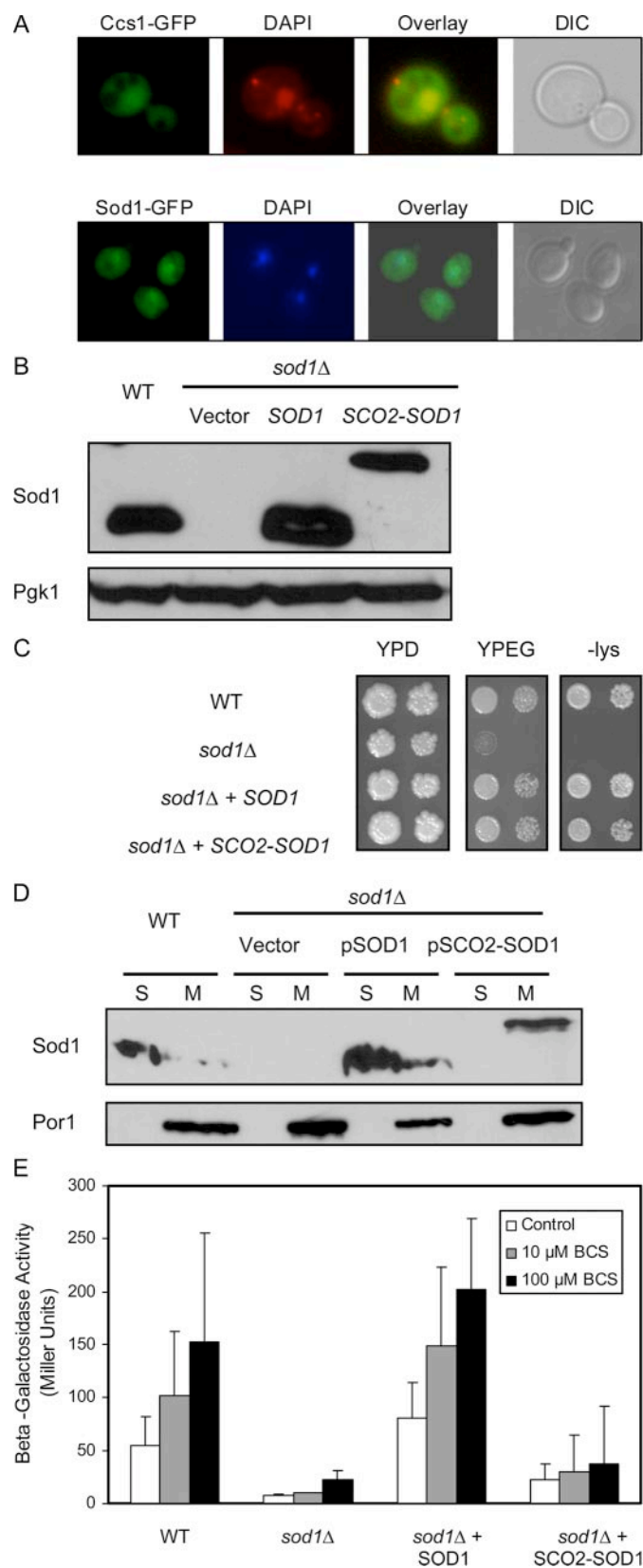


FIGURE 6. Ccs1 and Sod1 proteins partially localize to the yeast nucleus. *A*, after staining with the DNA dye 4',6-diamidino-2-phenylindole (DAPI), Ccs1-GFP and SOD1-GFP cells were examined by fluorescence microscopy to visualize the whole cell (differential interference contrast; DIC), the nucleus (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

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. 2*A*). 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. 2*B*). 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. 7*A*). Moreover, the Mac1-TAP 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. 7*B*). 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

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 fractionated 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 (*Por1*) 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 μ M or 100 μ M BCS, and β -galactosidase assays were performed.

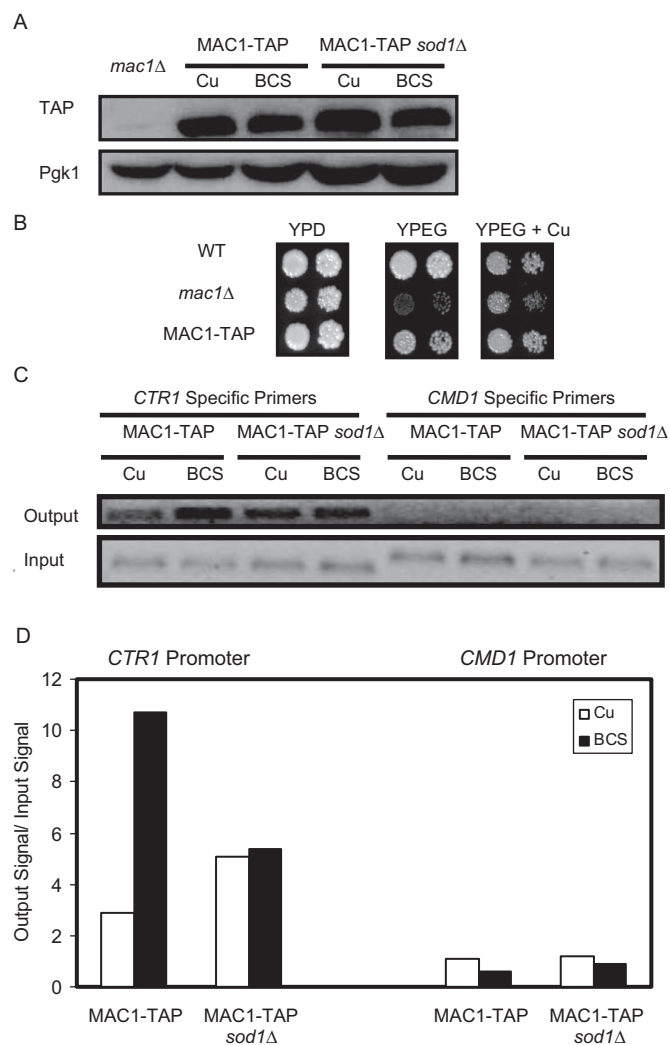


FIGURE 7. Sod1 is required for copper deficiency-induced Mac1 DNA binding. *A*, immunoblot analysis with an antibody against the TAP tag (*TAP*) indicates that either WT or *sod1Δ* cells with a genomic TAP-tag of the *MAC1* gene (*MAC1-TAP*) express similar levels of the Mac1 protein. Expression of 3-phosphoglycerate kinase (*Pgk1*) was used as a loading control. *B*, serial dilutions of WT, *mac1Δ*, and *MAC1-TAP* demonstrate that WT and *MAC1-TAP* cells can grow on nonfermentable carbon sources (YPEG), whereas *mac1Δ* cells show a severe growth defect on YPEG. *C*, enrichment of the *CTR1* promoter DNA by ChIP PCR is evident after treatment of *MAC1-TAP* with 500 μ M BCS for 15 min. Isogenic *MAC1-TAP sod1Δ* cells do not demonstrate significant enrichment of the *CTR1* promoter. There is no apparent binding of Mac1 at the calmodulin (*CMD1*) promoter. *D*, quantification of ChIP results from *C*.

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^{1+} 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 histi-

dine 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^{1+} ions in a tetracopper cluster (61). A Mac1 genetic variant, *MAC1^{up1}*, harbors a histidine to glutamine replacement and both constitutively binds CuRE elements *in vivo* and constitutively activates target gene transcription. The observation that *Mac1^{up1}* 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 *sod1Δ* supports the notion that the destruction or sequestration of superoxide anion *per se*, is not the critical step.

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Given that the superoxide anion disproportionation reaction carried out by Sod1 also results in the generation of hydrogen peroxide (H_2O_2), it is possible that Sod1-generated H_2O_2 serves as an important signal or reactant for Mac1 activation under conditions of copper deficiency. H_2O_2 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 H_2O_2 -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 H_2O_2 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 H_2O_2 -mediated zinc ejection and a concomitant conformational change (70–72). Given that the constitutively active Mac1^{up} 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 H_2O_2 by Sod1 could facilitate cysteine oxidation, thereby enhancing the lability of bound copper. Interestingly, our experiments indicate that Mac1^{up1} 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,⁴ 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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