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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 proven fruitful for the study of Cu/Zn SODs 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 baker's yeast. *C. albicans* SOD1 contains a proline at position 144 predicted to dictate dependence on CCS1. By mutating this proline, *C. albicans* SOD1 gained activity in baker's yeast 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 baker's yeast. *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 specie-specific barrier in CCS-SOD interactions which may reflect the vastly different lifestyles of the pathogenic versus non-infectious yeast.

Keywords

superoxide; copper; yeast; pathogen; SOD1

Introduction

From *E. coli* to humans, the copper and zinc containing superoxide dismutase (Cu/Zn SOD or SOD1) enzyme participates in reactive oxygen metabolism by disproportionating superoxide anion to hydrogen peroxide and oxygen [1, 2]. The chemistry is carried out by a copper ion at the active site while the zinc co-factor provides more of a structural role. In addition to metal co-factors, an intramolecular disulfide in each Cu/Zn SOD monomer stabilizes the quaternary structure [3]. The maturation process for Cu/Zn SOD in which the apo-reduced polypeptide is converted to an active metallated enzyme has been thoroughly

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investigated. In eukaryotes, this maturation requires a helper protein known as CCS for the copper chaperone for SOD that acts to insert copper and oxidize the disulfide. The zinc is acquired through an unknown mechanism. CCS was originally identified in the 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 *C. elegans* which has evolved with no CCS accessory factor for its Cu/Zn SOD [6].

The CCS copper chaperone harbors three distinct domains that work in concert to capture copper, to dock with Cu/Zn SOD 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 Cu/Zn SOD [7, 9–11]. A central domain II exhibits homology to the Cu/Zn SOD 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 Cu/Zn SOD 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 the baker's yeast *S. cerevisiae*, Cu/Zn SOD molecules from *Drosophila*, the *Arabidopsis* plant and humans can all be activated by the yeast CCS1 copper chaperone [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 Cu/Zn SOD, and in most eukaryotes the SOD 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 Cu/Zn SOD polypeptide, namely the position corresponding to proline 144 in *S. cerevisiae* SOD1. In studies with baker's yeast, P144 strongly inhibits activation of SOD1 *in vivo* without the CCS1 copper chaperone [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-P residue at position 144 (eg., Ser, Leu, Gln) enables some disulfide oxidation in the absence of CCS1, and the SOD1 can be activated without CCS1 [5, 24, 26, 27]. Thus far, P144 has only been noted in Cu/Zn SOD molecules of certain Ascomycota fungi; all plant and animal SOD1 molecules contain non-proline residues at the equivalent position, and these Cu/Zn SODs can be activated with or without a copper chaperone [5]. Yet even in these higher organisms, the CCS copper chaperone pathway is the preferred mechanism for Cu/Zn SOD activation [5].

The pathogenic fungus *Candida albicans* represents an interesting model system to study Cu/Zn SOD activation. Unlike all other eukaryotes studied to date, this pathogen has uniquely evolved with two cytosolic SODs: a Cu/Zn containing 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 baker's yeast 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 specie-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 YPD (yeast extract, peptone, 2% dextrose) or minimal (SC) synthetic complete medium [34], and *S. cerevisiae* strains were grown in selecting SC medium to maintain episomal plasmids. *S. cerevisiae* *sod1* 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 allowed to grow for 2–3 days either in air or in anaerobic culture jars.

The *S. cerevisiae* *sod1* ::*KanMX4* and *ccs1* ::*KanMX4* strains are commercially available derivatives of BY4741, *MATa his3 1 leu2 0 met15 0 ura3 0* (Open Biosystems). The JG100 *sod1* ::*URA3 ccs1* ::*KanMX4* strain was created by deleting *SOD1* in the *ccs1* ::*KanMX4* *S. cerevisiae* mutant using the *sod1* ::*URA3* disruption plasmid pAR010b.

The *C. albicans* CA-IF100 and *sod1* / , *sod2* / and *sod3* / strains *C. albicans* strains were kind gifts of K. Kuchler [35]. The *CCS1/ccs1* heterozygous (JG101) and the *ccs1* / 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(GTCATCCCCACTACTTGTGCTG) and reverse OJG143 (GCTTGGCGTAATCATGGTCATAGGATCTAATGGAAGGATAATGG) primers were used to amplify nucleotides –629 to –24 of *CCS1* from SC5314 genomic DNA; forward OJG144 (CGTGACTGGGAAAACCCCTGGCTTGAAACAGTGGAGTTACTCTTC) and reverse OJG146 (CGTTCTGGTCTCACTCCG) primers amplified sequences + 45 to +618 with respect to the *CCS1* Stop codon. Markers were created using forward OJG142 (CCATTATCCTTCCATTAGATCCTATGACCATGATTACGCCAAGC) and reverse OJG145 (GAAGAGTAACTCCACTGTTTCAAGCCAGGGTTTTCCAGTCACG)

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 (CAGATTCTTCTTCTTCTCCGC) oligos. To create the heterozygous *ccs1* / *CCS1* mutant strain JG101, SN78 was transformed with the *LEU2* fusion product by lithium acetate [37]. The homozygous *ccs1* / strain JG103 was created by transforming JG101 with the *HIS1* fusion. Deletions were confirmed by PCR. To reintroduce *CCS1*, the *ccs1* / 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 very low (2.5 μ M) methionine 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 *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 oligo-directed mutagenesis using pJG100 as 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 in *S. cerevisiae*, respectively have been described [39, 40]. The *sod1::URA3* plasmid pAR010b is designed to delete *S. cerevisiae* *SOD1* sequences –233 to +500 using a *sod1* 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 –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 [41]. For native gel electrophoresis, 30 µg lysate protein was subjected to electrophoresis (50 mA) on pre-cast 10% Tris-glycine gels (Novex). We noted that *C. albicans* WT SOD1 loses activity with prolonged electrophoresis on native gels; hence all studies with this SOD were carried with 90 min electrophoresis at 4°C. Prolonged electrophoresis conditions were carried out over 150 min at 4°C. SOD activity in native gels was monitored by Nitroblue tetrazolium (NBT) staining [42]. To eliminate Cu/Zn SOD activity, gels were soaked in 5 mM H₂O₂ prior to NBT staining as described [43]. Immunoblot analysis was conducted with 15–30 µg whole cell lysate protein run on precast NuPage 4–12% Bis-Tris gels (Novex) at 200 V, followed by transfer onto PVDF membranes using iBlot (Novex). A universal anti-Cu/Zn SOD antibody (originally directed against *C. elegans* Sod-1 [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 are 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 the Odyssey infrared imaging system (Licor 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 early stationary stage exhibit three soluble SOD enzymes that easily resolve by native gel electrophoresis. These include the mitochondrial Mn-SOD2, cytosolic Mn-SOD3 and the cytosolic Cu/Zn 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 and manganese containing SODs can be distinguished based on in-gel peroxide sensitivity [43], and as seen in Fig. 1 middle, copper containing SOD1 activity was lost with hydrogen peroxide treatment whereas both Mn-SODs were resistant.

To study *C. albicans* SOD1 further, we employed the baker's yeast expression system that has proven fruitful for examining maturation of Cu/Zn SOD 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 baker's yeast. The coding region for *C. albicans* SOD1 was placed under control of the *S. cerevisiae* SOD1 promoter and was expressed in a *S. cerevisiae* *sod1* strain; lysates were analyzed by the gel assay. On native gels, SOD1 easily resolves from the Mn-SOD2 of *C. albicans* (Fig. 2b top lane 4) but not from *S. cerevisiae* Mn-SOD2 (lane 2). The prolonged electrophoresis needed to resolve *S. cerevisiae* SOD2 and SOD1 is detrimental to WT *C. albicans* SOD1 activity as described in more detail below (see Fig. 7); thus our analysis of *C. albicans* SOD1 typically requires shorter durations of electrophoresis (as in Fig. 1 and Fig. 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 baker's yeast where SOD1 co-migrates with *S. cerevisiae* 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 background could be detected with *C. albicans* SOD1 expressed in baker's yeast (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 employed a growth test. Specifically *S. cerevisiae* cells devoid of SOD1 activity cannot grow in air without lysine due to superoxide damage to lysine biosynthetic enzymes [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, *sod1* 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 baker's yeast was surprising given the fact that Cu/Zn SOD 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 the *S. cerevisiae* CCS1 copper chaperone. 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 *sod1* strains of baker's yeast, 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 *ccs1* strains (Fig. 3b, lanes 1; Fig. 3c). The activity seen with CCS-independent P144 mutants, but not with WT SOD1 indicates that *C. albicans* SOD1 cannot be activated by the *S. cerevisiae* CCS1 copper chaperone.

To date, a CCS copper chaperone has not been described for *C. albicans*. Upon 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 hCCS (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 upon integration of the cDNA for CaO19.11929 (Fig. 4b, lane 4). Based on 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 co-expressed with *C. albicans* *SOD1* in a *ccs1 sod1* null strain of baker's yeast. Results were compared to strains co-expressing *C. albicans* *SOD1* with CCS molecules from *S. cerevisiae* (44% identity to *C. albicans* *CCS1*), human (32% identity) and *Drosophila* (28% identity). Activity of *C. albicans* SOD1 was

monitored by both the sensitive lysine dependency test (Fig. 5a) and by the native gel assay (Fig. 5b). Although *C. albicans* SOD1 is inactive when co-expressed with *S. cerevisiae* CCS1, expression of its partner *C. albicans* CCS1 bestowed activity to this SOD1 by both the aerobic lysine auxotrophy test and the native gel assay (Fig. 5a and 5b). *C. albicans* CCS1 is clearly functioning as a copper chaperone, as it activates WT *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 WT *C. albicans* SOD1 (Fig. 5). Thus, *C. albicans* SOD1 is best activated by its native CCS1 partner and by human hCCS, but not at all by the closely related CCS1 from baker's yeast.

We also conducted the converse experiment and tested whether *C. albicans* CCS1 can activate expression of baker's yeast 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 to 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 baker's yeast as it activates *C. albicans* SOD1 in the same expression system (Fig. 5).

The lack of apparent reactivity between baker's yeast CCS1 and *C. albicans* SOD1 (Fig. 5) was quite unexpected, particularly since baker's yeast CCS1 reacts well with Cu/Zn SOD 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 conserved among other eukaryotic Cu/Zn SOD molecules. Attention was drawn to H139 in *C. albicans* SOD1, which is a highly conserved Asn in other organisms (Fig. 7a). In studies that have been conducted with human SOD1, N139 helps form solvent-exposed hydrogen bonds to residues in the electrostatic loop, and N139K and N139D are documented SOD1 mutations in familial amyotrophic lateral sclerosis, although there is no obvious mis-folding of apo protein *in vitro* [47, 48]. In fungal SOD1, position 139 shares close proximity to P144 and C146 that control CCS-dependence and the SOD1 disulfide (Fig. 7a). A H139N substitution was introduced in *C. albicans* SOD1 and the protein expressed in *S. cerevisiae*. H139N SOD1 polypeptide levels were similar to that of WT *C. albicans* SOD1 (Fig. 7b bottom). Moreover, H139N SOD1 exhibits precisely the same CCS1 preference as is seen with WT *C. albicans* SOD1: the enzyme was active when co-expressed with *C. albicans* CCS1 (Fig. 7b top, lane 5) but not with *S. cerevisiae* CCS1 (lane 6). Although CCS1 selectivity was unchanged, the H139N mutation may affect SOD1 stability. As seen in Fig. 7b middle, activity of the H139N SOD1 was resistant to prolonged periods of native gel electrophoresis. This stability during electrophoresis precisely mirrors what is seen with WT Cu/Zn SOD from baker's yeast, humans, *Drosophila* and nematodes, all of which naturally contain N139 [6, 20, 24]. The stable activity gained with H139N *C. albicans* 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. Up until now, there has been great flexibility reported for CCS interactions

with Cu/Zn SOD. *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: Cu/Zn SOD molecules from humans, plants and insects are all strongly activated by baker's yeast 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 CCS or both.

Although *C. albicans* SOD1 cannot react with baker's yeast CCS1, it appears compatible with human hCCS (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 co-exists 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.

Cu/Zn SOD molecules are notoriously stable molecules with relatively long half lives *in vivo*, and 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 WT Cu/Zn SOD molecules from other organisms, including humans, *Drosophila*, *C. elegans* and *S. cerevisiae*, yet is reminiscent of the Cys-150 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 Asn in other Cu/Zn SODs. Unlike other eukaryotes, *C. albicans* expresses two cytosolic SODs and there is a switch from expression of *SOD1* to *SOD3* as cells enter 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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Abbreviations used

NBT	nitroblue tetrazolium
YPD	yeast extract peptone
SC	synthetic complete
CCS	copper chaperone for superoxide dismutase

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Figure 1. Active SOD1 enzyme from the *C. albicans* native host

The WT *C. albicans* strain CA-IF100 or the indicated homozygous SOD deletion mutants [35] were grown to early stationary phase in YPD, conditions where both cytosolic SOD1 and SOD3 are active. Cell lysates were prepared and analyzed by (top and middle) native gel electrophoresis and NBT 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 minutes. “ $+H_2O_2$ ”: Prior to NBT staining, gel was treated with 5 mM H_2O_2 to eliminate Cu/Zn SOD activity [43]. The positions of active SOD1, SOD2 and SOD3 enzyme on the native gels are shown, as well as the positions of the SOD1 and SOD3 polypeptides on the immunoblot.

Figure 2. *C. albicans* SOD1 expressed in the baker's yeast *S. cerevisiae*
 (a) Sequence alignment of SOD1 molecules from *C. albicans* and *S. cerevisiae* as determined by Clustal Omega. Asterisks and dots indicate amino acid identity and similarity, respectively. Copper binding residues are marked by C and yellow highlights; zinc binding by Z and aqua; the shared zinc and copper binding histidine is marked in green; disulfide cysteines by DS in fuchsia. Arrow points to P144 that dictates CCS-dependence.
 (b) Whole cell lysates were prepared from either WT *C. albicans* (lane 4) or from a *S. cerevisiae* *sod1*⁻ strain (lanes 1–3) expressing SOD1 from either *S. cerevisiae* (Sc) or *C. albicans* (Ca) or not expressing SOD1 (–). Lysates were subject to SOD activity analysis (top and middle) and immunoblotting for SOD1 (bottom) as in Fig. 1. Native gels were treated with H₂O₂ 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 main text). *S. cerevisiae* SOD1 is often seen as a doublet on native gels as indicated by brackets.
 (c) 10⁵, 10⁴, 10³ and 10² cells of the *S. cerevisiae* strains described in b were spotted onto SC medium containing or lacking lysine and grown either aerobically (+O₂) or in anaerobic culture jars (–O₂) as described in Materials and Methods.

Figure 3. Gain of *C. albicans* SOD1 activity in *S. cerevisiae* with mutations in P144

The *S. cerevisiae* *sod1* single mutant (a) or *sod1 ccs1* double mutant (b,c) were 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 (–). Cell lysates were analyzed for SOD activity by the native gel assay (a and b,c top) and for SOD1 protein by immunoblot (b,c bottom) as in Fig. 1 except native gels were subjected to prolonged electrophoresis (150 minutes) to resolve the active P144 SOD1 mutants from *S. cerevisiae* SOD2 (see 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].

(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- and C-terminus of CCS molecules. (b) The indicated strains of *C. albicans* were grown to mid log in low methionine SC 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. Strains utilized are the SN78 WT for *CCS1* (+/+); the heterozygous JG101 (+/-) and homozygous JG103 (-/-) *ccsI* deletion strains; -/-:CCS1 represents JG103 with *CCS1* integrated at the RP10 locus as described in Materials and Methods.

Figure 5. *C. albicans* SOD1 is activated by its partner *C. albicans* CCS1

The *sod1 ccs1* double mutant of *S. cerevisiae* expressing WT *C. albicans* SOD1 and co-expressing CCS molecules from *C. albicans* (Ca), *S. cerevisiae* (Sc), *D. melanogaster* (Dm) and humans (Hs) or no CCS (–) were tested for (a) lysine independent growth as in Fig. 2c using 10³ and 10² cells; and (b) for SOD activity and SOD1 protein levels as in Fig. 2b.

Figure 6. Activation of *S. cerevisiae* SOD1 by *C. albicans* CCS1

The *sod1 ccs1* strain expressing *S. cerevisiae* SOD1 and co-expressing CCS1 molecules from *C. albicans* (Ca) or *S. cerevisiae* (Sc) no CCS (-) were tested for (a) lysine independent growth as in Fig. 2c using 10⁴, 10³ and 10² cells; and (b) for SOD activity and SOD1 protein levels as in Fig. 3.

Figure 7. The unique H139 of *C. albicans* SOD1

(a) Shown is an alignment of the C-terminal region of Cu/Zn SOD 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 shading mark P144 for CCS-dependence and the C146 disulfide cysteine, respectively. (b) The *sod1 ccs1* *S. cerevisiae* strain expressing WT or H139 mutant *C. albicans* SOD1 and co-expressing *C. albicans* (Ca) or *S. cerevisiae* (Sc) CCS1, or no CCS (–) were subjected to the native gel assay for SOD activity (top and middle) and immunoblot for SOD1 (bottom). “short” (90 min) and “prolonged” (150 min) indicates duration of native gel electrophoresis as in Fig. 2b and 3 respectively.