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ORIGINAL PAPER

Species-specific activation of Cu/Zn SOD by its CCS copper chaperone in the pathogenic yeast *Candida albicans*

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Abstract *Candida albicans* is a pathogenic yeast of important public health relevance. Virulence of *C. albicans* requires a copper and zinc containing superoxide dismutase (SOD1), but the biology of *C. albicans* SOD1 is poorly understood. To this end, *C. albicans* SOD1 activation was examined in baker's yeast (*Saccharomyces cerevisiae*), a eukaryotic expression system that has proven fruitful for the study of SOD1 enzymes from invertebrates, plants, and mammals. In spite of the 80 % similarity between *S. cerevisiae* and *C. albicans* SOD1 molecules, *C. albicans* SOD1 is not active in *S. cerevisiae*. The SOD1 appears incapable of productive interactions with the copper chaperone for SOD1 (CCS1) of *S. cerevisiae*. *C. albicans* SOD1 contains a proline at position 144 predicted to dictate dependence on CCS1. By mutation of this proline, *C. albicans* SOD1 gained activity in *S. cerevisiae*, and this activity was independent of CCS1. We identified a putative *CCS1* gene in *C. albicans* and created heterozygous and homozygous gene deletions at this locus. Loss of *CCS1* resulted in loss of SOD1 activity, consistent with its role as a copper chaperone. *C. albicans* CCS1 also restored activity to *C. albicans* SOD1 expressed in *S. cerevisiae*. *C. albicans* CCS1 is well adapted for activating its partner SOD1 from *C. albicans*, but not SOD1 from *S. cerevisiae*. In spite of the high degree of homology between the SOD1 and CCS1 molecules in these two fungal species, there exists a species-specific barrier in CCS–SOD interactions which may reflect the vastly different lifestyles of the pathogenic versus the noninfectious yeast.

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Introduction

From *Escherichia coli* to humans, the copper and zinc containing superoxide dismutase (SOD) enzyme, also known as SOD1, participates in reactive oxygen metabolism by disproportionating superoxide anion to hydrogen peroxide and oxygen [1, 2]. The chemical reaction is conducted by a copper ion at the active site, whereas the zinc cofactor provides more of a structural role. In addition to metal cofactors, an intramolecular disulfide in each SOD1 monomer stabilizes the quaternary structure [3]. The maturation process for SOD1 in which the apo-reduced polypeptide is converted to an active metallated enzyme has been thoroughly investigated. In eukaryotes, this maturation requires a helper protein known as the copper chaperone for SOD (CCS) that acts to insert copper and oxidize the disulfide. The zinc is acquired through an unknown mechanism. CCS was originally identified in baker's yeast (*Saccharomyces cerevisiae*) as CCS1 [4], and is now known to span nearly all eukaryotic phyla; however, it is absent in bacteria [5]. A peculiar exception is the nematode *Caenorhabditis elegans*, which has evolved with no CCS accessory factor for its SOD1 [6].

CCS harbors three distinct domains that work in concert to capture copper, to dock with SOD1, and to transfer the metal and oxidize the disulfide [7, 8]. The N-terminal domain I is similar to the ATX1 family of soluble copper chaperones, which includes a CXXC copper binding site that participates in copper capture from an upstream source and insertion of copper into SOD1 [7, 9–11]. A central domain II exhibits homology to the SOD1 target and serves

to physically dock CCS to apo-SOD1 [12–15]. The C-terminal domain III harbors a CXC motif that plays a critical role in oxidation of the SOD1 disulfide and may also bind copper [7, 9, 16–19]. This structural paradigm of CCS has been well conserved with minor exceptions. For example, certain insect CCS molecules (e.g., CCS from *Drosophila melanogaster*) lack the N-terminal CXXC copper site [20], and the fission yeast *Schizosaccharomyces pombe* CCS carries a fourth cysteine-rich domain at the C-terminus that is used in copper buffering [21]. In spite of these differences, CCS–SOD1 interactions are well conserved across diverse species. When expressed in *S. cerevisiae*, SOD1 molecules from *Drosophila*, *Arabidopsis*, and humans can all be activated by the yeast CCS1 [20, 22–24]. The converse is also true: *S. cerevisiae* SOD1 is well activated by expression of plant, mammalian, and *S. pombe* CCS molecules [4, 21, 25], and partially by *Drosophila* CCS lacking the N-terminal CXXC copper site [20].

CCS is not the sole means for activating SOD1, and in most eukaryotes it can also acquire copper and oxidize the disulfide through a CCS-independent method (reviewed in [5]). The dependence on CCS can be dictated by a single residue in the SOD1 polypeptide, namely, the position corresponding to proline 144 in *S. cerevisiae* SOD1. In studies with *S. cerevisiae*, P144 strongly inhibits activation of SOD1 in vivo without CCS1 [5, 24, 26, 27]. P144 appears to restrict SOD1 disulfide oxidation and dimer formation without the aid of CCS1 [5, 26, 28]. However, substitution with a non-proline residue at position 144 (e.g., serine, leucine, or glutamine) results in some disulfide oxidation in the absence of CCS1, and SOD1 can be activated without CCS1 [5, 24, 26, 27]. Thus far, P144 has been noted only in SOD1 molecules of certain *Ascomycota* fungi; all plant and animal SOD1 molecules contain non-proline residues at the equivalent position, and these SOD1 molecules can be activated with or without a copper chaperone [5]. Yet even in these higher organisms, the CCS pathway is the preferred mechanism for SOD1 activation [5].

The pathogenic fungus *Candida albicans* is an interesting model system to study SOD1 activation. Unlike all other eukaryotes studied to date, this pathogen has uniquely evolved with two cytosolic SODs: SOD1 and a highly irregular manganese-containing SOD, known as SOD3 [29–31]. The rationale for dual SODs with distinct metal requirements is not known; however, it may reflect the unique lifestyle of this organism, which can switch from a harmless commensal yeast in humans to an invasive infectious form [32]. The *C. albicans* pathogen requires SOD1 for virulence [33], but little is known about the biology of this important enzyme for pathogenesis. As with other *Ascomycota* fungi, *C. albicans* SOD1 contains P144 and is therefore predicted to require a copper chaperone, but a CCS molecule has not been described for *C. albicans*.

In this study, we examine the expression of *C. albicans* SOD1 in the native *C. albicans* host as well as the heterologous *S. cerevisiae* expression system. We find that in spite of its close homology to *S. cerevisiae* SOD1, *C. albicans* SOD1 is not capable of activation by the *S. cerevisiae* CCS1. We identified a single CCS-encoding gene in *C. albicans* that is necessary to activate *C. albicans* SOD1 in both the native *C. albicans* host and the heterologous *S. cerevisiae* system. Through our comparative studies of CCS molecules from insects, humans, and fungi, we describe a species-specific barrier to CCS activation of SOD1 in the *C. albicans* pathogen.

Materials and methods

Yeast strains and growth conditions

C. albicans yeast strains were cultured at 30 °C either in an enriched yeast extract–peptone–2 % dextrose medium or in a minimal synthetic complete (SC) medium [34], and *S. cerevisiae* strains were grown in SC medium to maintain episomal plasmids. *S. cerevisiae sod1Δ* mutants were maintained in anaerobic culture jars (BBL GasPak) in medium containing 15 mg/L ergosterol and 0.5 % Tween 80 to support anaerobic growth. For tests of *S. cerevisiae* aerobic lysine auxotrophy, serial dilutions of cells (10^5, 10^4, 10^3, and 10^2 in number) were spotted onto SC medium containing or lacking lysine and cells were allowed to grow for 2–3 days either in air or in anaerobic culture jars.

The *S. cerevisiae sod1Δ::KanMX4* and *ccs1Δ::KanMX4* strains are commercially available derivatives of BY4741, *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (Open Biosystems). The JG100 *sod1Δ::URA3 ccs1Δ::KanMX4* strain was created by deleting *SOD1* in the *ccs1Δ::KanMX4 S. cerevisiae* mutant using the *sod1Δ:URA3* disruption plasmid pAR010.

The *C. albicans* CA-IF100, *sod1Δ/Δ*, *sod2Δ/Δ*, and *sod3Δ/Δ* strains were kind gifts from K. Kuchler [35]. The *CCS1/ccs1Δ* heterozygous (JG101) and the *ccs1Δ/Δ* homozygous (JG103) *C. albicans* mutants were constructed from parent SN78 using disruption marker cassettes and fusion PCR, as previously described [36]. The first round of PCR involved generation of upstream and downstream *CCS1* PCR products as well as *HIS1* and *LEU2* markers. Forward OJG141(GTCATCCCCACTACT TGTGCTG) and reverse OJG143 (GCTTGGCGTAATC ATGGTCATAGGATCTAATGGAAGGATAATGG) primers were used to amplify nucleotides −629 to −24 of *CCS1* from SC5314 genomic DNA; forward OJG144 (CGTGACTG GGAAAACCCTGGCTTGAAACAGTGGAGTTACTCTT C) and reverse OJG146 (CGCTTCTGGTCTCACTCCG) primers amplified sequences +45 to +618 with respect to the

CCS1 stop codon. Markers were created using forward OJG142 (CCATTATCCTTCCATTAGATCCTATGACCA TGATTACGCCAAGC) and reverse OJG145 (GAAGAGT AACTCCACTGTTTCAAGCCAGGGTTTTCCCAGTCA CG) primers to amplify *Candida dubliniensis HIS1* or *Candida maltosa LEU2* with templates pSN52 or pSN40, respectively [36]. Fusion reaction mixtures were assembled with Phusion polymerase (NEB) and products from the first round of PCR as templates; primers included the forward OJG147 (CTACTTGTGCTGCATCCCTCC) and the reverse OJG148 (CAGATTCTTCTTCTTCTTCCGC) oligonucleotides. To create the heterozygous *ccs1Δ/CCS1* mutant strain JG101, SN78 was transformed with the *LEU2* fusion product by lithium acetate [37]. The homozygous *ccs1Δ/Δ* strain JG103 was created by transforming JG101 with the *HIS1* fusion. Deletions were confirmed by PCR. To reintroduce *CCS1*, the *ccs1Δ/Δ* homozygous diploid JG103 was transformed with the *CCS1* rescue construct pJG111 digested with *Stu*I, integrating *CCS1* under the *MET3* promoter at the *RP10* locus [38]. Cells were grown in SC–cysteine medium containing a very low concentration of methionine (2.5 μM) to maximize *CCS1* expression [38].

Plasmids

Plasmid pLS108 is a *CEN LEU2* vector that expresses *S. cerevisiae SOD1* sequences −674 to +584, derived by introducing a *Bgl*II/*Nde*I fusion at the *SOD1* start codon in pLS101 [39]. Plasmid pJG099 was derived from pLS108 by converting the *Sna*BI site following the *SOD1* stop codon to *Nco*I. To generate the pJG100 plasmid for expressing *C. albicans SOD1* in *S. cerevisiae*, the coding region for *C. albicans SOD1* was PCR-amplified from strain SC5314 genomic DNA using an upstream primer with an *Nde*I site at the start codon fused to 13 nucleotides of the first exon joined in frame to 18 nucleotides of the second exon. The downstream primer contained an *Nco*I site immediately following the *C. albicans SOD1* stop codon. The PCR product was digested with *Nde*I and *Nco*I and ligated to pJG099 cut with these same enzymes, replacing the *S. cerevisiae SOD1* coding region with that of *C. albicans SOD1*. Vectors for expressing the P144A (pJG101), P144L (pJG102), P144Q (pJG103), and H139N (pJG104) derivatives of *C. albicans SOD1* were created by oligonucleotide-directed mutagenesis using pJG100 as a template. The *CEN HIS3* pJG110 vector for expressing *C. albicans CCS1* in *S. cerevisiae* was constructed as previously done for pLJ375 expressing *D. melanogaster* CCS [20]. The coding region for *C. albicans CCS1* was amplified using 5′ *Mlu*I and 3′ *Eco*RI sites, and the PCR product digested with these enzymes was inserted into the same sites in pLJ366 [20], effectively replacing the *S. cerevisiae CCS1* coding region with that of *C. albicans CCS1*. The pLS113 and pPS015 *CEN HIS3* vectors for expressing *S. cerevisiae* and human CCS molecules, respectively, in *S. cerevisiae* have been described [39, 40]. The *sod1::URA3* plasmid pAR010 is designed to delete *S. cerevisiae SOD1* sequences −233 to +500 using a *sod1Δ* deletion cassette in pRS306 precisely as has been described for pJAB002 [27].

The *CCS1* rescue construct pJG111 for expression in *C. albicans* was generated by amplifying *CCS1* gene sequences from −11 to the stop codon from SC5314 genomic DNA using primers that engineered *Bam*HI and *Pst*I sites at upstream and downstream positions. The products digested with *Bam*HI and *Pst*I were inserted into these same sites of pCaExp for expression under the *C. albicans MET3* promoter [38]. All plasmids were confirmed by DNA sequencing.

Biochemical analysis

Whole cell lysates from *S. cerevisiae* or *C. albicans* were prepared by glass bead homogenization as described in [41]. For native gel electrophoresis, 30 μg lysate protein was subjected to electrophoresis (50 mA) on precast 10 % tris(hydroxymethyl)aminomethane–glycine gels (Novex). We noted that wild-type *C. albicans* SOD1 loses activity with prolonged electrophoresis on native gels; hence, all studies with this SOD were done with 90 min of electrophoresis at 4 °C. Prolonged electrophoresis was conducted over 150 min at 4 °C. SOD activity in native gels was monitored by nitroblue tetrazolium staining [42]. To eliminate SOD1 activity, gels were soaked in 5 mM H_2O_2 prior to nitroblue tetrazolium staining as described in [43]. Immunoblot analysis was conducted with 15–30 μg whole cell lysate protein run on precast NuPage 4–12 % [bis (2-hydroxyethyl)amino]tris(hydroxymethyl)methane gels (Novex) at 200 V, followed by transfer onto poly(vinylidene difluoride) membranes using iBlot (Novex). A universal anti-SOD1 antibody (originally directed against *C. elegans* SOD1 [6]) was used at 1:10,000 dilution. An antibody directed against *C. albicans* SOD3 was generated using the synthetic peptide EKISLPKIDWALDALEPY and a 90-day rabbit protocol (Pierce Antibodies). When both SOD1 and SOD3 were analyzed, anti-SOD3 (1:10,000 dilution) was added first, followed by washing and incubation with anti-SOD1, then subsequent reaction with anti-rabbit secondary antibody. Immunoblots were visualized by an Odyssey infrared imaging system (LI-COR Biosciences).

Results and discussion

SOD activity from the *C. albicans* pathogen can be monitored using a native gel assay. In Fig. 1, soluble lysates from *C. albicans* grown to the early stationary stage exhibit

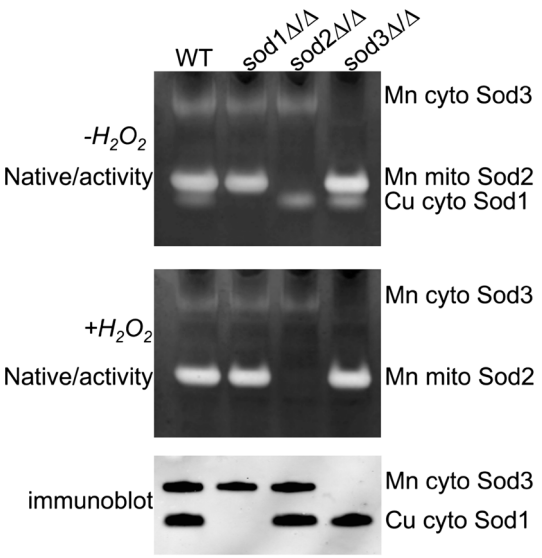

Fig. 1 Active copper and zinc containing superoxide dismutase (SOD1) from the *Candida albicans* native host: The wild-type (*WT*) *C. albicans* strain CA-IF100 or the indicated homozygous superoxide dismutase (SOD) deletion mutants [35] were grown to early stationary phase in yeast extract–peptone–2 % dextrose, conditions where both cytosolic SOD1 and SOD3 are active. Cell lysates were prepared and analyzed by (*top* and *middle*) native gel electrophoresis and nitroblue tetrazolium staining for SOD activity, and (*bottom*) denaturing gel electrophoresis and immunoblotting using anti-SOD1 and anti-SOD3 antibodies as described in "Materials and methods." Electrophoresis of native gels proceeded for 90 min. $+H_2O_2$ prior to nitroblue tetrazolium staining, the gel was treated with 5 mM H_2O_2 to eliminate SOD1 activity [43]. The positions of active SOD1, SOD2, and SOD3 enzymes on the native gels are shown, as are the positions of the SOD1 and SOD3 polypeptides on the immunoblot. *cyto* cytosolic, *mito* mitochrondrial

three soluble SOD enzymes that easily resolve by native gel electrophoresis. These include the mitochondrial manganese-containing SOD2, cytosolic manganese-containing SOD3, and cytosolic copper and zinc containing SOD1. Verification of the three soluble SODs was obtained using strains with individual mutations in the corresponding genes (Fig. 1, top). Additionally, the copper-containing and the manganese-containing SODs can be distinguished on the basis of in-gel peroxide sensitivity [43], and as seen in Fig. 1, middle, copper-containing SOD1 activity was lost with hydrogen peroxide treatment, whereas both manganese-containing SODs were resistant.

To study *C. albicans* SOD1 further, we used the *S. cerevisiae* expression system, which has proven fruitful for examining maturation of SOD1 from invertebrates, plants, and humans [6, 20, 23, 24]. Given the 70 % identity between *C. albicans* and *S. cerevisiae* SOD1 molecules (Fig. 2a), we anticipated faithful activation of *C. albicans* SOD1 in *S. cerevisiae*. The coding region for *C. albicans SOD1* was placed under control of the *S. cerevisiae SOD1* promoter and was expressed in an *S. cerevisiae sod1Δ* strain; lysates were analyzed by the gel assay. On native gels, SOD1 easily resolves from the manganese-containing SOD2 of *C. albicans* (Fig. 2b, top, lane 4) but not from *S. cerevisiae* manganese-containing SOD2 (lane 2). The prolonged electrophoresis needed to resolve *S. cerevisiae* SOD2 and SOD1 is detrimental to wild-type *C. albicans* SOD1 activity as described in more detail later (see Fig. 7); thus, our analysis of *C. albicans* SOD1 typically requires shorter durations of electrophoresis (as in Figs. 1, 2b, top). Under these conditions, *C. albicans* SOD1 expressed in its native host is easily discerned from the endogenous *C. albicans* SOD2 (Fig. 2b, lane 4). Yet in *S. cerevisiae*, where SOD1 co-migrates with SOD2, SOD1 activity can be detected as the peroxide-inhibitable fraction, as seen with endogenous *S. cerevisiae* SOD1 (Fig. 2b, lane 2). Surprisingly, no activity over the background could be detected with *C. albicans* SOD1 expressed in *S. cerevisiae* (Fig. 2b top, lane 3), even though the SOD1 polypeptide was produced (Fig. 2b, bottom, lane 3). As a more sensitive and reliable assay for SOD1 activity in vivo, we used a growth test. Specifically, *S. cerevisiae* cells devoid of SOD1 activity cannot grow in air without lysine because of damage to lysine biosynthetic enzymes caused by superoxide [44, 45]. Aerobic growth without lysine requires as little as 2 % of normal SOD1 activity [22] and is therefore a highly sensitive indicator of in vivo SOD1 activity. As seen in Fig. 2c, *sod1Δ* cells expressing *C. albicans* SOD1 failed to grow aerobically on medium lacking lysine. There are no signs of enzyme activity with *C. albicans* SOD1 expressed in *S. cerevisiae*.

The inactivity of *C. albicans* SOD1 in *S. cerevisiae* was surprising given the fact that SOD1 molecules from more distant species (e.g., *Drosophila*, *C. elegans*, plants, and mammals) are all abundantly active in this heterologous system [6, 20, 23, 24]. As one possibility, *C. albicans* SOD1 might be incapable of interacting with *S. cerevisiae* CCS1. To release *C. albicans* SOD1 from any CCS requirement, we mutated P144 needed for CCS dependence (Fig. 2a). As seen in Fig. 3a, P144A, P144L, and P144Q alleles of *C. albicans* SOD1 were abundantly active when expressed in *sod1Δ* strains of *S. cerevisiae*, and activity was resistant to prolonged electrophoresis on native gels. P144 mutations have previously been shown to promote disulfide oxidation [26], and this impact on the disulfide may account for the stabilization observed during electrophoresis. In any case, the gain of *C. albicans* SOD1 activity with P144 mutations was independent of CCS because activity was identical in $CCS1^+$ versus *ccs1Δ* strains (Fig. 3b, c). The activity seen with CCS-independent P144 mutants, but not with wild-type SOD1, indicates that *C. albicans* SOD1 cannot be activated by *S. cerevisiae* CCS1.

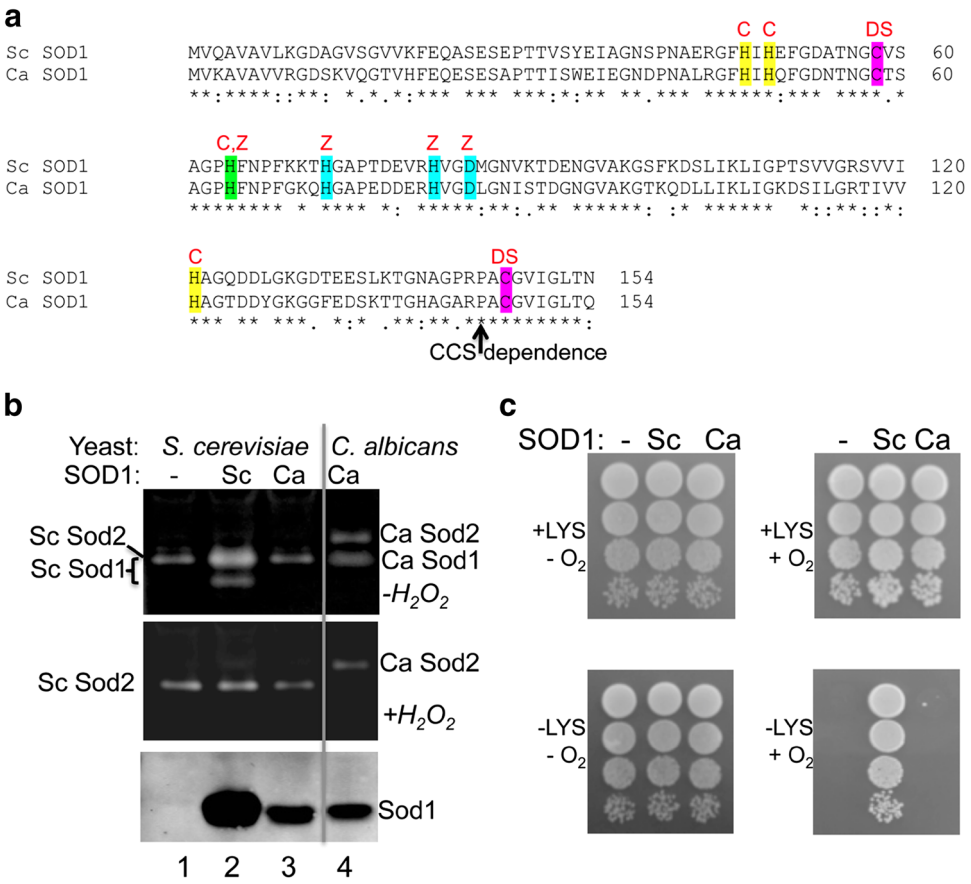

Fig. 2 *C. albicans* SOD1 expressed in *Saccharomyces cerevisiae*: **a** Sequence alignment of SOD1 molecules from *C. albicans* (*Ca*) and *S. cerevisiae* (*Sc*) as determined by Clustal Omega. *Asterisks* and *dots* indicate amino acid identity and similarity, respectively. Copper binding residues are marked by *C* and in *yellow*, zinc binding is marked by *Z* and in *aqua*, the shared zinc and copper binding histidine is marked in *green*, and disulfide cysteines are marked by *DS* and in *fuchsia*. The *arrow* points to P144, which dictates a copper chaperone for SOD (*CCS*) dependence. **b** Whole cell lysates were prepared from either WT *C. albicans* (*lane 4*) or from an *S. cerevisiae sod1Δ* strain (*lanes 1–3*) expressing SOD1 from either *S. cerevisiae* (*Sc*) or *C. albicans* (*Ca*) or not expressing SOD1 (*dash*). Lysates were subjected to SOD activity analysis (*top* and *middle*) and immunoblotting for SOD1 (*bottom*) as in Fig. 1. Native gels were treated with H_2O_2 where indicated as in Fig. 1. The positions of the various SOD enzymes from *C. albicans* or *S. cerevisiae* on the native gels are marked accordingly. Under these short (90-min) electrophoresis conditions, *S. cerevisiae* SOD2 co-migrates with the major band of SOD1 (see the main text). *S. cerevisiae* SOD1 is often seen as a doublet on native gels as indicated by *brackets*. **c** Cells (10^5, 10^4, 10^3, and 10^2) of the *S. cerevisiae* strains described in **b** were spotted onto synthetic complete medium containing or lacking lysine and grown either aerobically ($+O_2$) or in anaerobic culture jars ($-O_2$) as described in "Materials and methods"

To date, a CCS has not been described for *C. albicans*. On inspection of the *Candida* Genome Database, we identified a single potential CCS encoding loci in *C. albicans* (hypothetical protein CaO19.11929) that shares 43 % identity with CCS1 of *S. cerevisiae* and 32 % identity with human CCS (Fig. 4a). Conserved features include the MXCXXC copper binding site at the N-terminus and the CXC site at the C-terminus (Fig. 4a). To determine whether the putative *C. albicans* CCS functions as a copper chaperone, homozygous and heterozygous gene deletions were introduced at the CaO19.11929 locus in *C. albicans*. As seen in Fig. 4b, lane 3, the homozygous null strain for CaO19.11929 exhibits a dramatic loss in SOD1 activity. Polypeptide levels of *C. albicans* SOD1 were also somewhat lowered as has been reported with CCS gene deletions in other organisms [20, 46]. These changes in SOD1 were reversed on integration of the complementary DNA for CaO19.11929 (Fig. 4b, lane 4). On the basis of these findings and others described below, we have denoted *C. albicans* CaO19.11929 as *CCS1* (Genbank accession number KF040455).

We tested whether *C. albicans* CCS1 could restore activity to *C. albicans* SOD1 expressed in *S. cerevisiae*. The coding region for *C. albicans CCS1* was placed under control of the *S. cerevisiae CCS1* promoter and was coexpressed with *C. albicans SOD1* in a *ccs1Δ sod1Δ* null strain of *S. cerevisiae*. The results were compared with those for strains coexpressing *C. albicans SOD1* with CCS molecules from *S. cerevisiae* (44 % identity to *C. albicans* CCS1), humans (32 % identity), and *Drosophila* (28 %

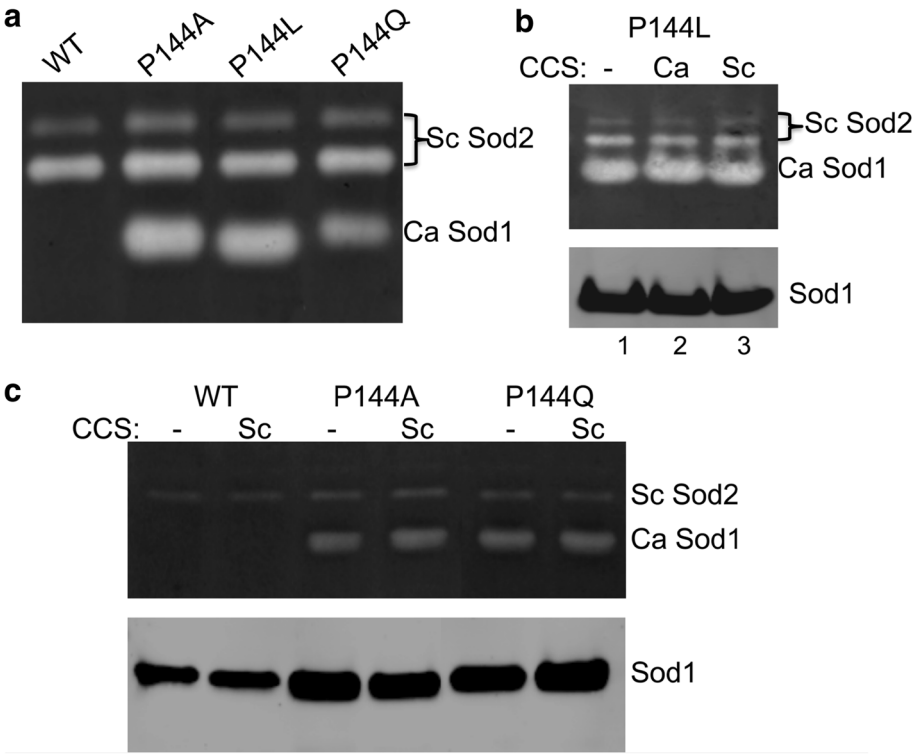

Fig. 3 Gain of *C. albicans* SOD1 activity in *S. cerevisiae* with mutations in P144. The *S. cerevisiae sod1Δ* single mutant (**a**) or *sod1Δ ccs1Δ* double mutant (**b**, **c**) was transformed with vectors for expressing either WT *C. albicans* SOD1 or the indicated P144 mutant alleles of *C. albicans* SOD1. **b**, **c** Cells also expressed CCS1 from either *S. cerevisiae* (*Sc*) or *C. albicans* (*Ca*) or no CCS (*dash*). Cell lysates where analyzed for SOD activity by the native gel assay (**a** and **b** *top*, **c** *top*) and for SOD1 protein by immunoblot (**b** *bottom*, **c** *bottom*) as in Fig. 1 except native gels were subjected to prolonged electrophoresis (150 min) to resolve the active P144 SOD1 mutants from *S. cerevisiae* SOD2 (see the main text). *S. cerevisiae* SOD2 can often be seen running as a doublet on native gels as indicated by *brackets*. The faster migration of P144L SOD1 from *C. albicans* has also been seen with P144L SOD1 from *S. cerevisiae* [26]

identity). Activity of *C. albicans* SOD1 was monitored by both the sensitive lysine dependency test (Fig. 5a) and the native gel assay (Fig. 5b). Although *C. albicans* SOD1 is inactive when coexpressed with *S. cerevisiae* CCS1, expression of its partner *C. albicans* CCS1 bestowed activity to this SOD1 as determined by both the aerobic lysine auxotrophy test and the native gel assay (Fig. 5). *C. albicans* CCS1 is clearly functioning as a copper chaperone, as it activates wild-type *C. albicans* SOD1 (Fig. 5), but has no effect on the CCS-independent P144L SOD1 mutant (Fig. 3b, lane 2). It is noteworthy that in addition to *C. albicans* CCS1, the copper chaperone from humans was also capable of activating wild-type *C. albicans* SOD1 (Fig. 5). Thus, *C. albicans* SOD1 is best activated by its native CCS1 partner and by human CCS, but not at all by the closely related CCS1 from *S. cerevisiae*.

We also conducted the converse experiment and tested whether *C. albicans* CCS1 can activate expression of *S. cerevisiae* SOD1. As seen in Fig. 6, *C. albicans* CCS1 was capable of conferring some activity to *S. cerevisiae* SOD1, but the actual level of activity seems poor compared with that obtained with *S. cerevisiae* CCS1 (Fig. 6b). Although there is no antibody available to monitor levels of the *C. albicans* CCS1 polypeptide, this copper chaperone is certainly expressed in *S. cerevisiae* as it activates *C. albicans* SOD1 in the same expression system (Fig. 5).

The lack of apparent reactivity between *S. cerevisiae* CCS1 and *C. albicans* SOD1 (Fig. 5) was quite unexpected, particularly since *S. cerevisiae* CCS1 reacts well with SOD1 molecules from far distant species, including plants, insects, and humans [20, 23, 24]. As one possibility, there may be some unique attribute to *C. albicans* SOD1 that dictates this barrier in spite of 80 % similarity between *S. cerevisiae* and *C. albicans* SOD1 molecules. To begin to address this, we searched for residues that are unique to *C. albicans* SOD1, but are conserved among other eukaryotic SOD1 molecules. Attention was drawn to H139 in *C. albicans* SOD1, which is a highly conserved asparagine in other organisms (Fig. 7a). In studies that have been conducted with human SOD1, N139 helps form solvent-exposed hydrogen bonds with residues in the electrostatic loop, and N139K and N139D are documented SOD1 mutations in familial amyotrophic lateral sclerosis, although there is no obvious misfolding of apoprotein

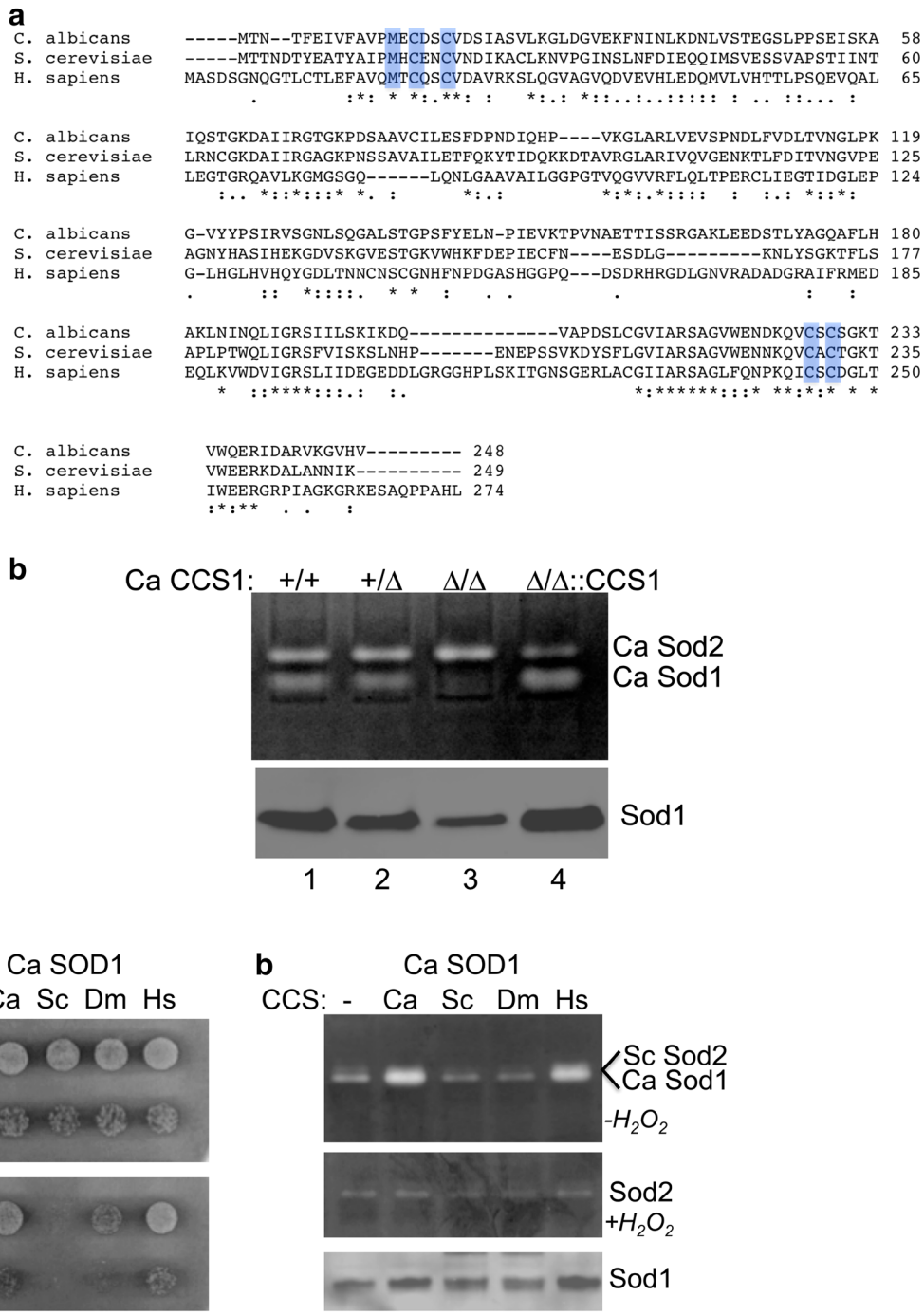

Fig. 4 A single CCS-encoding gene in *C. albicans*. **a** Sequence alignment of CCS1 molecules from *C. albicans*, *S. cerevisiae*, and humans as determined by Clustal Omega. *Asterisks* and *dots* indicate amino acid identity and similarity, respectively; *shaded areas* demark conserved copper binding cysteines in the N-terminus and C-terminus of CCS molecules. **b** The indicated strains of *C. albicans* were grown to mid log phase in low-methionine synthetic complete medium as described in "Materials and methods," and cell lysates were subjected to SOD activity (*top*) and SOD1 protein (*bottom*) analyses as in Fig. 1. The strains used were the SN78 WT for *CCS1* (+/+), and the heterozygous JG101 (+/*Δ*) and homozygous JG103 (*Δ*/*Δ*) *ccs1* deletion strains; *Δ/Δ:CCS1* represents JG103 with *CCS1* integrated at the *RP10* locus as described in "Materials and methods"

Fig. 5 *C. albicans* SOD1 is activated by its partner *C. albicans* CCS1. The *sod1Δ ccs1Δ* double mutant of *S. cerevisiae* expressing WT *C. albicans* SOD1 and coexpressing CCS molecules from *C. albicans* (*Ca*), *S. cerevisiae* (*Sc*), *Drosophila melanogaster* (*Dm*), and humans (*Hs*) or no CCS (*dash*) were tested **a** for lysine-independent growth as in Fig. 2c using 10^3 and 10^2 cells, and **b** for SOD activity and SOD1 protein levels as in Fig. 2b

in vitro [47, 48]. In fungal SOD1, position 139 shares close proximity to P144 and C146 that control the CCS dependence and the SOD1 disulfide (Fig. 7a). An H139N substitution was introduced in *C. albicans* SOD1 and the protein expressed in *S. cerevisiae*. H139N SOD1 polypeptide levels were similar to that of wild-type *C. albicans* SOD1 (Fig. 7b, bottom). Moreover, H139N SOD1 exhibits precisely the same CCS1 preference as is seen with wild-type *C. albicans* SOD1: the enzyme was active when coexpressed with *C. albicans* CCS1 (Fig. 7b, top, lane 5) but not when coexpressed with *S. cerevisiae* CCS1 (Fig. 7b, top, lane 6). Although CCS1 selectivity was

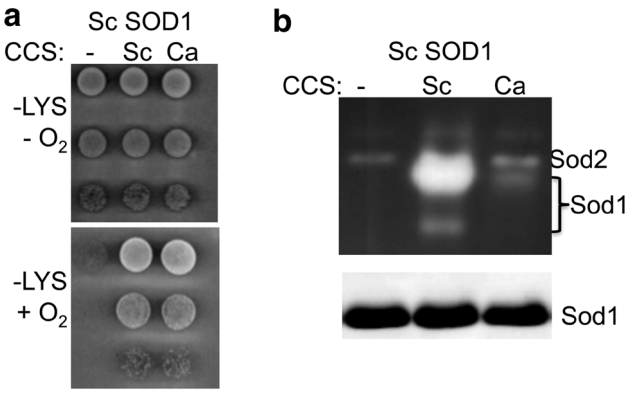

Fig. 6 Activation of *S. cerevisiae* SOD1 by *C. albicans* CCS1. The *sod1Δ ccs1Δ* strain expressing *S. cerevisiae* SOD1 and coexpressing CCS1 molecules from *C. albicans* (*Ca*) or *S. cerevisiae* (*Sc*) or no CCS (*dash*) were tested **a** for lysine-independent growth as in Fig. 2c using 10^4, 10^3, and 10^2 cells, and **b** for SOD activity and SOD1 protein levels as in Fig. 3

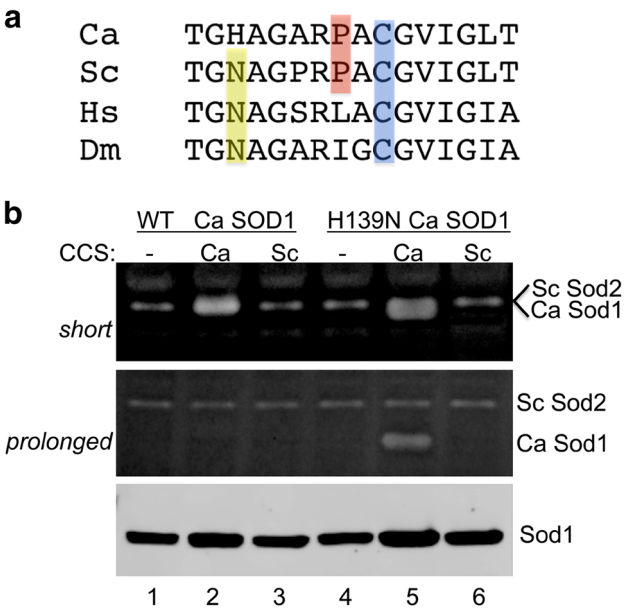

Fig. 7 The unique H139 of *C. albicans* SOD1. **a** An alignment of the C-terminal region of SOD1 molecules from *C. albicans* (*Ca*), *S. cerevisiae* (*Sc*), humans (*Hs*), and *D. melanogaster* (*Dm*). *Yellow* marks the conserved N139 in diverse SOD1 molecules that is a histidine in the case of *C. albicans* SOD1. *Red* and *blue* mark P144 for CCS dependence and the C146 disulfide cysteine, respectively. **b** The *sod1Δ ccs1Δ S. cerevisiae* strain expressing WT or H139 mutant *C. albicans* SOD1 and coexpressing *C. albicans* (*Ca*) or *S. cerevisiae* (*Sc*) CCS1 or no CCS (*dash*) was subjected to native gel assay for SOD activity (*top* and *middle*) and immunoblot for SOD1 (*bottom*). *Short* (90 min) and *prolonged* (150 min) indicate the duration of the native gel electrophoresis as in Figs. 2b and 3, respectively

unchanged, the H139N mutation may affect SOD1 stability. As seen in Fig. 7b, middle, the activity of the H139N SOD1 was resistant to prolonged periods of native gel electrophoresis. This stability during electrophoresis precisely mirrors what is seen with wild-type SOD1 from *S. cerevisiae*, humans, *Drosophila*, and nematodes, all of which naturally contain N139 [6, 20, 24]. The stable activity gained with *C. albicans* H139N SOD1 could reflect alterations in the aforementioned hydrogen-bonding network.

Together these studies support an unanticipated species specificity in the activation of *C. albicans* SOD1. Until now, great flexibility has been reported for interactions of CCS with SOD1. *S. cerevisiae* SOD1 reacts well with CCS molecules from humans, plants, and the *Ascomycota* fungus *S. pombe* [4, 21, 25]. The converse is also true: SOD1 molecules from humans, plants, and insects are all strongly activated by *S. cerevisiae* CCS1 [20, 22, 23]. The barrier between *C. albicans* SOD1 and *S. cerevisiae* CCS1 is not understood, but could involve blockages in CCS–SOD physical interactions, in copper insertion, or in disulfide oxidation. This could occur at either the level of SOD1 or the level of CCS or both.

Although *C. albicans* SOD1 cannot react with *S. cerevisiae* CCS1, it appears compatible with human CCS (Fig. 5). Unlike fungal CCS molecules, domain II of human CCS exhibits remarkably high sequence homology to the SOD1 target [49], and this feature may promote docking with the heterologous *C. albicans* SOD1. Since *C. albicans* naturally coexists with human cells, there is a distant possibility that at some point during its life cycle, *C. albicans* SOD1 encounters the copper chaperone of its human host.

SOD1 molecules are notoriously stable, with relatively long half-lives in vivo, and their activity remains stable in vitro over a range of conditions, including prolonged electrophoresis on native gels. However, *C. albicans* SOD1 exhibits a progressive loss in activity during native gel electrophoresis that is not seen with wild-type SOD1 molecules from other organisms, including humans, *Drosophila*, *C. elegans*, and *S. cerevisiae*, yet is reminiscent of the C150-containing variant of *Xenopus laevis* SOD1 [50, 51]. With *C. albicans* SOD1, the instability involves sequences at the C-terminus, including P144 and an unusual H139 that is a highly conserved asparagine in other SOD1 enzymes. Unlike other eukaryotes, *C. albicans* expresses two cytosolic SODs and there is a switch from expression of *SOD1* to expression of *SOD3* as cells enter the deep stationary phase [29]. We are currently examining whether the instability uncovered here in vitro is important to the turnover of SOD1 in vivo as the cell makes the switch to manganese-containing SOD3.

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