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Expanded role of the Cu-sensing transcription factor Mac1p in *Candida albicans*

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Abstract

Summary—As part of the innate immune response, the host withholds metal micronutrients such as Cu from invading pathogens, and microbes respond through metal starvation stress responses. With the opportunistic fungal pathogen *Candida albicans*, the Cu sensing transcription factor Mac1p governs the cellular response to Cu starvation by controlling Cu import. Mac1p additionally controls reactive oxygen species (ROS) homeostasis by repressing a Cu-containing superoxide dismutase (*SOD1*) and inducing Mn-containing *SOD3* as a non-Cu alternative. We show here that *C. albicans* Mac1p is essential for virulence in a mouse model for disseminated candidiasis and that the cellular functions of Mac1p extend beyond Cu uptake and ROS homeostasis. Specifically, *mac1Δ/Δ* mutants are profoundly deficient in mitochondrial respiration and Fe accumulation, both Cu-dependent processes. Surprisingly, these deficiencies are not simply the product of impaired Cu uptake; rather *mac1Δ/Δ* mutants appear defective in Cu allocation. The respiratory defect of *mac1Δ/Δ* mutants was greatly improved by a *sod1Δ/Δ* mutation, demonstrating a role for *SOD1* repression by Mac1p in preserving respiration. Mac1p down-regulates the major Cu consumer SOD1 to spare Cu for respiration that is essential for virulence of this fungal pathogen. The implications for such Cu homeostasis control in other pathogenic fungi are discussed.

Abbreviated Summary—Across numerous fungi, the Cu-sensing transcription factor Mac1p induces transcription of the Cu import machinery during Cu limitation. In the opportunistic fungal pathogen *Candida albicans,* Mac1p has the added function of regulating homeostasis of reactive oxygen species involving down-regulation of the major Cu consumer Sod1p. This regulation of Sod1p has the added purpose of sparing Cu for cytochrome C oxidase essential for mitochondrial respiration and for virulence in a murine model of disseminated candidiasis.

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Author contributions

E.M.C., B.P.C., and V.C.C. conceptualization; E.M.C., V.M.B., B.P.C., and V.C.C. acquisition, analysis and interpretation; E.M.C. and V.C.C. writing of the manuscript.

Conflicts of Interest

There are no conflicts of interest to declare.

Keywords

Candida albicans; copper; iron; cell respiration; superoxide dismutase

Introduction

Transition metals represent a double-edged-sword to biological systems; they are required for life but also can become toxic if not properly managed. Because of this dual nature, homeostasis of transition metals in biological systems is tightly controlled. Cu is one such metal that has this dual property of being both essential and potentially toxic. Cu is required as a redox cofactor for numerous enzymes involved in oxygen chemistry, but can also have deleterious side chemistry (Turski & Thiele, 2009, Festa & Thiele, 2011, Tan *et al.*, 2014, Chillappagari *et al.*, 2010, Liochev & Fridovich, 2002). Unicellular microbes are particularly vulnerable to fluxes in environmental metals and have evolved sophisticated means of regulating Cu homeostasis.

In the bakers' yeast *Saccharomyces cerevisiae* Cu homeostasis is controlled by two Cu binding transcription factors: Ace1p and Mac1p (Keller *et al.*, 2005). Ace1p senses high, toxic Cu concentrations and activates Cu detoxification machinery involving genes encoding Cu-binding metallothioneins (Culotta *et al.*, 1994, Thiele, 1988). In times of Cu limitation, Mac1p activates transcription of genes for Cu uptake including the high affinity Cu permeases and cupric reductases (Gross *et al.*, 2000). Variations on this theme of sensing and responding to extremes in Cu can be found throughout yeast species. The pulmonary fungal pathogen *Cryptococcus neoformans* uses the Cu-sensing transcription factor Cuf1p which bears homology to both *S. cerevisiae* Ace1p and Mac1p. Cuf1p activates both Cu uptake and Cu detoxification genes in response to Cu limitation and Cu excess, respectively (Garcia-Santamarina *et al.*, 2018, Ding *et al.*, 2011, Waterman *et al.*, 2007, Waterman *et al.*, 2012). On the other hand the pathogenic and saprotrophic fungi *Aspergillus fumigatus* has retained separate Ace1/Mac1 systems but in this fungus, AfMac1p has evolved to independently upregulate genes involved in Fe uptake as well as Cu uptake (Park *et al.*, 2018, Kusuya *et al.*, 2017, Cai *et al.*, 2017, Park *et al.*, 2017). The opportunistic human fungal pathogen *Candida albicans* also has separate Mac1p and Ace1p Cu regulators and the core functions of Mac1p have been retained, namely transcriptional activation of Cu uptake genes including the *CTR1* Cu permease and *FRE7* cupric reductase (Woodacre *et al.*, 2008). However, *C. albicans* Mac1p has additionally evolved to regulate anti-oxidant systems including superoxide dismutase enzymes (SOD).

SOD metalloenzymes utilize redox active co-factors such as Mn and Cu to disproportionate superoxide anion free radicals and therefore play important roles in metabolism of reactive oxygen species (ROS) (Sheng *et al.*, 2014). Eukaryotes typically contain a Cu/Zn-SOD1 in the cytosol and mitochondrial intermembrane space, and a distinct Mn-SOD2 in the mitochondrial matrix. Mitochondrial SOD1 and SOD2 ensure protection against respiratory chain superoxide, while cytosolic Cu/Zn-SOD1 can participate in cell signaling involving ROS (Weisiger & Fridovich, 1973, Broxton & Culotta, 2016, Sturtz *et al.*, 2001, Jaarsma *et al.*, 2001, Okado-Matsumoto & Fridovich, 2001, Reddi & Culotta, 2013, Montllor-Albalate

et al., 2019, Juarez *et al.*, 2008). Apparently unique to *C. albicans* and closely related fungi is a second Mn containing Sod3p in the cytosol (Lamarre *et al.*, 2001). During Cu limitation, *C. albicans* Mac1p transcriptionally represses Cu-Sod1p and induces Mn-Sod3p to maintain cytosolic levels of SOD (Li *et al.*, 2015, Broxton *et al.*, 2018, Broxton & Culotta, 2016). Additionally, Mac1p induces a mitochondrial alternative oxidase (*AOX2*) to compensate for loss of mitochondrial Cu/Zn SOD1 (Woodacre *et al.*, 2008, Broxton & Culotta, 2016). *C. albicans* Mac1p clearly participates in ROS homeostasis as well as Cu uptake.

The Mac1-Cu response is utilized by *C. albicans* during pathogenesis. In a murine model of disseminated candidiasis, where the kidney is the major target organ, kidney Cu levels fluctuate such that at early time points Cu levels are high, but decrease later in infection (Li *et al.*, 2015, Mackie *et al.*, 2016, Besold *et al.*, 2018, Culbertson *et al.*, 2020). *C. albicans* is able to sense these changes in Cu and exhibits abundant expression of *SOD1* at early stages of infection, and then repression of *SOD1* and transcriptional induction of *SOD3* and *CTR1* later in infection when Cu levels decrease (Li *et al.*, 2015, Mackie *et al.*, 2016, Besold *et al.*, 2018). Clearly *C. albicans* senses a Cu starved microenvironment in murine kidneys and is responding using Mac1p. It was not known if this Mac1p response plays a role in virulence in this model. Furthermore, the full impact of Mac1p on cell physiology is not understood. Is the function of Mac1p limited to cell surface Cu uptake and ROS homeostasis? It was unclear what, if any, additional genes are Mac1p targets in *C. albicans.*

In these studies, we investigate the requirement for *C. albicans* Mac1p in pathogenesis and its expanded role in regulating metal homeostasis and anti-oxidant enzymes. We find that Mac1p does indeed contribute to pathogenesis in a murine model of disseminated candidiasis and that its cellular function extends beyond Cu uptake and reactive oxygen homeostasis. Cells lacking Mac1p are severely compromised in both mitochondrial respiration and Fe accumulation, both a product of disrupted Cu homeostasis. Loss of Cu uptake in *mac1Δ/Δ* mutants cannot totally explain these deficiencies; rather *mac1Δ/Δ* mutants are defective in intracellular distribution of Cu to essential targets including Cu-requiring cytochrome oxidase (COX) for respiration. We provide evidence that Mac1p control of anti-oxidant genes through down-regulation of *SOD1* not only maintains oxidative stress protection, but also spares Cu for mitochondrial COX. Mac1p helps maintain respiration essential for pathogenesis in the face of limiting host copper.

Results and Discussion

The mac1 strain has a virulence defect in a murine model of disseminated candidiasis

We sought to determine whether the Mac1p response to Cu during *C. albicans* invasion of the kidney was essential for pathogenesis. For these studies, we generated a *mac1Δ/Δ* mutation in a *C. albicans* clinical isolate lacking auxotrophic markers. Both *mac1Δ/+* heterozygous and *mac1Δ/Δ* homozygous mutations were generated in the SC5314 background using the SAT1 flipper technique (Reuss *et al.*, 2004). We validated that *mac1Δ/Δ* homozygotes, but not *mac1Δ/+* heterozygotes, behaved similarly to a *mac1Δ/Δ* homozygous deletion in the SN152 background (Li *et al.*, 2015), and are unable to switch from Cu containing Sod1p to Mn containing Sod3p during Cu starvation, as induced by the Cu(I) chelator bathocuproinedisulfonic acid (BCS) (Fig. 1A, lane 7). This defect was

corrected by integrating a single copy of *MAC1* into its native locus (Fig. 1A, lane 9). We used both the *mac1Δ/Δ* deletion strain and the reintegrated *MAC1* rescue for virulence studies in a murine model of disseminated candidiasis where the kidneys are the major target organs and where tissue Cu levels decline during infection (MacCallum & Odds, 2005, Li *et al.*, 2015, Mackie *et al.*, 2016, Besold *et al.*, 2018, Culbertson *et al.*, 2020). As seen in Fig. 1B, female mice infected with WT *C. albicans* by lateral tail vein injection succumbed to the infection by 9 days. The *mac1Δ/Δ* strain displayed a stark virulence defect (Fig. 1B) with *mac1Δ/Δ* infected mice displaying a mean survival of 15 days post-infection. Virulence was fully restored by reintegration of *MAC1* (Fig. 1B). Thus, Mac1p plays an important role in fungal virulence in disseminated candidiasis. Recent studies by Khemiri have shown role for *C. albicans* Mac1p in adherence to a human colon epithelial cell line *in vitro*, consistent with the importance of Mac1p in fungal growth and cell invasion (Khemiri *et al.*, 2020).

The effects of mac1Δ/Δ mutations on fungal copper accumulation and mitochondrial respiration

The basis for the virulence defect of *mac1Δ/Δ* strains was unclear. We therefore examined in detail the effects of *MAC1* loss in cultures of *C. albicans in vitro*. Aside from regulating *SOD1* and *SOD3*, Mac1p has been reported to induce the Cu permease gene *CTR1* when cells are deprived of extracellular Cu (Woodacre *et al.*, 2008). To directly monitor *CTR1* expression we examined transcript levels by qRT-PCR. In enriched YPD media that was not depleted for Cu, *CTR1* mRNA levels in the *mac1Δ/Δ* mutant were significantly lower than that of WT cells (Fig. 2A). Therefore Mac1p not only upregulates *CTR1* with Cu starvation, but is also needed for basal levels of *CTR1* expression when extracellular Cu is available. Consistent with this low basal level of *CTR1* expression, *mac1Δ/Δ* mutants displayed ≈25–30% of the total intracellular Cu of WT cells (Fig. 2B, also see Figs. 3C and S1A). This low level of intracellular Cu in *mac1Δ/Δ* cells is similar to WT cells treated with 400 μM of the Cu chelator BCS (Fig. 2B).

We tested whether this low level of intracellular Cu in *mac1Δ/Δ* mutants impacted mitochondrial respiration, a Cu-dependent process essential for virulence *of C. albicans* (Bambach *et al.*, 2009, Becker *et al.*, 2010, Aoki *et al.*, 1990). Cytochrome c oxidase (COX) of the electron chain is a Cu-requiring complex (Turski & Thiele, 2009, Festa & Thiele, 2011) and can be monitored by measuring the rate of cyanide sensitive-oxygen consumption. As seen in Fig. 2C, COX-respiration in the *mac1Δ/Δ* strain was drastically reduced compared to the WT strain. This defect is indeed due to Cu deficiency, as oxygen consumption levels were restored to WT levels in cells supplemented with high Cu in the growth medium (Fig. 2C).

Since *C. albicans* is dependent on respiration for growth *in vivo* (Bambach *et al.*, 2009, Becker *et al.*, 2010, Aoki *et al.*, 1990), we tested whether the lowered oxygen consumption in *mac1Δ/Δ* cells impacted respiration-dependent growth *in vitro*. *C. albicans* relies on respiration in the presence of non-fermentable carbon sources such as glycerol and ethanol. As seen in Fig. 2D top, *mac1*Δ/Δ strains failed to grow when glycerol and ethanol are provided as sole carbon sources, compared to growth on glucose that supports both fermentation and respiration. This requirement of Mac1p for growth on non-fermentable

carbon sources is consistent with findings of *mac1Δ/Δ* mutants in an independent *C. albicans* strain background (BWP17) (Marvin *et al.*, 2004). As with oxygen consumption, the respiratory defect of *mac1Δ/Δ* mutants was totally rescued by supplementation with Cu salts (Fig. 2D bottom). Thus, the respiratory deficiency of *mac1Δ/Δ* mutants as seen in Fig. 2C is indeed sufficient to block respiration-dependent growth as might occur *in vivo* during infection.

To test if low intracellular Cu by itself can explain the respiratory deficiency of *mac1Δ/Δ* cells, we quantified COX-respiration in WT cells that accumulate virtually identical low levels of total intracellular Cu through treatment with 400 μM BCS (Fig. 2B). Surprisingly, these Cu-depleted WT cells showed no defect in respiration (Fig. 2E). Unlike *mac1Δ/Δ* mutants, COX-respiration in WT cells appears resilient to Cu deprivation. Hence, the respiration defect of *mac1Δ/Δ* mutants cannot be explained by low levels of total intracellular Cu. Instead, the mutants may be unable to properly allocate Cu to COX in the mitochondria.

Contribution of SOD1 to the respiratory defect of mac1Δ/Δ cells

We addressed whether the apparent *mac1Δ/Δ* defect in Cu allocation to COX is due to improper regulation of *SOD1*. Sod1p is an abundant cellular protein that can account for 10–20% of the total intracellular Cu in yeast strains (Rae *et al.*, 1999). Since *mac1Δ/Δ* mutants cannot repress *SOD1* gene expression, Sod1p polypeptide levels remain unaffected in these Cu starved cells (Fig. 1A, lane 7, and (Li *et al.*, 2015)). Most importantly, Sod1p retains a certain level of activity in *mac1Δ/Δ* cells (Fig. 3A, lane 3). In spite of very low intracellular Cu, Sod1p is still receiving Cu in *mac1Δ/Δ* cells.

To test whether the residual Cu bound to Sod1p in *mac1Δ/Δ* mutants is preventing Cu activation of COX, we generated a *mac1Δ/Δ sod1Δ/Δ* double mutant using a CRISPR based approach for *C. albicans* (Nguyen *et al.*, 2017). When we assayed COX respiration, we found that deletion of *SOD1* significantly ameliorated the oxygen consumption defect of *mac1Δ/Δ* mutants (Fig. 3B). This rescue of respiration by the *sod1Δ* mutation occurred without any increases in total intracellular Cu (Fig. 3C). Therefore, the presence of residual Sod1p in Cu-starved *mac1Δ/Δ* cells appears to function as a Cu sink, sequestering Cu away from COX. This notion of Sod1p limiting Cu accessibility to COX is consistent with our previous studies in WT cells where inappropriate over-expression of *SOD1* lowered COX respiration (Broxton & Culotta, 2016). We conclude that Mac1p in *C. albicans* has evolved to maintain COX activity essential for growth and pathogenesis in at least two ways: by inducing Ctr1p-dependent Cu transport and by down-regulating Sod1p, a major Cu consumer. The regulation of anti-oxidant enzymes by *C. albicans* Mac1p not only serves to maintain oxidative stress protection and ROS signaling during Cu starvation (Broxton *et al.*, 2018, Broxton & Culotta, 2016), but also facilitates Cu sparing for COX to help maintain mitochondrial respiration. A similar mechanism may extend to other organisms as well. In *C. neoformans,* the Cuf1p Cu sensor represses Cu/Zn SOD1 during Cu starvation (Garcia-Santamarina *et al.*, 2018), which may also be part of a Cu sparing pathway for maintaining respiration or other essential Cu-dependent processes. The notion of Cu sparing for mitochondrial respiration has also be proposed for certain marine microorganisms. During

Cu limitation, the photosynthetic algae *Chlamydomonas reinhardtii* degrades Cu containing plastocyanin for photosynthesis and Cu is prioritized for mitochondrial COX (Kropat *et al.*, 2015, Merchant *et al.*, 1991, Merchant & Bogorad, 1986). Down regulation of plastocyanin during Cu limitation has also been observed in diatoms (Hippmann *et al.*, 2017).

Evidence for non-Sod1 factors in Mac1p-maintenance of respiration

It is important to note that loss of *SOD1* only partly rescues COX-dependent respiration in *mac1Δ/Δ* mutants, but does not restore oxygen consumption to WT levels (Fig. 3B). Is this level of restored oxygen consumption sufficient to support respiration-dependent growth? As seen in Fig. 3D, deletion of *SOD1* did not restore growth of *mac1Δ/Δ* strains on non-fermentable carbon sources without Cu supplements (Fig. 3D column 1); however the *mac1Δ/Δ sod1Δ/*Δ mutant did exhibit improved growth compared to the *mac1Δ/Δ* strain when low levels of Cu salts were added to the growth media (Fig. 3C, column 2–6). Thus, loss of *SOD1* can improve respiration-dependent growth of a *mac1Δ/Δ* strain, but only under specific conditions of Cu availability. We also tested the effects of Sod1p loss in the disseminated model of candidiasis, where respiration is essential for virulence (Bambach *et al.*, 2009, Becker *et al.*, 2010, Aoki *et al.*, 1990) and where the kidney is the major site of infection (MacCallum & Odds, 2005). Previous studies have shown that Cu availability becomes low in the kidney during *C. albicans* invasion (Li *et al.*, 2015, Mackie *et al.*, 2016, Culbertson *et al.*, 2020, Besold *et al.*, 2018), and with these Cu-limited conditions, loss of *SOD1* may not be sufficient to support respiration-dependent growth. Indeed, as shown in Fig. 3E, there was no significant difference between mouse survival in mice infected with *mac1Δ/Δ* or *mac1Δ/Δ sod1Δ/Δ* strains. However, as a potential caveat to these studies, *sod1Δ/Δ* strains by themselves are defective in virulence in this mouse model (Hwang *et al.*, 2002) and consistent with this, we observed a slight trend towards decreased virulence in the *mac1Δ/Δ sod1Δ/Δ* strain compared to the single *mac1Δ/Δ* mutant. In any case, our cell culture studies (Fig. 3C and 3D) demonstrate that Mac1p repression of *SOD1* is just one method for preserving COX-respiration and other factors may contribute as well.

mRNA profile changes associated with mac1Δ/Δ mutations

To search for other factors that may contribute to the *mac1Δ/Δ* defect, we used an RNA-seq approach to identify possible targets of Mac1p regulation. For these studies, we compared *mac1Δ/Δ* to WT cells treated with 400 μM of the Cu(I) chelator BCS. As seen in Fig. S1A, these two experimental groups have similarly low levels of intracellular Cu (see also Fig. 2B). Any variations in gene expression patterns can therefore be ascribed specifically to the loss of Mac1p, not variations in Cu accumulation.

The possible targets of Mac1p-repression were defined as those genes repressed ≈ 3-fold or greater with BCS, but either unchanged or elevated in a *mac1Δ/Δ* strain. As shown in Fig. S1B, only three genes fit this category and of these, the most strongly repressed by Cu starvation is *SOD1*. Similar findings were obtained with *C. neoformans,* where the most prominent target of Cuf1-repression during Cu starvation is *SOD1* (Garcia-Santamarina *et al.*, 2018). In the case of *C. albicans,* other genes down-regulated by BCS but not in *mac1Δ/Δ* mutants include *AAH1*, which encodes an adenine deaminase (Uhl *et al.*, 2003, Garcia-Sanchez *et al.*, 2005), and *FGR23,* implicated in the regulation of filamentous growth (Uhl *et*

al., 2003) (Fig. S1B). Neither *AAH1* or *FGR23* are predicted to encode cuproproteins, nor contain consensus sequences for Mac1p binding (Woodacre *et al.*, 2008, Khemiri *et al.*, 2020) downstream of the start site as is the case for *SOD1* (Li *et al.*, 2015), or in upstream promoter sequences. It is therefore possible that *SOD1* is the only gene repressed by Mac1p in *C. albicans*, highlighting the importance of *SOD1* repression during Cu starvation. As a possible caveat to our search of consensus binding sequences, *C. albicans* Mac1p may be promiscuous in its DNA recognition site, as has been observed for *C. neoformans* Cuf1p (Garcia-Santamarina *et al.*, 2018) and *A. fumigatus* Mac1p (Kusuya *et al.*, 2017, Park *et al.*, 2018).

We also interrogated possible gene targets of Mac1p activation. Such genes should be upregulated upon treatment with BCS but not in *mac1Δ/Δ* strains. Indeed, we identified many known targets of Mac1p including genes for Cu uptake (*CTR1, FRE7, FRE30*), and genes that substitute for Sod1p loss (*SOD3, AOX2*) (Fig. S1C) (Woodacre *et al.*, 2008, Li *et al.*, 2015, Khemiri *et al.*, 2020). Additionally genes encoding hexose sugar transporters and hyphal specific genes (e.g., *ECE1, HWP1, ALS3*) were among those uniquely induced by BCS (Fig. S2A and Table S1); none of which contained obvious Mac1p binding consensus sequences. We have previously shown that *sod1Δ/Δ* deletions induce hexose transporters, reflecting a role of Sod1p in glucose signaling (Broxton *et al.*, 2018); the absence of Sod1p in BCS-treated WT cells (Fig. 1A) likely accounts for induction of sugar transporters in these cells. The induction of hyphal specific genes is consistent with some development of filamentation observed in Cu-starved WT cells, but not in the *mac1Δ/Δ* strain (Fig. S2B). Cu starvation is known to induce filamentation in *C. albicans* in a Mac1p-dependent manner (Marvin *et al.*, 2004, Huang *et al.*, 2006).

Fe-starvation state of mac1Δ/Δ mutants

RNA-seq analysis indicated that *mac1Δ/Δ* mutants are not only starved for Cu but also Fe. Specifically, we observed a large number of genes induced in *mac1Δ/Δ* cells consistent with a transcriptional Fe-starvation stress response. Gene Ontology (GO) analysis revealed that many genes induced in the *mac1Δ/Δ* strain were related to ribonucleoprotein complex biogenesis, which is regulated by the HAP43/CCAAT binding protein Fe regulatory complex of *C. albicans* (Fig. S2A) (Singh *et al.*, 2011). We observed strong induction of genes involved in reductive Fe uptake, heme acquisition and the siderophore transporter *SIT1* (Fig. 4A), all of which are known be induced under Fe starvation stress conditions (Chen *et al.*, 2011). While this manuscript was in its final stages of preparation, Khemiri et. al. published a study showing a similar high induction of specific Fe transport genes in *mac1Δ/Δ* cells from the background strain SN125 (Khemiri *et al.*, 2020).

Regulation of Fe transport genes has also been reported with *A. fumigatus* Mac1p (Afmac1), however the role of Afmac1 in Fe homeostasis appears opposite to that of *C. albicans* Mac1p. AfMac1 is an activator of Fe uptake genes and loss of Afmac1p results in a decreased expression of Fe transporters (Park *et al.*, 2018), unlike the pronounced induction of the Fe transport regulon with *C. albicans mac1Δ/Δ* mutations (Fig. 4A). *C. albicans* Mac1p is either a repressor of Fe uptake or the effects of *mac1Δ/Δ* are an indirect consequence of Cu starvation. We favor the latter. Cu is important for Fe acquisition and in

yeast species including *C. albicans,* FET multicopper oxidases drive Fe uptake into the cell (Ziegler *et al.*, 2011, Mamouei *et al.*, 2017). Consistent with this requirement for Cu in Fe uptake we find that *C. albicans mac1Δ/Δ* mutants accumulate extraordinarily low levels of intracellular Fe (Fig. 4B), explaining the strong induction of the Fe starvation stress response at the mRNA level (Fig. 4A). Moreover, this Fe deficiency of *C. albicans mac1Δ/Δ* mutants is totally reversed by Cu supplements (Fig. 4B), unlike AfMac1p control of *A. fumigatus* Fe transport which occurs independent of Cu status (Park *et al.*, 2018). With *C. albicans*, the Fe starvation stress state of *mac1Δ/Δ* mutants appears secondary to defects in Cu homeostasis and the inability of these cells to populate FET oxidases with Cu.

Fe and Cu deficiency and the respiratory defect of mac1Δ/Δ mutants

With such low Fe in *mac1Δ/Δ* mutants, we questioned if limited Fe bioavailability could be contributing to the respiration defect of these cells. Both Complex I and Complex II of the electron transport chain make use of Fe-S clusters (Paul *et al.*, 2017) and Complex II, III, and IV all make use of Fe in the form of heme (Kim *et al.*, 2012). To test the impact of low Fe on *mac1Δ/Δ* respiration, we sought to uncouple the Fe and Cu deficiencies of this mutant. Through Fe titrations we found that supplementation of 350 μM ferrous ammonium sulfate to the growth media restored Fe levels in the *mac1Δ/Δ* mutant to near WT levels (Fig. 5A), with no elevation in intracellular Cu (Fig. 5B). This restoration of intracellular Fe pools also alleviated the Fe starvation stress response of this mutant as demonstrated by reversal of the strong *mac1Δ/Δ* induction of the *CFL4* and *SOD4* gene targets of the Sef1/Sfu1p Fe regulatory system (Fig. 5C,D) (Chen *et al.*, 2011). However, amelioration of the *mac1Δ/Δ* Fe deficiency had no effect on the poor respiration of these mutants (Fig. 5E), compared to the strong rescue of respiration with Cu supplements (Fig. 2C). The *mac1Δ/Δ* respiratory phenotype is overwhelmingly driven by deficiencies in Cu, not Fe.

Evidence for mac1Δ/Δ defects in Cu allocation for Fe uptake

It is important to note that the Fe starvation state of *mac1Δ/Δ* cells cannot wholly be explained by low total Cu. The same Fe stress response is not mirrored in WT cells treated with BCS (Fig. S2A, Fig. 4A), even though these cells accumulate similarly low levels of Cu (Fig. 2B, S1A). Only a small number of the Fe transport genes were induced in BCS-WT cells, including *CFL2, CFL5, FET31,* and the level of induction was greatly reduced compared to that of *mac1Δ/Δ* cells (Fig. 4A). Just *SOD4,* which is not involved in Fe transport but is part of the Fe starvation regulon (Chen *et al.*, 2011, Schatzman *et al.*, 2020, Sorgo *et al.*, 2013), is induced to similar levels in BCS-treated WT versus *mac1Δ/Δ* cells (Fig. 4A). Furthermore, BCS-treated WT cells accumulate substantially higher levels of Fe compared to *mac1Δ/Δ* cells (Fig. 6A). These comparisons to Cu-starved WT cells demonstrate that the severe Fe deficiency of *mac1Δ/Δ* cells is not simply due to low total Cu. As with COX respiration, the *mac1Δ/Δ* mutant appears impaired in proper allocation of Cu for Fe uptake.

Since the respiratory defect of *mac1Δ/Δ* cells was improved upon loss of the Sod1p Cu consumer (Fig. 3B), we tested if a deletion in *SOD1* would likewise restore Fe accumulation. Significantly and unlike respiration, deletion of *SOD1* had no effect on the Fe deficiency of the *mac1*Δ/Δ mutant (Fig. 6B). Mac1p repression of *SOD1* helps spare Cu for

respiration but not for Fe uptake. Other Cu consumers may be preventing Cu allocation for Fe uptake and one family of genes that are suspect are the FETs themselves. *Candida* has 5 annotated FETs: Fet3p, Fet31p, Fet33p, Fet34p, and Fet99p (Muzzey *et al.*, 2013). Of these, Fet34p and Fet99p are reported to be the most important for Fe acquisition (Mamouei *et al.*, 2017) and both of the corresponding genes are strongly induced in *mac1Δ/Δ* strains as part of the Fe starvation stress response (Fig. 4A). Of the FETs less critical for Fe uptake (Fet3p, Fet33p, Fet31p; (Mamouei *et al.*, 2017)), *FET31* is 100x fold induced in *mac1Δ/Δ* mutants, while *FET3* and *FET33* are not substantially changed (Fig. 4A). It is possible that Cu binding to highly abundant Fet31p may preclude proper Cu allocation to the more essential FETs for Fe uptake. Other possible Cu consumers of the cell include the putative amine oxidases Amo1p and Amo2p and although neither is induced in *mac1Δ/Δ* strains (Table S1), they may contribute to poor Cu availability. Additionally, we cannot exclude the possibility that Cu is sequestered in organelles such as the vacuole in the *mac1Δ/Δ* strain. These mechanisms for poor Cu allocation might also contribute in part to the respiratory defect of *mac1Δ/Δ* strains, on top of the major impact of retaining Sod1p in these Cu-starved cells.

Conclusions:

Across numerous fungal species, the Cu sensor Mac1p/Cuf1p controls cell surface uptake of Cu to ensure that intracellular Cu levels remain ample under conditions where extracellular Cu is low (Marvin *et al.*, 2004, Woodacre *et al.*, 2008, Li *et al.*, 2015, Jungmann *et al.*, 1993, Labbe *et al.*, 1997, Garcia-Santamarina *et al.*, 2018, Jiang *et al.*, 2011, Ding *et al.*, 2011, Kusuya *et al.*, 2017, Cai *et al.*, 2017, Park *et al.*, 2017). Our studies demonstrate that in addition to this role in Cu uptake, Mac1p in *C. albicans* participates in Cu allocation and prioritizing Cu for processes such as Fe uptake and cellular respiration. This role in Cu allocation is accomplished in part through down-regulation of a major Cu consumer Sod1p and re-directing Cu towards COX for mitochondrial respiration. Previously, Mac1p control of *C. albicans* anti-oxidant genes *SOD1, SOD3* and *AOX2* was shown to maintain oxidative stress protection and ROS signaling during times of Cu limitation (Broxton & Culotta, 2016, Broxton *et al.*, 2018). We now show this regulation of antioxidant genes has the added purpose of sparing Cu for respiration that is essential for pathogenesis of *C. albicans.* These findings with Mac1p may not be unique to *C. albicans*. *C. neoformans* also represses *SOD1* during Cu limitation (Garcia-Santamarina *et al.*, 2018) and the Cu may be re-purposed for other cuproenzymes essential for pathogenesis.

Experimental Procedures

Fungal strains and culture conditions

Cultures of *C. albicans* were maintained at 30°C in YPD (1% yeast extract, 2% peptone, 2% dextrose). Where indicated, cultures were supplemented 400 μM of the Cu(I) chelator BCS. Tests for respiration-dependent growth used non-fermentable carbon source containing YPGE medium (2% bacto-peptone, 1% yeast extract, 3% glycerol, 2% ethanol) and both YPD and YPGE plates were made with 2% bactoagar. YPD and YPGE plates supplemented with Cu contained 100 μM $CuSO_4$. YPM media (1% yeast extract, 2% peptone, 2% maltose) was used to stimulate flippase activity. When noted, Fe was supplemented to liquid cultures

as ferrous ammonium sulfate (Sigma Chemical Co.)) and Cu was added as $CuSO_4$ (Sigma-Aldrich)

C. albicans clinical isolate SC5314 was used as the WT and parental strain from which all mutants were derived. Construction of the *mac1Δ/Δ* mutant by the SAT1-flipper method was as follows using primers described in Table S2: genomic DNA from strain SC5314 was used to amplify *MAC1* residues −441 to −205 and +1423 to +1593. These were inserted respectively into the SacI and NotI and the XhoI and KpnI sites of the pSFS2 plasmid (Reuss *et al.*, 2004), generating plasmid pEC-M1L. The *mac1* deletion cassette from pEC-M1L was mobilized by KpnI and SacI digestion and used to transform SC5314 by electroporation. Cells were plated onto YPD containing 200 mg/mL nourseothricin sulfate (NAT) (Gold Bio), and NAT resistant colonies selected and verified for presence of the NAT cassette by PCR. Positive colonies were then grown overnight in YPM media to induce excision of NAT resistance by flippase. Cells were then plated onto 25 mg/mL NAT containing YPD media and NAT sensitive colonies were selected. The resultant *mac1Δ/+* heterozygous mutant strain EC003 was verified by PCR. To delete the second allele, *MAC1* sequences −205 to −1 and +1201 to +1419 were amplified by PCR and inserted into the SacI and NotI, and XhoI and KpnI sites in pSFS2 to generate the plasmid pEC-M1S. The deletion cassette from pEC-M1S was liberated with KpnI and SacI digestion and used to transform the *mac1Δ/+* strain EC003. NAT resistant colonies were selected and NAT resistance excised as above. Deletion of the second *MAC1* allele was verified by PCR, resulting in the *mac1Δ/Δ* strain EC004.

A single copy of *MAC1* was introduced in the *mac1Δ/mac1Δ* strain EC004 as follows: *MAC1* genomic sequences from −205 to +1419 were amplified and inserted into pSFS2 at SacI and NotI sites. *MAC1* residues +1209 to 1419 were inserted into XhoI and KpnI sites of this plasmid to generate the pEC-M1R plasmid, which was then linearized with SacI and KpnI and transformed into the *mac1Δ/Δ* strain EC004, generating the *mac1Δ/Δ::MAC1* re-integrant strain EC005.

A CRISPR protocol optimized for *C. albicans* was used as previously described (Nguyen *et al.*, 2017) to generate the *mac1Δ/Δ sod1Δ/Δ* and a *mac1Δ/Δ* control strain using oligonucleotides listed in Table S3. Homozygous null mutations were introduced using a donor DNA oligo 100 bp in length composed of residues −50 to −1 upstream and 50 base pairs directly downstream of the coding regions of *MAC1* and *SOD1*, respectively. Guide RNAs to *MAC1* and *SOD1* were designed using Benching Software. Both *SOD1 and MAC1* were simultaneously deleted in *C. albicans* strain SC5314 to generate strain EC007 and the *mac1Δ/Δ* single homozygous mutant strain EC006 was generated similarly. Strains were confirmed by PCR and DNA sequencing.

Fungal cells were photographed using dark field microscopy using a Nikon Infinity 1 microscope at 40x magnification.

Murine Virulence Studies

Murine studies were carried out according to the National Institutes of Health guidelines for the ethical treatment of animals. This protocol was approved by the Institutional Animal

Care and Uses Committee of the Johns Hopkins University medical institutions, protocol number M013M264. Nine-week old BALB/c female mice per strain in groups of ten were infected with 2×10^5 *C. albicans* cells in 100 μL by lateral tail injection as previously described (Conti *et al.*, 2014). Female mice were used as no difference between sexes has been noted in regards to the Cu response seen in the kidney during infection (Besold *et al.*, 2018, Li *et al.*, 2015, Culbertson *et al.*, 2020). The fungal cells for infection were obtained by growth overnight in YPD to OD_{600} ~15; cells were harvested by centrifugation, washed twice in sterile 1x phosphate buffered saline (PBS), enumerated on a hemocytometer and diluted to 2×10^6 cells mL^{-1} in PBS. Mouse survival following injection was monitored daily up to 30 days.

Metal measurements

For Cu analysis, cells were grown in YPD to OD_{600} of ≈2.0. 10–20 OD_{600} cell units were harvested by centrifugation and washed twice with 10 mM Tris, 1 mM EDTA, pH 8, and twice with MilliQ deionized water and cell recovery determined by measuring OD_{600}. Cells were digested with 200 μL 10% nitric acid (Fisher Chemical) at 100°C, diluted to a final concentration of 2% nitric acid in MilliQ water and then Cu content was measured using an AAnalyst graphite furnace atomic absorption spectrometer (AAS) (PerkinElmer). Metal content was normalized to cell number.

For Fe measurements, samples were subjected to a bathophenanthroline disulfonate (BPS) based assay as previously described (Tamarit *et al.*, 2006). Cells for Fe analysis were prepared in nitric acid as described above for AAS. A plate-based version of the assay was adopted in which reactions contained 100 μL of standard or cell sample, 75 μL of water, 40 μL of 38 mg ml^{-1} sodium ascorbate, and 32 μL of a 1/3 saturated ammonium acetate solution. A first measurement of absorbance at 535 and 680 nm was then measured on an Eon (Biotek) plate reader. After the first spectrophotometric readings, 3 μL of 34 mg mL^{-1} BPS was added to each well. A second set of absorbance measurements at 535 and 680 nm was then measured. The BPS signal was determined as $X = ((A_{535}\text{-}A_{680})_{sample+BPS} - (A_{535}\text{-}A_{680})_{sample\text{-}BPS}) - ((A_{535}\text{-}A_{680})_{blank+BPS} - (A_{535}\text{-}A_{680})_{blank\text{-}BPS})$.

Oxygen Consumption Assays for COX respiration

Oxygen consumption measurements were conducted on whole cells similarly to published protocols (Li *et al.*, 2011, Broxton & Culotta, 2016) with modifications. Cells were grown in YPD to an OD_{600} of ≈2.0, harvested by centrifugation and resuspended to an OD_{600} of 5.0 and allowed to grow for 1 hour in fresh YPD at 30°C. 500 μL of the 5 mL culture were added to 1 mL of YPD in a Clark-type electrode (Hanstech Oxythem Plus) in a magnetically stirred, thermostatically controlled 1.5 mL chamber at 30°C. Oxygen saturation was measured over the course of 3–5 minutes and the change in oxygen saturation over time was used to determine the oxygen consumption rate. To validate oxygen consumption dependent on COX-mitochondrial respiration, 10 mM potassium cyanide (KCN) was added to inhibit COX respiration as described (Li *et al.*, 2011). For all respiration experiments 3 cultures for each strain served as the biological replicates and for each biological replicate 2 technical replicates of oxygen consumption were averaged.

Analyses of SOD protein and enzyme activity

For immunoblots and SOD activity gels, cells were grown to an OD_{600} of ≈2 in 10 mL YPD. 10 OD_{600} cell units were harvested, washed in water and resuspended in 100 μL of a lysis buffer containing 5 mM EDTA, 5 mM EGTA, 50 mM NACL, 10% glycerol, and 0.1% Triton X-100 in 10 mM sodium phosphate, pH 7.8. An equal volume of 0.5 mm zirconium oxide beads (Research Products International) was added and the samples were vortexed three times for a minute and a half at 4°C. Samples were then centrifuged for 10 minutes at 14,000xg. Supernatant was removed and protein concentration was determined via Bradford method. The resultant protein lysates were used for immunoblot analysis and SOD activity.

Immunoblot analysis for Sod1p and Sod3p followed published procedures (Gleason *et al.*, 2014). Briefly 10–15 μg of the samples were subjected to denaturing gel electrophoresis on 4–12% Bis-Tris acrylamide gels (Thermo Fisher). Proteins were then transferred to a PVDF membrane and blocked in 2% non-fat milk (VWR Life Science). Blots were probed with anti-SOD1(Jensen & Culotta, 2005) at a 1:10000 dilution and anti-SOD3 (Gleason *et al.*, 2014) at 1:5000. Primary antibody incubation was followed by incubation with secondary goat anti-rabbit IgG Alexa Flour 680 antibody at 1:10,000 (Thermofisher Scientific). Blots were imaged on an Odyssey infrared imaging system (LI-COR Biosciences) at 700 nm.

For analysis of SOD activity, lysates were run on native non-reducing 10% Tris-glycine gels at 50 mA for 90 minutes at 4°C. Gels were then stained with 35 mL of a potassium phosphate buffered solution containing Nitro Blue Tetrazolium (Sigma-Aldrich), riboflavin (Acros Organics) and 35 μL of TEMED (Invitrogen) as previously published (Gleason *et al.*, 2014). Gels were incubated in this solution for 1 hour in the dark with gentle shaking. Gels were then placed in dH_2O, exposed to light and photographed.

RNA analysis by qRT-PCR and RNA-seq

To prepare RNA for qRT-PCR or RNA-seq, triplicate cultures were grown 16 hours to an OD_{600} of ≈ 2.0 in YPD. RNA was extracted from at least five OD_{600} cell units via an acid-phenol protocol as previously described (Collart & Oliviero, 2001). Briefly cells were lysed for an hour at 65°C with vortexing every 5 minutes in 400 μL of acid phenol pH 4.5 (Ambion AM9720), 350 μL of 3M sodium acetate pH 5.5 (Thermo Fisher Scientific), and 1% SDS. Samples were subjected to three organic extractions with acid-phenol, precipitated by ethanol and nucleic acids treated with DNAse by Rapidout (ThermoFisher Scientific K2981). For qRT-PCR, cDNA was synthesized using a RevertAid First Strand cDNA (ThermoFIsher Scientific K1622). cDNA samples were diluted 1:10 in DEPC-treated water before qRT-PCR analysis with PowerUp SYBR Green Master Mix (ThermoFisher Scientific). qRT-PCR was performed on a QuantStudio 3 (Applied Biosystems) instrument. Cycle threshold values (Ct values) were normalized to *TUB2*. Relative expression was calculated using the dCT method. Amplicons of ~200 bp were obtained from the primers for *TUB2*, *CTR1, CFL4*, and *SOD4* shown in the Table S4. For RNA-seq, RNA samples were processed by Novogene Corporation Inc. for paired-end sequencing on an Ilumina HiSeq 2500. Reads were aligned to the *C. albicans* reference genome (SC5314) using TopHat2 (Kim *et al.*, 2013). Aligned reads were then used to generate read counts for each gene and the DE-seq package from Bioconductor was used for statistical analysis of differential gene

expression (Anders & Huber, 2010). The RNA-seq data that support the findings of this study will be deposited to the NCBI sequence read archive (SRA) under BioProject accession no. PRJNA649282 and will be openly available.

Software and Statistics

Differences were considered statically significantly if comparisons for P values were ≤ 0.05 using a one-way analysis of variance (ANOVA) with a Tukey posttest as determined by Graphpad Prism 7. Mouse survival was analyzed using a log rank test (Mantel Cox) to query any statistical differences. A gene was considered differentially expressed if its FDR for differential expression was <0.05. For GO analysis we used the Candida Genome Data Base's GO Term Finder and report GO terms with an FDR of less than 25%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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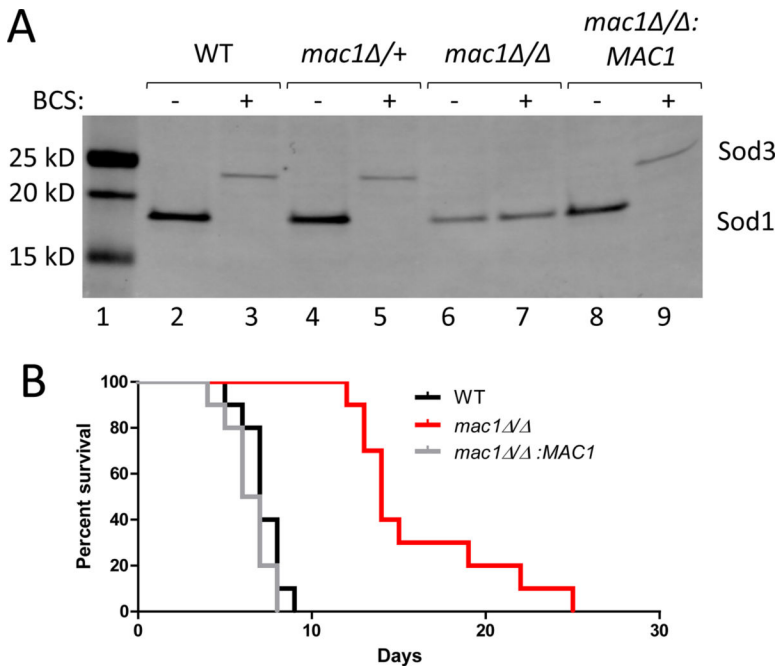

Figure 1. Generation of a *mac1Δ/Δ* mutant and virulence in a murine model of disseminated candidiasis

(A) Shown is immunoblot analysis of Sod1p and Sod3p for the indicated *C. albicans* strains using anti-SOD1 and anti-SOD3 antibodies as described in *Experimental Procedures*. (B) Survival curves are shown with groups of 10 female mice infected with 2×10^5 cells of the indicated strains as outlined in *Experimental Procedures*. There was a statically significant difference ($p<0.0001$) between mice infected with WT and those infected with *mac1Δ/Δ* but not between WT and *mac1Δ/Δ:MAC1* ($p=0.15$). Statistical significance of survival curves was determined by the log-rank (Mantel-Cox) test. Strains utilized: WT, SC5314; *mac1Δ/+,* EC003; *mac1Δ/Δ,* EC004; *mac1Δ/Δ:MAC1,* EC005.

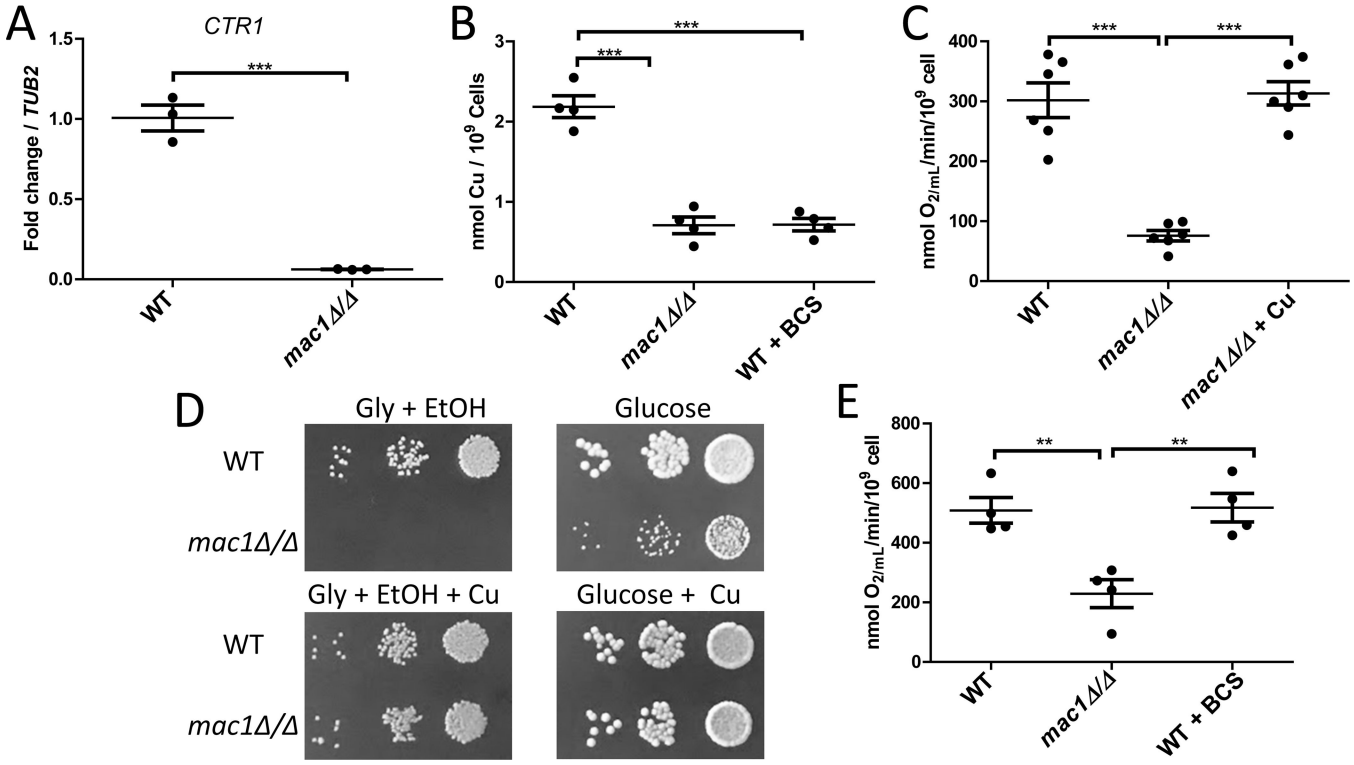

Figure 2. The Cu deficiency and respiratory defects of *mac1Δ/Δ* strains

(A) Expression of *CTR1* mRNA was analyzed by qRT-PCR and results normalized to *TUB2.* Results are shown for three independent cultures. Expression of *CTR1* in the *mac1Δ/Δ* strain was significantly different as determined by two-tailed student's T test (p=0.0003). (B) Total cellular Cu levels were measured by AAS in the indicated strains. Shown are the results of 4 independent cultures across 2 experimental trials. (C,E) KCN-inhibitable oxygen consumption was measured by a Clark-type electrode in the indicated strains. "+ Cu" and "+BCS" indicates cells cultured and assayed for oxygen consumption in the presence of 50 μM $CuSO_4$ and 400 μM BCS, respectively. Results are from 4–6 cultures across 2–3 experimental trials. (D) 5 μL of a cell solution containing 0.01, 0.001, and 0.0001 OD_{600}/mL of the designated strains were plated on YPGE ("Gly + EtOH") or YPD ("Glucose") plates. Where indicated, media was supplemented with 100 μM $CuSO_4$. Statistical significance for B,C,E was determined by one way ANOVA with Tukey posttest; **p≤0.01, ***p≤0.001. The absence of a bracket indicates that comparisons are not significantly different. Strains utilized are as described in Fig. 1. With all the data points, the wide bar reflects the mean value and the standard error of the mean (SEM) is shown with error bars.

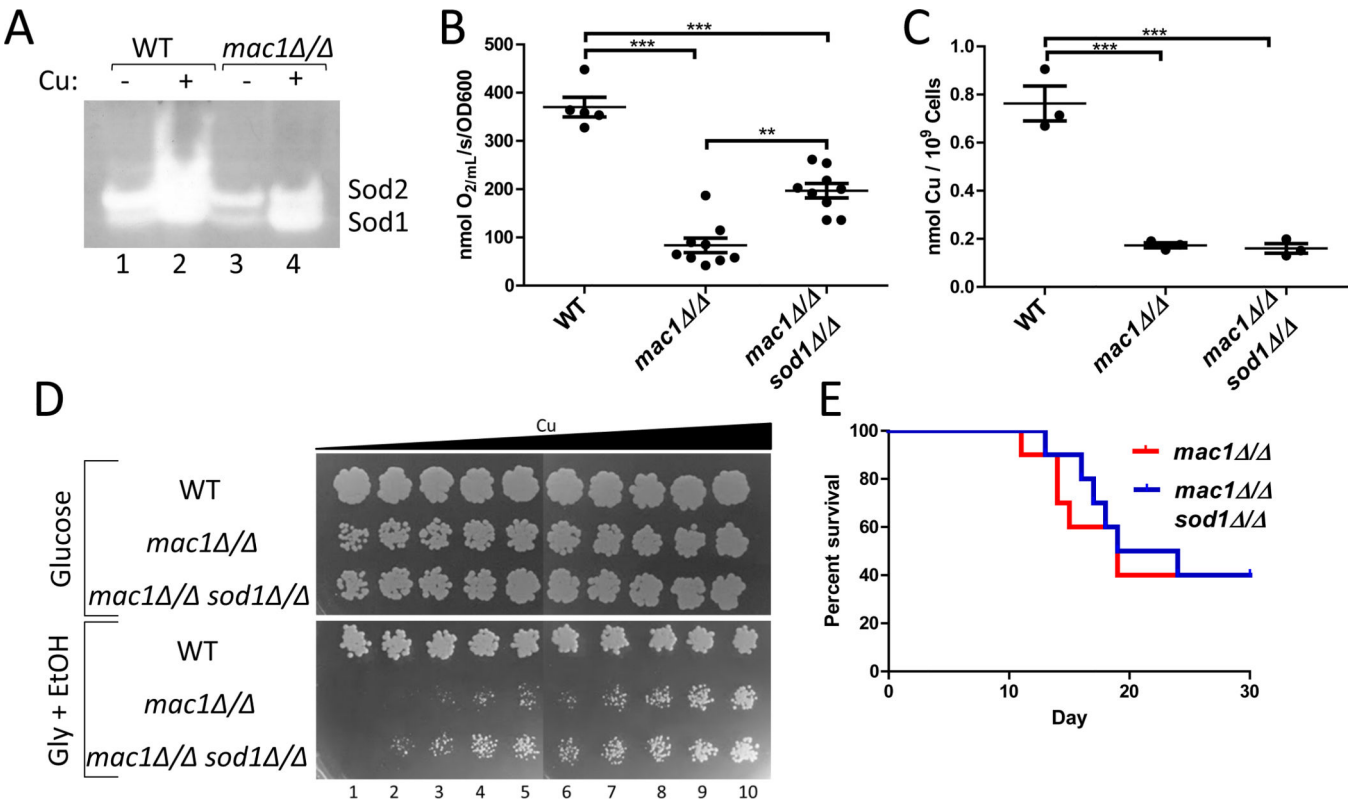

Figure 3. Deletion of *SOD1* helps alleviate the respiratory deficiency of the *mac1Δ/Δ* strain

(A) SOD activity was analyzed by the native gel assay as described in *Experimental Procedures.* Shown are results from cells grown in the presence or absence of 1 mM $CuSO_4$. The *mac1Δ/Δ* strain exhibits Sod1 activity even without Cu supplements to the growth media. (B,C) Oxygen consumption (B) and total Cu levels (C) were measured in the indicated strains as described in Fig. 2C and 2B, respectively. Statistical significance was determined by one-way ANOVA with Tukey posttest; **p≤0.01, ***p≤0.001. The absence of a bracket indicates that comparisons are not significantly different. The individual data points represent 5–9 or 3 independent cultures in parts B or C respectively; the wide bar shows the mean and error bars represent SEM. (D) 10 μL of a cell solution containing 0.0005 OD_{600}/mL of the indicated strains in PBS and increasing concentrations of Cu was spotted onto YPD (“Glucose”) or YPGE (“Gly + EtOH”). The cell solutions for columns 2–10 were supplemented with 50 μM, 75 μM, 100 μM, 125 μM, 150 μM, 175 μM, 200 μM, 250 μM, and 1000 μM $CuSO_4$, respectively. (E) Shown are survival curves of groups of 10 female mice infected with 2×10^5 cells of the designated strains. There was no statically significant difference (p=0.51) between female mice infected with *mac1Δ/Δ* and those infected with *mac1Δ/Δ sod1Δ/Δ* strains. Statistical significance of survival curves was determined by the log-rank (Mantel-Cox) test. Strains utilized: WT, SC5314; *mac1Δ/Δ,* EC006; *mac1Δ/Δ sod1Δ/Δ,* EC007.

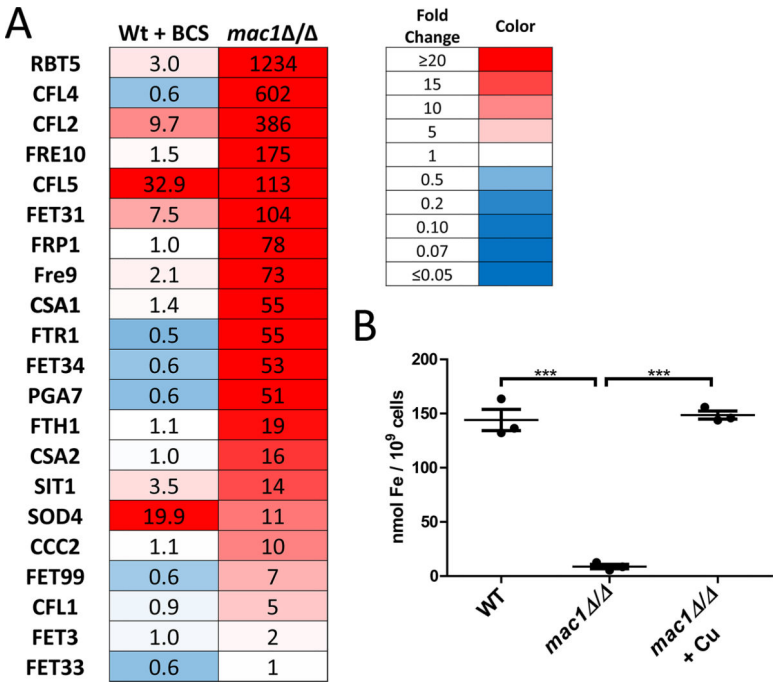

Figure 4. *mac1Δ/Δ* mutants are severely starved for Fe

(A) The fold change in mRNA levels as determined by RNA-seq is shown for genes involved in Fe transport in *C. albicans* WT + BCS and in *mac1Δ/Δ* cells, both compared to untreated WT cells. (B) Intracellular levels of Fe were measured by a colorimetric BPS-based assay as described in *Experimental Procedures.* Where designated, cultures were supplemented with 100 μM $CuSO_4$ ("+ Cu"). Values are from 3 independent cultures. Statistical significance was determined by one-way ANOVA with Tukey posttest; ***$p \leq 0.001$. Strains utilized: WT, SC5314; *mac1Δ/Δ,* EC004.

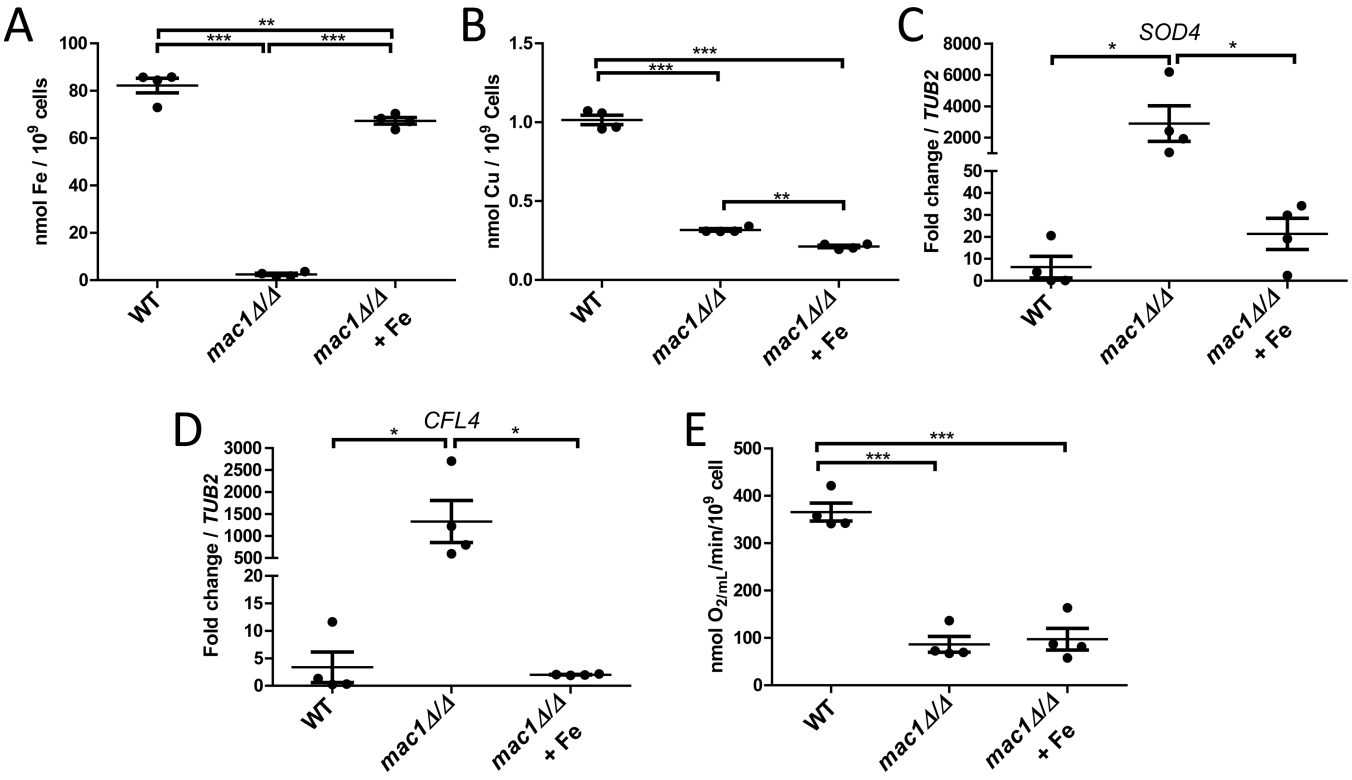

Figure 5. The *mac1Δ/Δ* deficiency in Fe does not contribute to the *mac1Δ/Δ* respiratory defect

Intracellular levels of Fe (A) or of Cu (B) were measured as in Fig. 4B and 2B, respectively. (C,D) mRNA levels of *SOD4* and *CFL4* was monitored by qRT-PCR in the indicated strains and values normalized to *TUB2*. (E) KCN-inhibitable oxygen consumption was measured as in Fig. 2C. Values are from 4 independent cultures over 2 experimental trials. Where designated, cultures were supplemented with 350 μM ferrous ammonium sulfate ("+ Fe"). Statistical significance was determined by one-way ANOVA with Tukey posttest; *p≤0.05, **p≤0.01, ***p≤0.001. The absence of a bracket indicates that comparisons are not significantly different. Among the data points, bar represents the mean with error bars showing the SEM. Strains utilized: WT, SC5314; *mac1Δ/Δ,* EC004.

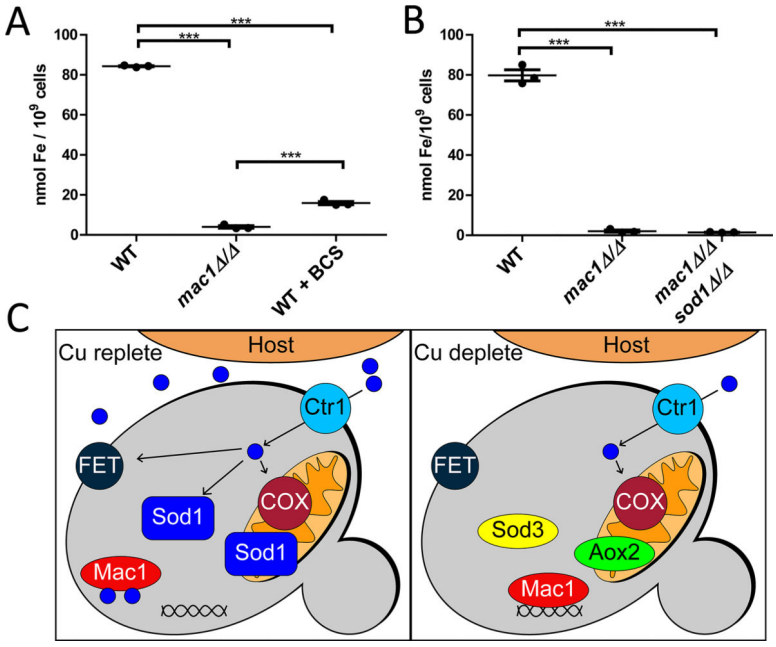

Figure 6. The metal deficiencies of *mac1Δ/Δ* cells and a proposed model for Mac1p in Cu homeostasis

(A,B) Intracellular Fe concentrations of the indicated strains was measured as in Fig. 4B. Statistical significance was determined by one-way ANOVA with Tukey posttest; ***p≤0.001. The absence of a bracket indicates that comparisons are not significantly different. Within the data points representing three independent cultures, wide bar represents mean with error bars showing the SEM. Strains utilized: WT, SC5314; *mac1Δ/Δ,* EC004 (part A) or EC006 (part B); *mac1Δ/Δ sod1Δ/Δ,* EC007. (C) Shown is a model for how *C. albicans* Mac1p regulates Cu homeostasis during infection. In the early stages of kidney infiltration (left), host Cu availability is high and there is sufficient Cu for FET multicopper oxidases for Fe uptake, for Cu/Zn Sod1p and for COX for mitochondrial respiration. At later stages (right), the host limits Cu availability to *C. albicans.* Mac1p is activated and induces Cu import as has been shown for numerous fungi (Woodacre *et al.*, 2008, Marvin *et al.*, 2004, Jungmann *et al.*, 1993, Gross *et al.*, 2000, Kusuya *et al.*, 2017, Cai *et al.*, 2019). Additionally, *C. albicans* Mac1p represses *SOD1*, and induces *SOD3* and *AOX2* to maintain ROS homeostasis in the cytosol and mitochondria, respectively. The repression of the major Cu consumer Sod1p serves to maintain high COX respiration in the mitochondria through a Cu sparing mechanism. *C. albicans* Mac1p also helps spare a limited amount of Cu for Fe uptake, through a mechanism that does not involve *SOD1* repression.