Copper Acquisition and Utilization in Fungi

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Abstract

Fungal cells colonize and proliferate in distinct niches, from soil and plants to diverse tissues in human hosts. Consequently, fungi are challenged with the goal of obtaining nutrients while simultaneously elaborating robust regulatory mechanisms to cope with a range of availability of nutrients, from scarcity to excess. Copper is essential for life but also potentially toxic. In this review we describe the sophisticated homeostatic mechanisms by which fungi acquire, utilize, and control this biochemically versatile trace element. Fungal pathogens, which can occupy distinct host tissues that have their own intrinsic requirements for copper homeostasis, have evolved mechanisms to acquire copper to successfully colonize the host, disseminate to other tissues, and combat host copper bombardment mechanisms that would otherwise mitigate virulence.

Keywords

INTRODUCTION

Copper is an essential trace element for virtually all forms of eukaryotic life. The ability of ionic copper to cycle between an oxidized (Cu^{2+}) and reduced (Cu^+) state is exploited by many enzymes to catalyze biochemical processes including aerobic respiration, superoxide detoxification, and iron acquisition (119). However, labile copper ions inactivate other metalloenzymes by metal displacement, and inappropriately engage with intracellular metallophilic ligands provided by sulfur, oxygen, and nitrogen, and drive the generation of reactive oxygen species (ROS) through Fenton-like chemistry (56, 57, 109). The acquisition, distribution, storage, and efflux of copper must be tightly regulated to satisfy cellular demands under a range of environmental conditions while minimizing toxicity due to copper accumulation beyond homeostatic capacity. Indeed, like any eukaryotic cell, fungal cells must import, buffer, route, compartmentalize, utilize, detoxify, and sense different levels of

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copper that are critical for their fitness in distinct environmental niches where they thrive, or where they simply survive under conditions of deficiency (Figure 1). Although many of the fundamental mechanisms for copper homeostasis have been discovered in fungi, and are conserved in mammals, fungi have transcriptional responses to copper availability that are distinct from those occurring in metazoans. Here we review fundamental concepts and mechanisms in copper acquisition, utilization, and regulation in fungi and highlight recent advances in the burgeoning field of fungal copper biology as it relates to host-pathogen interactions and virulence.

COPPER TRANSPORT ACROSS MEMBRANES

Fungi can assume many forms, such as hyphae, spores, and yeast cells, but in all forms they are surrounded by both a carbohydrate-rich cell wall and a plasma membrane. The fungal plasma membrane is largely impermeable to copper, creating the need for a copper uptake system that facilitates the import of copper across the lipid bilayer and into the cytosol. Moreover, copper transport must occur across a number of distinct membranes, such as that which demarcates the secretory compartments, the mitochondrial inner membrane, and specialized membranes in fungal spores (Figure 2). Low-affinity copper uptake systems such as the Fe^{2+}, Cu^+, and Zn^{2+} transporter Fet4 in the yeast *Saccharomyces cerevisiae* have been reported (75); however, an ideal copper transporter would have both high affinity and high specificity for copper, so as to acquire copper when it is scarce, respond to changing needs for copper, and ignore other charged ions. Given that copper can engage with multiple biological ligands, a copper transporter should outcompete other ligands in the local environment to coordinate to copper, yet release copper once it has been transported to the desired destination. Fungi express a family of dedicated, high-affinity copper transporters (Ctr) (39, 40, 122, 124, 175). Indeed, the Ctr proteins are not only specific for copper but also exquisitely selective, which is due to transporting the reduced form of copper, Cu^+, rather than Cu^{2+} (76). Ctr family transporters are characterized by the presence of three transmembrane domains per polypeptide, with a characteristic Met-X_3-Met motif located in the second transmembrane domain that is absolutely necessary for Cu^+ transport (131). Biochemical, genetic, and structural studies demonstrate that three Ctr protein monomers assemble as a homotrimer to form a functional transporter unit (3, 43, 131).

Ctr family members function both at the plasma membrane to import Cu^+ and on the vacuolar membrane to mobilize luminal Cu^+ stores. *S. cerevisiae* encodes two plasma membrane Ctr family members, Ctr1 and Ctr3, that possess an extracellular domain with methionine- and cysteine-rich motifs, respectively, that are proposed to concentrate copper at the transmembrane pore (Figure 2) (93, 131). Although the extreme amino termini of these metal-binding regions are dispensable for copper transport, a conserved Met/Cys-X-Met motif near the first transmembrane domain forming the pore is strictly required for function. These residues have been proposed to form a thiol-rich track, along with the Met-X_3-Met motif of the second transmembrane domain, that funnels Cu^+ into the pore formed by the homotrimeric transporter (82). The ability of Ag^+, a metal ion that is isoelectric to Cu^+, to compete for uptake and the requirement in *S. cerevisiae* for members of a ferric/cupric metalloreductase family of proteins located on the cell surface support the notion that Ctr proteins transport Cu^+ (76). Other fungi also possess cupric reductases and two functionally

redundant plasma membrane Ctr proteins, with *Cryptococcus neoformans* expressing Ctr1 and Ctr4 and *Schizosaccharomyces pombe* harboring Ctr4 and Ctr5 (18, 175). However, rather uniquely, *S. pombe* Ctr4 and Ctr5 form obligatory heteromultimers that constitute a functional transporter (175). Loss of either Ctr4 or Ctr5 renders the other subunit unable to drive high-affinity Cu^+ uptake, likely owing to an inability to be properly trafficked to the plasma membrane. In contrast, *S. cerevisiae* and *C. neoformans* require that both plasma membrane transporters be absent for the cells to manifest a copper-deficiency growth phenotype due to loss of copper-dependent enzyme activity (45, 93).

In addition to the Ctr1/3 Cu^+ transporters on the plasma membrane, *S. cerevisiae* Ctr2 and *S. pombe* Ctr6 mediate the transport of intracellular copper from the vacuolar lumen to the cytoplasm (21, 136). In keeping with a putative Cu^+ substrate, Ctr2 requires the vacuolar-localized Fre6 reductase for the mobilization of copper stores to the cytosol, as a *fre6* mutant phenocopies a *ctr2* mutant (137). Further supporting Cu^+ as substrate, a truncated Ctr2 variant also containing a W7R mutation mislocalizes to the plasma membrane and imports copper in a manner that is dependent on the cell surface Fre1 reductase (137). It is interesting that while expression of the homologous gene *CTR6* in *S. pombe* is elevated in response to copper deficiency, *S. cerevisiae CTR2* expression is induced in response to iron deficiency, perhaps reflecting its requirement largely under conditions where extracellular copper pools are not available to Ctr1/Ctr3 (21, 137).

A more recently characterized *S. pombe* copper transporter, Mfc1, is a member of the major facilitator superfamily (MFS) of transporters and possesses 12 helical transmembrane domains connected by short hydrophilic loops (13). An exception is loop 6, which is greatly enlarged and functions to separate the protein into two connected halves. This allows for interdomain movement between the amino and carboxyl halves required for substrate transport (172). Of note, amino acid sequence analysis of Mfc1 shows four pairs of methionine residues that could function to coordinate copper during transport. Interestingly, the *MFC1* gene is highly expressed during meiosis, and Mfc1 localizes to forespore membranes that accrue the products of meiosis. Presumably this is to ensure that the developing spores have an adequate supply of copper, since the copper-dependent amine oxidase Cao1, which localizes primarily in the forespores, shows a dramatic decrease in activity in cells lacking *MFC1* (128). Normal meiotic progression is also delayed in cells lacking *MFC1* when copper is limited (13). Further study will be required to determine the nature of copper transported by Mfc1, whether it depends on a cupric reductase, and whether other fungal and mammalian species contain a homologous copper transporter.

INTRACELLULAR COPPER BUFFERING

Once Cu^+ enters the cytoplasm, cells are immediately faced with the challenge of buffering free Cu^+ to preclude the inappropriate metalation of proteins with solvent-exposed copper-ligands and its participation in uncontrolled Fenton reactions. Moreover, the metal ion must be directed to its appropriate intracellular destinations with some specificity. In fact studies suggest that, in general, cells contain virtually no free copper, with the majority existing in a buffered pool (132). One component of this copper buffering pool is a family of small, cysteine-rich, metal-binding proteins known as metallothioneins (MTs) (101, 168).

The *CUP1* gene from *S. cerevisiae* encodes, arguably, the best-characterized fungal MT and was initially identified by investigation into naturally occurring yeast strains resistant to high levels of copper (24). Copper-sensitive cells were not able to grow on plates containing 0.3 mM $CuSO_4$, whereas isogenic copper-resistant cells were able to grow on plates containing 1.75 mM $CuSO_4$. Subsequent stepwise selection yielded strains capable of growth in up to 12 mM $CuSO_4$, and molecular analysis of these strains revealed that they had undergone tandem amplification of the *CUP1* gene (53).

The Cup1 protein is capable of binding up to eight Cu^+ ions, with each trigonally coordinated to å sulfur atom of cysteine residues at a distance of 2.23 Å, as determined by X-ray absorption analysis (66). Although largely unstructured in the apo form, the Cu^+ - and Ag^+ -bound three-dimensional solution structures of Cup1 have been determined by ^{1}H two-dimensional NMR spectroscopy (23, 126). Although lacking any α-helix or β-sheet motifs, the peptide backbone folds around a single metal cluster in two large parallel loops. The Cu^+ -bound structure of another MT, from the yeast *Neurospora crassa*, has also been investigated by ^{1}H-NMR spectroscopy (29). The polypeptide backbone structure is markedly different from that of *S. cerevisiae* MT, presumably because spacing of the Cu^+ - coordinating cysteine residues is different. This, and the lack of structure in the absence of Cu^+, suggests that the overall tertiary structures of MTs are almost completely determined by metal coordination.

Aside from directly sequestering and preventing copper toxicity in *S. cerevisiae*, overexpression of *CUP1* can partially rescue growth phenotypes of yeast lacking copper-zinc superoxide dismutase (SOD1) (149). These and other experiments suggest that MTs can play a role in cellular defense against oxidative stress, as well as copper detoxification (47, 74, 151). A second MT-like protein encoded by *S. cerevisiae*, Crs5 (33), is less effective than Cup1 in copper buffering but detoxifies excess copper (86). Other fungi such as *Candida glabrata* and *C. neoformans* also encode MTs that provide powerful cytosolic Cu^+ -buffering capabilities (44, 113).

Sod1 is highly abundant in the cytosol of *S. cerevisiae* and in addition to its role in protection against oxidative stress due to disproportionation of oxygen radicals, it is also a copper-buffering agent (35). This function appears to be independent superoxide scavenging activity, since *SOD1* is able to protect cells from copper toxicity in anaerobic environments, where there is little superoxide. Also, the *PMR1*, *BSD*, and *ATX1* genes that suppress oxygen toxicity in cells lacking *SOD1* fail to suppress copper sensitivity in the same cells.

A powerful source of intracellular copper buffering is also provided by the tripeptide glutathione (GSH), synthesized from cysteine, glutamic acid, and glycine in a two-step mechanism involving the enzymes glutamate cysteine ligase and glutathione synthetase (54, 55, 107). While serving as a general cellular antioxidant, GSH also binds metals including Cu^+ through thiol ligands (32). Cellular fractionation of both *N. crassa* and *S. cerevisiae* demonstrates that a measurable portion of copper is GSH bound (67, 103). In vitro experiments suggest that GSH-Cu chelate complexes can donate bound Cu^+ to known Cu^+ - binding proteins such as apo-hemocyanin, MT, and the copper chaperone Atx1 (25, 51, 115), suggesting a potential copper-dissemination role. Free amino acids such as methionine,

cysteine, and histidine have also been suggested to participate in intracellular copper buffering (123, 173).

COPPER TRANSPORT TO THE SECRETORY COMPARTMENT

Beyond intracellular copper buffering, it is critical to distribute copper to physiologically relevant proteins and compartments to drive copper-dependent biochemical reactions that take place inside fungal cells or in their local environment. Several fungal enzymes that traverse the secretory pathway—such as the multi-copper ferroxidase Fet3, critical for iron acquisition; laccase, required for melanin biogenesis; and secreted lytic polysaccharide monooxygenases (LPMOs) that degrade extracellular complex carbohydrates—require copper for proper folding, secretion, and activity (7, 85, 158). Fet3 activity, which requires Cu^+, is a useful measure of the ability of copper to enter the secretory pathway, as *S. cerevisiae* lacking metalated Fet3 fails to grow under low-iron conditions. Interestingly, *S. cerevisiae* cells lacking the Tfp1 subunit of the vacuolar H^+-ATPase and defective in post-Golgi vesicle acidification, as well as cells lacking the post-Golgi localized CLC chloride (Cl^-) channel Gef1, are unable to grow on low-iron medium owing to inactive Fet3, but growth is rescued by exogenous copper (64). These observations prompted a model in which proper acidification of post-Golgi compartments is critical for Cu^+ loading onto Fet3. Since acidification leads to an increase in membrane potential, Gef1 may be necessary to counterbalance this with an influx of Cl^-. It has also been suggested that Cl^- acts as an allosteric effector required for proper copper metalation of Fet3 (41).

An exciting new discovery relevant to the copper metalation of Fet3 suggests that compartmentalization of K^+ in the *trans*–Golgi network (TGN), by the K^+/H^+ exchanger Kha1, plays a critical role in this process (169). *S. cerevisiae* cells lacking *KHA1* display a growth defect in nonfermentable carbon sources that can be rescued by either iron or copper supplementation and show a decrease in iron accumulation, indicative of defective Fet3. Although Kha1 is a proposed K^+/H^+ exchanger, no difference in cytosolic or TGN pH was observed between cells possessing Kha1 and those lacking Kha1. These results, along with in vitro experiments showing that K^+ promotes binding of copper to apo-Fet3 but not to the E487A or N49A mutants predicted to be deficient in K^+ binding, support the notion that the requirement of Kha1 for proper Fet3 activity is due to a requirement for K^+. Consistent with these results and the requirement for Kha1 for copper-dependent Fe acquisition, *KHA1* mRNA levels are increased by the iron-responsive transcription factors Aft1/2.

While Tfp1, Gef1, and Kha1 are required for Cu^+ loading onto Fet3, they do not directly transport Cu^+ into the secretory lumen. To accomplish this in a controlled manner, integral-membrane Cu^+-transporting ATPases, such as Ccc2 in *S. cerevisiae*, deliver Cu^+ into the secretory compartment, where it is loaded onto luminal copper-requiring proteins (58). Deletion of the *CCC2* gene results in a severe decrease in the activity of Fet3 and a concomitant iron deficiency (174). Ccc2 is, in general, conserved from bacteria to humans, and loss-of-function mutations in the corresponding human genes cause Menkes, or Wilson, disease (42).

Ccc2 operates in Cu^+ delivery to the secretory compartment using two cytosolic metal-binding domains (MBDs) that adopt a ferredoxin-like $\beta\alpha\beta\beta\alpha\beta$ fold capable of binding Cu^+ via a conserved Cys-X_2-Cys motif in which the two cysteine residues bind Cu^+ in a solvent-exposed, bidentate fashion (9, 10). These domains are necessary for in vivo activity, in that a yeast strain expressing mutant Ccc2 lacking both MBDs fails to grow on low-iron media, presumably owing to an inability to metalate Fet3 and support iron import (116). The MBDs are thought to function by receiving Cu^+ from an intracellular chaperone (see below) and passing Cu^+ to the transmembrane exit tunnel. A homolog of Ccc2 (LpCopA) from *Legionella pneumophila* has been crystalized and provides a framework for modeling the mechanism by which this family of copper-ATPases function (72). Cu^+ bound to the MBDs is first transferred to a Cu^+-binding site located on the cytoplasm-facing side of the transmembrane domains comprising glutamic acid, aspartic acid, and methionine residues. Notably, this pore entrance forms an α-helical platform that may serve as a docking station for the Cu^+-loaded MBD or a cytosolic Cu^+ chaperone. Owing to the relative low affinity of Cu^+ binding to carboxylate moieties of glutamic acid and aspartic acid residues, this binding event would be transient and result in Cu^+ transfer to the high-affinity intramembrane Cu^+-binding site made up of sulfur ligands contributed by two cysteines and a methionine residue, where the Cu^+ is bound in a stable trigonal planar coordination mediated by the three sulfur atoms at a distance of ~2.25Å, as determined by EXAFS analysis (111). Binding of Cu^+ to this high-affinity site triggers ATP hydrolysis and subsequent Cu^+ occlusion and transfer to a transient binding site composed of two methionines and a glutamic acid residue before dissociation and release into the secretory lumen (4). Catalytic activity requires the formation of an ATP-dependent acyl-phosphate intermediate at the essential aspartic acid residue in a conserved DKTGT motif to generate the force necessary to pump Cu^+ against a gradient (106).

COPPER CHAPERONES

Since the cellular copper buffering pool sequesters free copper from the cell, copper needs to be directed to the desired compartments. For this purpose, fungi have evolved highly specific and dedicated chaperones to direct copper trafficking. Efficient Cu^+ transfer to the secretory compartment, particularly under conditions of Cu^+ limitation, requires a metallochaperone for Cu^+ delivery to Ccc2. The metallochaperone for Ccc2 is Atx1, an ~8-kDa protein originally identified in a genetic screen as a multicopy suppressor of oxygen toxicity in cells lacking *SOD1* (104). Cells lacking *ATX1* show reduced Ccc2-mediated activation of Fet3, and a corresponding iron deficiency, a phenotype that can be rescued by the addition of exogenous copper (105). In keeping with the critical role for Cu^+ loading onto Fet3 in the secretory compartment, expression of the *ATX1* gene is induced under conditions of low iron, via the Aft1/2 iron-sensing transcription factors, rather than in response to low copper levels (105).

In keeping with the reactivity of copper ions when uncontrolled, Atx1 delivers Cu^+ to Ccc2 by directly interacting with the cytosolic MBDs in a fashion that allows facile, yet controlled, copper transfer (Figure 3a) (130). Nature has cleverly reutilized the ferredoxin-like $\beta\alpha\beta\beta\alpha\beta$ fold found in Ccc2, which is also adopted by Atx1, to drive protein-protein interactions along complementary surfaces (6, 140). As in the Ccc2 MBDs, Cu^+ binds to

Atx1 at an invariant Cys-X_2-Cys motif, essential for this transfer, in which the two cysteine residues bind copper in a solvent-exposed, bidentate fashion. Additionally, Atx1 possesses a highly charged lysine-rich surface required for the Atx1 Cu^+ transfer to Ccc2 (129). The Ccc2 MBDs possess a patch of negatively charged residues that form salt bridges with Atx1, orienting the proteins for the formation of metal-bridged coordination intermediates, which would both facilitate Cu^+ transfer and preclude nonspecific interactions with Cu^+ (5, 8, 22). The thermodynamic gradient for metal transfer is shallow ($K_{exchange}$ ~1.4), allowing for bidirectional transfer between Atx1 and Ccc2, which could function in the regulation of copper loading into the secretory compartment (11, 83, 170). Outside of interactions with Ccc2, the Atx1 homolog in *S. pombe* has been shown to mediate Cu^+ transfer to the amine oxidase Cao1, demonstrating that a single chaperone may metalate multiple proteins (125).

While Sod1 serves as a copper buffer to protect cells under elevated copper conditions, super-oxide disproportionation activity requires a dedicated chaperone to deliver the essential copper cofactor (36, 61, 80). The copper chaperone for Sod1 (Ccs) comprises three distinct domains (Figure 3b). Ccs domain 1 displays striking similarity to the Atx1 and Ccc2 MBD ferredoxin-like $\beta\alpha\beta\beta\alpha\beta$ fold (100). The conserved Cys-X_2-Cys motif within this domain binds Cu^+ but is largely required for Cu^+ loading of Sod1 under copper-limiting conditions. Domain 2 forms a Greek key β-barrel fold, similar to Sod1, and this domain is responsible for direct interactions with Sod1, forming a pseudodimer, and mutations abrogating this interaction prevent copper metalation and activation of the homodimeric Sod1 enzyme (98, 99). Domain 3, bearing a critical Cys-X-Cys motif, is responsible for Cu^+ binding and essential for copper transfer to Sod1.

Ccs and Sod1 were initially thought to reside in the cytosol, but it is now recognized that a significant fraction of Ccs and Sod1 exists in the mitochondrial intermembrane space (IMS) (Figure 4) (146). In contrast to its noncritical role in loading of Sod1, Ccs domain 1 plays an indispensable role in targeting the protein to mitochondria. After import into the IMS through the TOM (translocase of the outer membrane) complex, Ccs domain 1 interacts with the mitochondrial disulfide relay system protein Mia40, which is anchored to the mitochondrial inner membrane (135). Mia40 contains six conserved cysteine residues that are maintained in an oxidized state by the flavin-linked sulfhydryl oxidase Erv1 (114). Upon interaction with oxidized Mia40, a disulfide bond is transferred to the two cysteine residues of the Cys-X_2-Cys motif from Ccs domain 1 (92). This disulfide-containing form of Ccs is retained within the IMS, presumably owing to a stable folded conformation, as the TOM complex is only capable of transporting unfolded proteins. In the absence of Ccs, Sod1 fails to accumulate in the mitochondria, implicating the importance of Ccs in this compartment (146). The presence of Ccs in both the cytoplasm and the IMS suggests that it could serve as a mitochondrial metallochaperone providing copper for multiple mitochondrial functions. However, yeast lacking *CCS* retain wild-type mitochondrial copper levels and cytochrome *c* oxidase (COX) activity (30).

COPPER TRAFFICKING TO MITOCHONDRIA

COX is the terminal enzyme of the electron transport chain in mitochondria, playing a critical role in energy generation via oxidative phosphorylation. COX is composed of 12

subunits with 2, Cox1 and Cox2, requiring copper (Figure 4). The copper sites are buried within the structure, therefore requiring coordination between copper availability and the assembly of the complex. COX biogenesis requires >25 accessory proteins known as COX assembly factors. At least 9 of these factors (Cox11, Sco1, Sco2, Cox17, Coa6, Cmc1, Cmc2, Cox19, Cox23) facilitate insertion of the copper cofactors and trafficking of IMS copper.

Cox1 binds a single copper atom. The metal center is designated the CuB site and is located ~13 Å below the membrane surface (153). Experiments performed in *Rhodobacter sphaeroides* demonstrated that proper metalation of the CuB site requires the accessory factor Cox11 (78). The amino-terminal domain of Cox11 anchors the protein to the inner membrane, with the soluble carboxyl-terminal domain directly binding copper via three cysteine residues (27). *S. cerevisiae* cells lacking *COX11* show reduced COX activity and have lower levels of Cox1 polypeptide, demonstrating the importance of copper for Cox1 stability within the fully assembled holoenzyme (155).

Cox2 harbors a binuclear copper site (CuA) and is buried in the enzyme complex ~8 Å above the membrane surface into the IMS. Proper metalation of this site requires a dedicated accessory factor, Sco1, as yeast *sco1* mutants are devoid of COX activity and display dramatically decreased levels of Cox2 (71, 144). Similar to Cox11, the amino-terminal domain of Sco1 protrudes into the inner membrane, with the soluble carboxyl-terminal domain containing conserved cysteine residues that directly bind Cu^+ in a solvent-exposed manner (120). Mutation of these cysteine residues results in nonfunctional COX in *S. cerevisiae* (138). A Sco1 paralog, Sco2, is also involved in copper loading of COX, although its function in fungi is at least partially redundant with that of Sco1.

Although Cox11 and Sco1 directly metalate COX subunits, they both require the presence of another accessory protein, Cox17, to obtain Cu^+, and *S. cerevisiae cox17* mutants lack detectable COX activity (70, 81). In contrast to Cox11 and Sco1, Cox17 is a soluble protein that exists in both the cytosol and the mitochondrial IMS (20). This observation was initially understood to support the hypothesis that Cox17 may be responsible for ferrying Cu^+ into mitochondria; however, tethering of Cox17 to the inner membrane maintained normal COX activity and normal mitochondrial Cu^+ levels (112), indicating that Cox17 critically functions in this regard in the IMS.

Another recently described factor involved in copper loading of COX is Coa6 (68). *S. cerevisiae* lacking *COA6* displays decreased respiration and COX assembly. Addition of exogenous copper rescued these defects, suggesting a role for Coa6 in the Cu^+-delivery pathway. The presence of conserved $Cys-X_9-Cys-X_n-Cys-X_{10}-Cys$ motif in Coa6 is reminiscent of previously described Cu^+-binding motifs, and mutation of any of the four cysteine residues to alanine results in a protein incapable of restoring COX activity to wild-type levels in *S. cerevisiae* lacking endogenous *COA6*. The $Cys-X_9-Cys-X_n-Cys-X_{10}-Cys$ motif is also found in Cmc1, Cmc2, Cox19, and Cox23, and these proteins have been implicated in copper delivery in the IMS. Further studies will be required to uncover the molecular mechanisms by which these proteins are involved in COX metalation, both in yeast and in mammals.

A fascinating observation was that only a fraction of total mitochondrial copper was associated with Sod1 and COX (30, 62), with the majority found in a soluble, anionic copper ligand complex (CuL). The exact nature of the CuL complex awaits further investigation, but it is resistant to protease digestion, displays anionic characteristics, and has a fluorescence maximum at 360 nm, suggesting the ligand might be a metabolite or nucleotide. While CuL is found in the cytosol, the majority of Cu^+-bound CuL is in the mitochondrial matrix. Cleverly expressing *SOD1* and *CRS5* as Cu^+-chelating proteins that depleted the bioavailable matrix copper pool, Cobine et al. (31) suggested that matrix Cu^+ pools, rather than those originating from the cytosol to IMS, are destined for loading onto COX and mitochondrial Sod1 in the IMS. As the inner membrane is impermeable to most ions, systems to import copper from the IMS to the matrix, as well as to mobilize matrix copper pools back out into the IMS, must be operational.

Pic2 is a mitochondrial carrier family (MCF) protein, previously implicated in phosphate transport, that was recently demonstrated to transport copper into the mitochondrial matrix (159). *S. cerevisiae* cells lacking *PIC2* display growth inhibition and lower levels of Cox2 when Ag^+ is present in the growth media, as well as decreased COX activity when grown on a nonfermentable carbon source. Also, deletion of *PIC2* results in a decrease in total mitochondrial copper, and isolated mitochondria from *pic2* cells show decreased rates of copper uptake. *Lactococcus lactis* bacterial cells expressing the *S. cerevisiae* Pic2 protein display enhanced copper uptake when the transporter is presented with either free copper or the CuL complex isolated from *S. cerevisiae* mitochondria. Further investigation will be required to determine the nature by which Pic2 trans-ports copper, or CuL and the identity of the transporter that mobilizes copper out of the matrix to the IMS.

In response to copper starvation, *C. albicans* induces a switch from Cu,Zn *SOD1* to Mn *SOD3* while maintaining COX activity (26). This switch in Sod enzymes eliminates Cu,Zn Sod1 from the IMS, potentially rendering mitochondria susceptible to superoxide. However, superoxide is maintained at low levels by inducing expression of an alternative iron-dependent oxidase, Aox2, which is able to accept electrons directly from coenzyme Q and bypass a major source of IMS superoxide, complex III of the electron transport chain. Thus, when copper is scarce, *C. albicans* is capable of prioritizing available copper for COX at the expense of Cu,Zn Sod1.

COPPER-RESPONSIVE TRANSCRIPTION FACTORS

Reciprocal and Separable Responses to Copper Toxicity and Copper Starvation

The regulation of copper homeostasis in fungi is primarily controlled at the level of transcription by copper-responsive transcription factors. These fungus-specific transcription factors sense changes in copper status and appropriately activate the copper acquisition or detoxification genes under conditions of copper limitation or excess, respectively. Characteristic of these transcription factors are cysteine-rich regions, generally arranged as Cys-X-Cys and/or Cys-X_2-Cys repeats, which in some cases have been shown to bind Cu^+ as a direct mechanism to survey and respond to copper status (37, 88). Many fungi contain two copper-responsive transcription factors that reciprocally activate genes in response to copper starvation or toxicity.

Initially discovered in *S. cerevisiae*, Ace1 activates expression of the MT genes *CUP1* and *CRS5*, as well as *SOD1*, in response to excess copper (>1 μM) (Figure 5a) (33, 73, 150, 165). Ace1 contains a single cysteine-rich region directly downstream of its conserved minor groove DNA-binding domain (DBD) (60). This cysteine-rich region binds four Cu^+ atoms to form a tetra-copper-thiolate cluster (37, 148). Cluster formation triggers intramolecular conformational changes within the amino-terminal DBD that activates monomeric Ace1 DNA binding (60). Metal ion–ligand activation is highly dependent upon and specific for Cu^+ over other monovalent and divalent metal ions, with the exception of isoelectric Ag^+ (60). Within the Ace1 DBD lies a Cys_3-His zinc finger and basic KGRP amino acid motif required for establishing DNA minor groove contacts (46, 50, 154). Activated Ace1 recognizes specific metal response elements (MREs) in target gene promoters with the consensus sequence 5'-HTHNNGCTGD-3'(D = A, G, or T; H = A, C, or T), and MRE mutations result in reduced Ace1 occupancy and loss of copper-responsive gene expression (49, 60, 84). The importance of Ace1-mediated activation of copper detoxification genes is exemplified by the copper hypersensitivity phenotype of *ace1* cells, which fail to induce MT gene expression. Studies using an amino-terminus-truncated variant of Ace1 (residues 1–122), which preserves the cysteine-rich and KGRP DBD, demonstrated this domain is competent for Cu^+ cluster formation and MRE recognition both in vitro and in vivo (34, 59). However, target gene activation was abolished, indicating the amino-terminal domain confers DNA binding and specificity whereas the carboxyl-terminal domain is required for transcriptional activation (34).

Additional Ace1 homologs have been discovered in several other fungal species, including *AMT1* from the opportunistic pathogen *C. glabrata*, that function similarly to *S. cerevisiae* Ace1 (177). Expression of *AMT1* in *S. cerevisiae ace1* cells induces *S. cerevisiae CUP1* expression in a copper-dependent manner, ameliorating the copper sensitivity phenotype (152). Similarly, *S. cerevisiae* Ace1 activates *C. glabrata MTI* in a copper-dependent manner, demonstrating these proteins are not just structural but also functional homologs that recognize heterologous MREs (152). In contrast to Ace1, which is basally expressed irrespective of copper status, Amt1 is under positive autoregulatory feedback due to the presence of an MRE within its own promoter and is critical for eliciting an adequate copper detoxification response whereby Amt1 levels are amplified to activate expression of MT genes in this organism (176).

S. cerevisiae has a second copper-regulated transcriptional activator that responds to copper limitation: Mac1 activation induces expression of the high-affinity Cu^+ transporters encoded by *CTR1* and *CTR3* and the plasma membrane reductase encoded by *FRE1* during copper limitation (Figure 5a) (65, 90, 96). Mac1 contains two functionally distinct cysteine-rich repeats located within the carboxyl-terminal region that collectively have the potential to coordinate up to eight Cu^+ ions (87, 88). In contrast to Ace1, excess copper (>10 nM) results in Mac1 inactivation and potential degradation at even higher copper concentrations (10 μM) in vivo (178). This rapidly suppresses *CTR1/3* transcription, effectively providing a secondary countermeasure to prevent copper toxicity. Under copper limitation, activated Mac1 binds to copper-responsive elements (CuREs) in the promoters of target genes with a defined consensus sequence 5 -TTTGCTC-3, strikingly similar to Ace1 MREs (96, 171). Consistent with the role of Mac1 in copper acquisition, *mac1* cells display impaired iron

acquisition, respiratory defects, and sensitivity to ROS due to reduced copper accumulation and defective metalation of Fet3, COX, and Sod1, respectively (90). Interestingly, a single mutation of a terminal histidine to glutamine in one of the cysteine-rich motifs results in constitutive Mac1 activation and induction of *CTR1/3* and *FRE1*, regardless of copper status (90, 96). These cells display a copper hypersensitivity phenotype similar to that of *ace1* cells owing to the chronic expression of the copper-acquisition machinery (178). Although copper-binding mutants have been useful tools to study Mac1, we currently have a very limited understanding of exactly how Mac1 senses copper limitation.

Single-Component Copper-Responsive Factors

Utilization of two distinct copper-sensing transcription factors such as Ace1 and Mac1 is not conserved throughout the fungal kingdom. Evolutionarily distant fungi have evolved to use a single copper-responsive transcription factor exemplified by Cuf1. Cuf1 has been thoroughly investigated in *S. pombe*, and while its overall sequence homology to Ace1 or Mac1 is low, hallmarks from both proteins are conserved (95). The amino-terminal DBD shares a high degree of identity with Ace1 (51%), and the carboxyl-terminal domain contains a cysteine-rich region with identity to Mac1 (48%), therefore appearing chimeric in nature (95). Cuf1 function is analogous to that of Mac1: It is activated during copper limitation and induces expression of the plasma membrane copper transporters *CTR4* and *CTR5* and the vacuolar copper transporter *CTR6* by binding to *cis*-acting regulatory elements in their promoters (17, 21, 95, 141). Excess copper is sensed through the cysteine-rich region and results in Cuf1 inactivation (Figure 5b). Similar to *S. cerevisiae mac1* cells, *cuf1* cells display characteristic copper starvation phenotypes, which are reversed by cop-per supplementation. Interestingly, the *S. pombe* genome does not encode any known copper-detoxification MTs; therefore, there is no known role for Cuf1 in the copper detoxification response aside from transcriptional silencing of copper-uptake genes at high copper concentrations.

While Cuf1 is functionally analogous to Mac1, the two have key differences. Cuf1 binds DNA sequences with strong similarities to the Ace1/Amt1 MRE, termed copper-signaling elements (CuSEs), with the consensus sequence 5′-D(T/A)DDHGCTGD-3′ (D = A, G, or T; H = A, C, or T) (14). Studies of *S. cerevisiae ACE1* transcomplementation into *S. pombe cuf1* cells resulted in sustained activation of *CTR* transporter expression even at high copper concentrations (>100 μM) and a copper hypersensitivity phenotype. Additionally, a chimeric Cuf1 protein containing the amino-terminal 1–63 residues of *S. cerevisiae* Ace1 could rescue the copper starvation phenotype of *cuf1* cells, suggesting that although Cuf1 is functionally homologous to Mac1, its mode of DNA binding and recognition is analogous to that of Ace1 (14). Therefore, Cuf1 represents a class of transcription factors composed of two separate functional domains from Ace1/Mac1 of *S. cerevisiae*.

Further structure-function analysis of Cuf1 revealed additional regulatory mechanisms controlling Cuf1 function. The amino-terminal 11–53 residues of Cuf1 contain a noncanonical nuclear localization sequence (NLS) rich in positively charged amino acids required for nuclear import during copper limitation (15). Cuf1 also contains a leucine-rich nuclear export signal (NES) down-stream of the cysteine-rich region and is exported from the nucleus during copper excess via the nuclear exportin Crm1. A functional NES and

cysteine-rich domain are required for nuclear export, as mutations of either resulted in nuclear accumulation and unregulated expression of *CTR4/5/6* (16). However, nuclear accumulation is not sufficient for unregulated gene expression, as a *crm1+* mutant, which accumulates nuclear Cuf1, could still be inactivated by copper. From this data, a model has been proposed for Cuf1 regulation (Figure 5b).

The basidiomycete *C. neoformans* also encodes a protein with similarity to *S. pombe* Cuf1 (CnCuf1). CnCuf1 is unique in that it serves the dual regulatory functions of Mac1 and Ace1 for regulating both copper acquisition and copper detoxification (45). During copper limitation, CnCuf1 activates expression of the *CTR1* and *CTR4* copper transporters. As copper accumulates toward toxic levels, CnCuf1 switches its induction profile to express the copper-detoxifying MTs, *MT1* and *MT2*. Surprisingly, one study reported cytoplasmic retention of CnCuf1 at the cell periphery under high-copper conditions, despite *MT1/2* induction in a copper- and CnCuf1-dependent manner (89). Elucidation of how CnCuf1 senses and responds to both extremes in the copper spectrum requires further examination. Possibly, the presence of four cysteine-rich regions within the amino-terminal half of CnCuf1 increases binding capacity for copper compared to Ace1 or Mac1. Could each cysteine-rich region have a copper-binding affinity gradient per-mitting a fine-tuned response of CnCuf1 toward activating copper-acquisition or -detoxification genes? Additionally, a unique feature of CnCuf1 is a large (~500 amino acids) carboxyl-terminal domain with no known homology to other proteins. This large domain contains no cysteines; how-ever, it contains multiple methionine residues that could still participate in copper coordination. Perhaps the CnCuf1 amino-terminal and carboxyl-terminal domains separately regulate copper acquisition and detoxification, either by recognition of different *cis*-acting regulatory elements or by recruitment of different transcriptional accessory proteins required for target gene induction.

COPPER AND FUNGAL PATHOGENESIS

Host Copper Mobilization and the Initial Copper Burst

As frontline defenders in immunity, macrophages and neutrophils survey their environment for invading pathogens and activate to signal and recruit other immune cells in response to infection. Activated macrophages engulf foreign microbes, compartmentalizing them within the phagosome, which ultimately matures into a phagolysosome (160). The phagolysosome is a highly inhospitable and toxic environment, with low pH, high concentrations of ROS and reactive nitrogen species (RNS), and several proteases present (19, 143, 160). Whereas iron and zinc, two well-recognized nutritional factors required for virulence, are actively exported from the phagolysosome, copper is specifically delivered to this compartment to induce a state of copper toxicity (117). Accordingly, bacterial pathogens with mutations in copper detoxification P-type ATPase efflux pumps are more susceptible to macrophage killing within this compartment (1, 145, 166).

Macrophage activation by host-derived cytokines such as IFN-γ or bacteria-derived lipopolysaccharide induces expression of mammalian Ctr1 and stimulates copper uptake (166). This response is accompanied by increased expression and relocalization of the mammalian Cu^+-transporting P-type ATPase ATP7A (yeast Ccc2 homolog) from the Golgi

network to the phagolysosome membrane, effectively rerouting copper to pathogen-containing phagolysosomes (166). X-ray microprobe analyses have confirmed a significant increase in copper content in phagolysosomes of mycobacteria-infected macrophages (161). While evidence supports copper accumulation within the phagolysosome, the source of host-acquired copper is unknown. An immediate acute phase response to infection is increased serum circulation of the multi-copper oxidase ceruloplasmin (Cp), which is capable of binding six atoms of copper per Cp polypeptide and accounts for approximately 95% of total serum copper and thus could serve as a peripheral reservoir to supply copper to immune cells (28, 77). Alternatively, recruited immune cells may contain sufficient intracellular copper stores in endosomes or mitochondria, which could be mobilized to the phagolysosome during infection. While no direct link has been made for Cp supplying copper to immune cells, Cp has been shown to deliver copper to cells in both a Ctr1-dependent manner and a Ctr1-independent manner (91, 134). It is highly unlikely that free copper is a physiological source, as the toxic properties of unbound copper are just as detrimental to host cells.

Pathogen Response to Copper Bombardment

Successful pathogens have evolved effective mechanisms to circumvent host-imposed copper toxicity (Figure 6). Some fungal pathogens, such as *C. neoformans*, utilize copper-chelating MTs as a primary copper-resistance mechanism (44, 147). While mammalian and other metazoan MTs typically bind fewer than 10 Cu^+ ions per polypeptide, the *C. neoformans* MT1 and MT2 proteins coordinate 16 and 24 Cu^+ ions, respectively, providing an unprecedented efficient copper-scavenging mechanism (44). In vivo, live imaging of *C. neoformans*–infected mice has revealed an environment high in copper at the initial site of infection in the lungs, presumably within the phagosomes of resident alveolar macrophages (44). Copper-specific induction of MT1 and MT2 in the lungs is critical for *C. neoformans* survival in a pulmonary infection model, and data suggest these MTs serve redundant functions, as single deletions had no effect on virulence in a mouse model of infection. Virulence attenuation was dependent on deletion of both MTs, effectively removing a critical component of the cellular copper-buffering capacity and creating conditions permissive for copper toxicity (44). In addition, the virulence could not be returned if a MT in which all Cu^+ -coordinating cysteine residues were mutated to alanine were expressed, confirming the role of copper chelation.

The fungal pathogen *C. albicans* is an oral-genito-urinary pathogen that commonly colonizes the kidney, and mass spectrometry imaging studies demonstrated increased copper accumulation in infected kidney tissue at the onset of infection (108). *C. albicans* also expresses two MTs, Cup1 and Crd2, as intracellular copper buffers (121, 139). However, these MTs are much smaller, with a lower copper-buffering capacity, and appear to be a secondary measure of preventing copper toxicity (139, 164). Instead, unique to *Candida* species is the Ace1- and copper-dependent expression of *CRP1*, which encodes a plasma membrane P-type ATPase (164). Crp1 is distinct from the P-type ATPase Ccc2, as it instead actively transports excess copper from the cytoplasm to the extracellular environment, whereas Ccc2 is not found localized at the plasma membrane under any conditions tested (174). While P-type ATPase pumps are a common mechanism for copper resistance in

prokaryotes, this is the only known instance of a fungal pathogen with a dedicated copper efflux system. Accordingly, the MTs are dispensable when a functional Crp1 transporter is present, although *C. albicans* is extremely sensitive to low levels of copper in the absence of both detoxification mechanisms (139, 164). Recent reports have demonstrated Crp1 expression is required for full virulence and is highly induced during the initial stages of infection in the kidneys of mice (108).

Host Copper Sequestration During Late Stages of Infection

Nutritional immunity, the deliberate withholding of trace micronutrients by the host to the disad-vantage of invading pathogens, has been well described for iron, manganese, zinc, and calcium (38, 79, 118). Specific niches within the host are particularly limited in copper levels that are available to invading fungal pathogens, such as the brain and kidney for *C. neoformans* and *C. albicans*, respectively (102, 147). A copper-specific nutritional immunity response is evidenced during *C. albicans* infection of the kidney, where an initial rise in tissue copper concentrations subsequently diminishes as infection progresses (108). However, no bona fide host nutritional immunity mechanisms have yet been described for copper in host tissues.

Pathogen Response to Copper Deprivation

Given the requirement for several copper-dependent enzymes in fungal virulence, the ability to scavenge trace levels of copper from the host is paramount for establishing and maintaining an infection (52). In response to copper limitation in the host, *C. albicans* induces the expression of the *CTR1/3* transporters in a manner that correlates with decreased host tissue copper concentrations as infection progresses in the kidney, and deletion of the *CTR1* transporter results in a reduced capacity of *C. albicans* to colonize the kidneys in a mouse infection model (108). Interestingly, copper deprivation also impaired the ability of *C. albicans* to assimilate and utilize several carbon sources as well as altering core metabolic pathways (108). The ability to utilize alternative carbon sources during infection is a well-known virulence determinant, and Ctr1-mediated copper acquisition may play a role in promoting efficient carbon assimilation (12, 133).

In immune-compromised individuals, *C. neoformans*, which resists copper toxicity in alveolar macrophages, can disseminate from the lungs and colonize the brain, resulting in lethal cryptococcal meningoencephalitis (94). *CTR1/4* transporters are highly induced in the brains of infected mice and humans, in contrast to their lungs, supporting the notion that *C. neoformans* encounters copper limitation within this niche (147, 162). Accordingly, inactivation of the *CTR1/4* transporters resulted in reduced fungal burden and attenuation of virulence in mouse models of infection (147).

Copper-Dependent Enzymes Important for Pathogenesis

The pigment melanin, deposited on the fungal cell wall, is a phenolic polymer that has antioxidant and stress-protectant properties that may play a role in absorbing host-derived ROS/RNS (Figure 6). Melanin synthesis requires the copper-dependent enzyme laccase, and its expression in *C. neoformans* is modestly induced in response to elevated copper in vitro and in the cerebral spinal fluid of *C. neoformans*–infected rabbits (142, 167). Interestingly,

L-DOPA, an abundant molecule in the human brain used for epinephrine synthesis, is an extracellular melanin precursor utilized by *C. neoformans* laccase (48). In contrast, *Aspergillus fumigatus* can synthesize melanin de novo via the polyketide pathway that involves noncanonical secretion of melanin precursor biosynthesis enzymes, which are targeted to endosomes through posttranslational palmitoylation (156, 157). Melanin precursors are then converted to melanin extracellularly by classically secreted laccases for final deposition onto the cell surface (157). Recently, an additional role for melanin was reported: during *A. fumigatus* infection, it inhibited macrophage LC3-associated phagocytosis by prohibiting formation of an active NADPH oxidase complex (2). It will be interesting to discern whether this is a common characteristic of all fungal pathogen melanin or specific to that of *A. fumigatus*.

The initial ROS/RNS burst generated within phagosomes, particularly in the presence of high copper levels, is an effective strategy for killing microbes, and an efficient ROS detoxification system is critical for survival of bacterial and fungal pathogens within this hostile environment (26, 63). Although ROS are primarily handled by Cu,Zn Sod1 in the cytoplasm and Mn Sod2 in mitochondria, several fungi have evolved alternative mechanisms to detoxify host-derived ROS. This is exemplified in *C. albicans*, which encodes at least six *SOD* enzymes. Sod4, 5, and 6 are all secreted to the extracellular environment and tethered to the cell wall via a glycosylphosphatidylinositol linkage, strategically placed to detoxify superoxide before it enters cells. Structural and biochemical analysis revealed these enzymes are unique in that they are copper-only enzymes and acquire copper extracellularly, without a known chaperone (69, 127). Perhaps through mass action, the elevated copper levels within the phagosomal compartment of macrophages facilitate copper loading of the copper-only Sod enzymes. Additionally, due to the copper-restricted conditions encountered within certain niches of the host, *C. albicans* Mac1 directly represses Cu,Zn Sod1 and induces an alternative cytosolic Sod3, which utilizes manganese as a catalytic cofactor (97, 102, 110). *C. albicans* has thus evolved a mechanism for swapping metal cofactors for cytosolic Sod during copper limitation, and this switch from Sod1 to Sod3 has been demonstrated to occur in vivo as a critical virulence determinant (102).

CONCLUDING REMARKS

The baker's yeast *S. cerevisiae* has proven to be a powerful model organism for deciphering the copper biology of fungi. Many of the described functions in this model translate directly to other fungi and to higher eukaryotic organisms, underscoring a high level of conservation for the acquisition and distribution of copper. Given the diversity of the fungal kingdom, it is fascinating that different fungi have evolved distinct mechanisms for responding to copper excess or deficiency, likely specialized for their unique environments. However, gaps in our understanding of fungal copper biology remain, particularly mechanistic details of transport across cellular membranes in via the Ctr family of transporters that will ultimately require high-resolution structures and in vitro copper-transport reconstitution assays. Also, the nature and function of small-molecule copper ligands for copper delivery and compartmentalization await further investigation to discover their biogenesis and role in cellular copper homeostasis. A new and exciting area of investigation is the role of copper at the host-pathogen axis. Historically, iron and manganese have taken the spotlight in this

field; however, copper is now taking center stage. While certain copper-regulated genes in *S. cerevisiae* are also conserved in pathogenic fungi, it appears pathogens have expanded their copper homeostasis and utilization repertoire to enhance survival and virulence within the host. Understanding variations in copper-responsive mechanisms between fungal pathogens that infect distinct host niches with drastic differences in copper availability is likely to shed light on host copper bombardment and withholding mechanisms.

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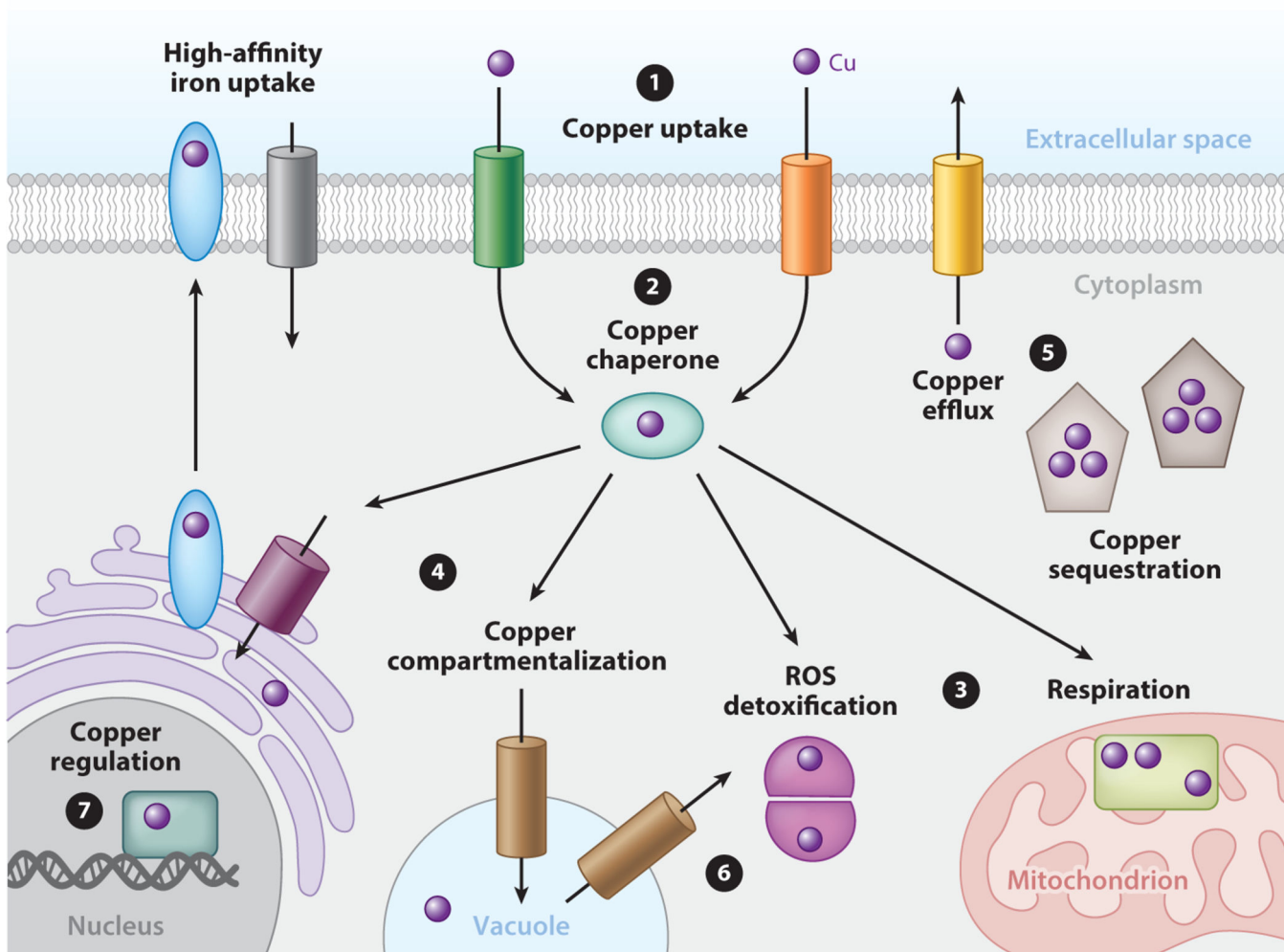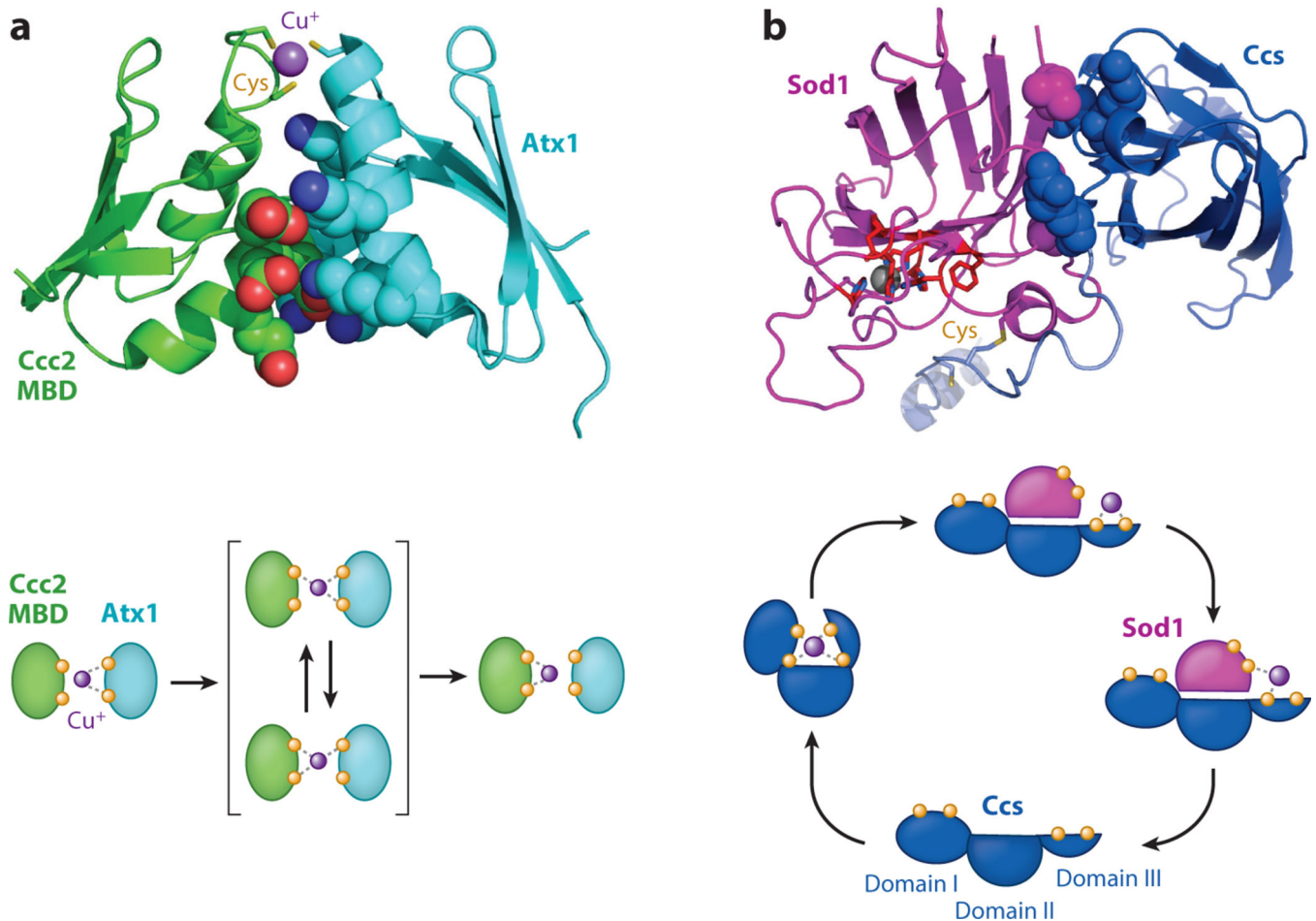
Figure 1.
Acquisition and distribution of copper in fungi. ❶. Dedicated high-affinity copper transporters at the plasma membrane facilitate the unidirectional import of copper (*purple spheres*) to the cytoplasm. ❷ Once internalized, copper is bound by copper chaperones or other buffering components that facilitate the delivery of copper to ❸ cytoplasmic and mitochondrial enzymes for copper-dependent activation or ❹ subcellular compartments for storage or loading into secretory copper-dependent enzymes. However, if copper accumulates beyond homeostatic capacities, fungi employ copper detoxification mechanisms such as ❺copper sequestration or efflux. ❻ Under conditions of extracellular copper deficiency cells mobilize vacuolar copper stores. ❼ In fungi, many of these processes are controlled at the level of transcription by copper-responsive transcription factors that regulate the expression of these components in response to intracellular copper status.
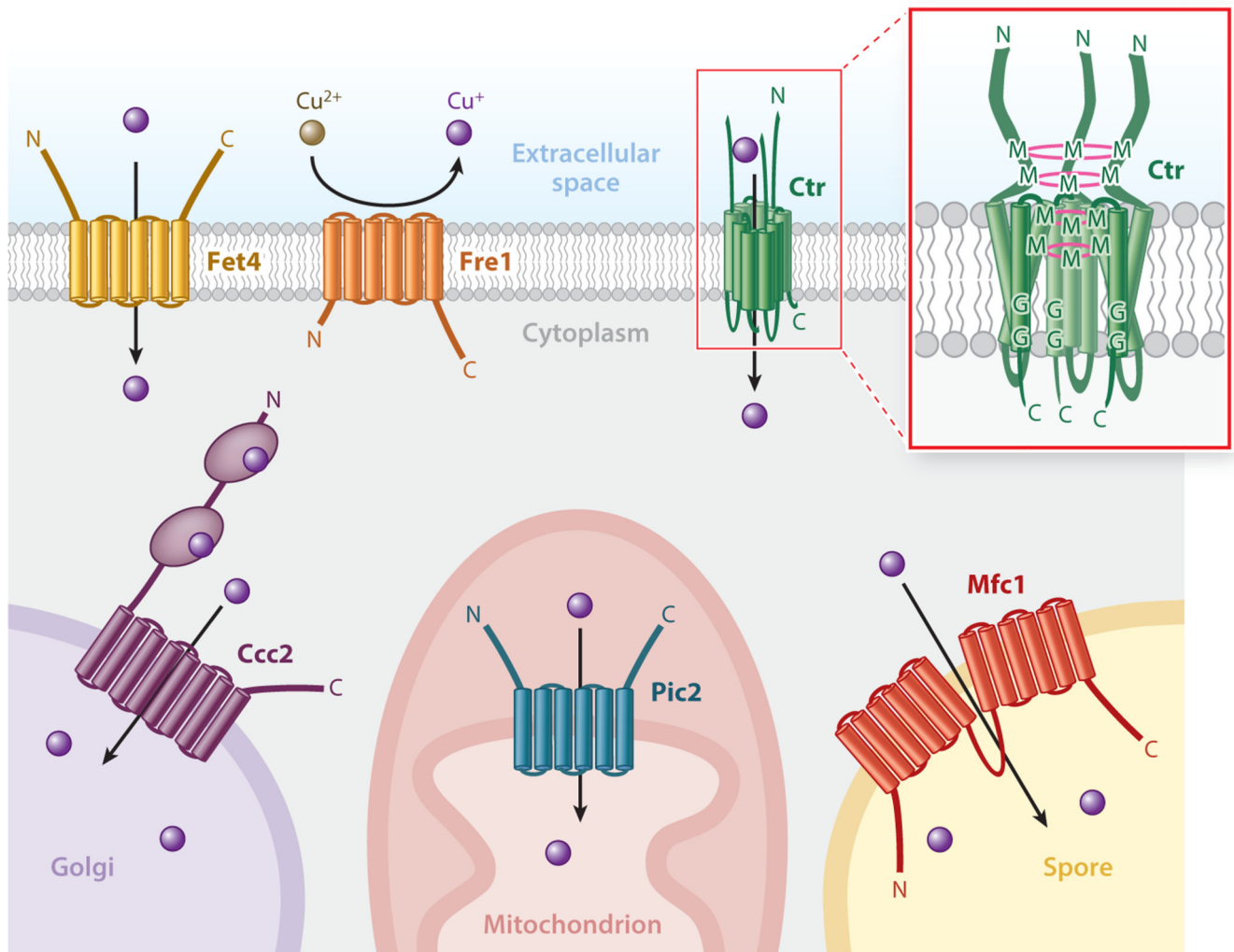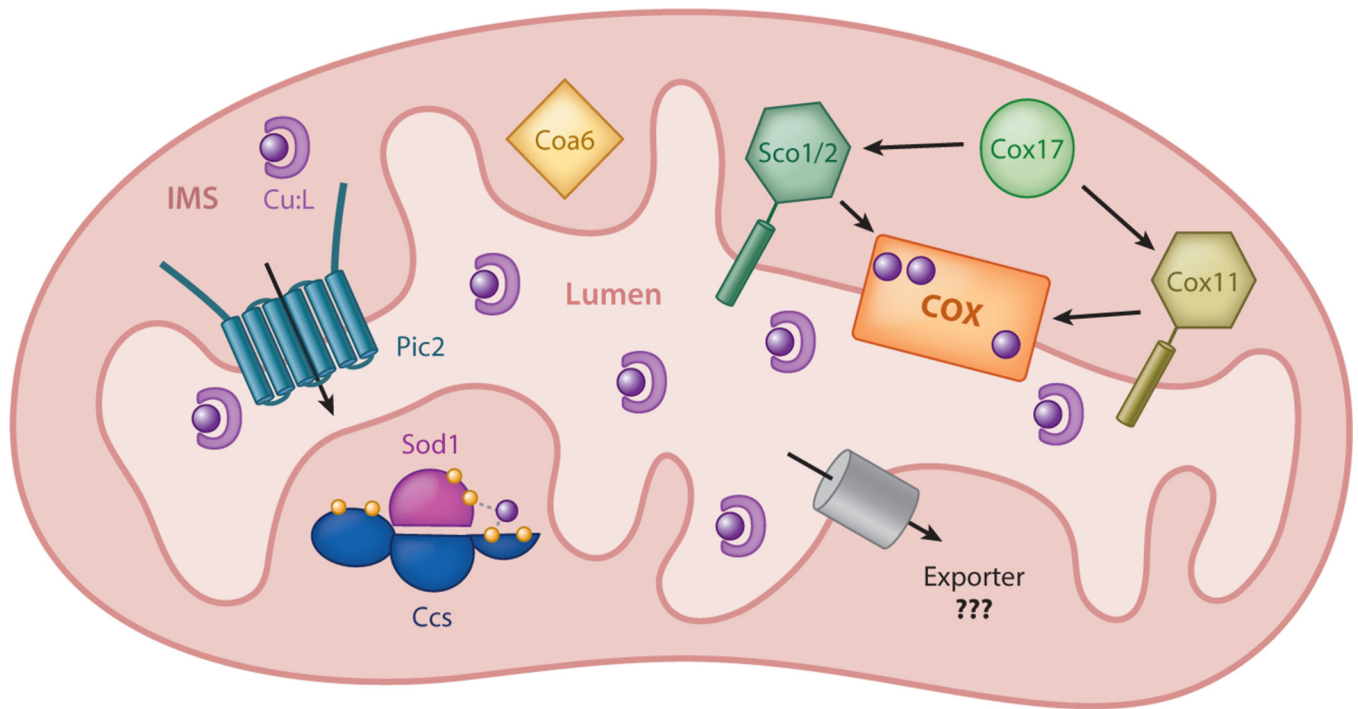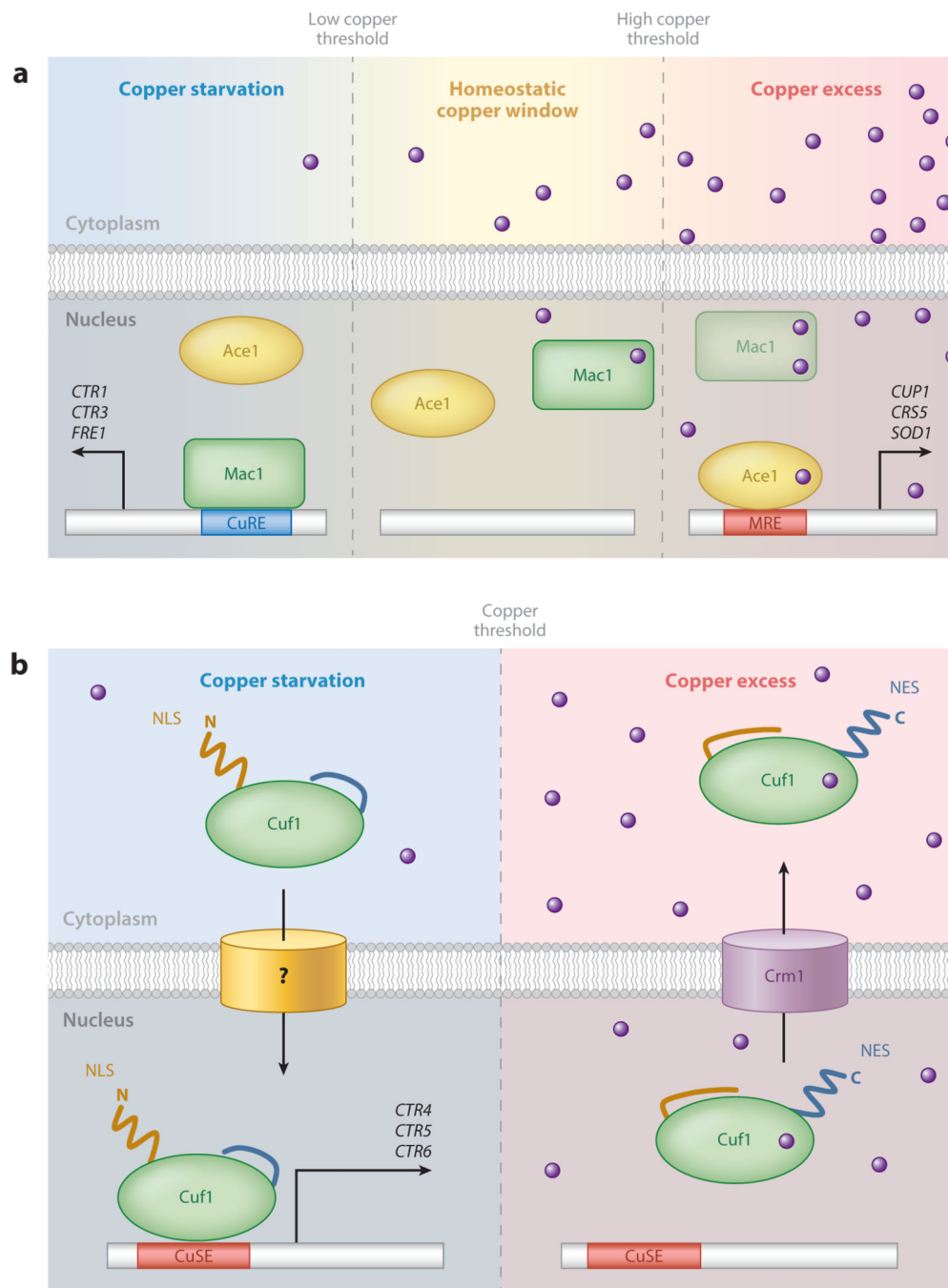
Figure 2.
Copper transport across membranes. The Fet4 iron and zinc transporter is capable of mediating low-affinity copper transport from the extracellular environment into the cytoplasm. The plasma membrane Ctr proteins, in combination with the cupric reductase Fre1, mediate high-affinity copper transport. (*Inset*) Ctr transporter organization as a homotrimer. Pink rings represent potential layers of copper coordination forming a selectivity filter at the mouth of the pore. Several additional transporters function to compartmentalize cytoplasmic copper. Mfc1 transports copper into the developing forespore during meiosis, Pic2 mediates copper transport into the mitochondrial matrix, and Ccc2 pumps copper into the Golgi network. Abbreviations: C, carboxyl terminus; G, glycine residue; M, methionine residue; N, amino terminus.

Figure 3.

Metallochaperone-mediated copper transfer. (*a*) Structure of Atx1 (*cyan*) bound to a metal-binding domain (MBD) of Ccc2 (*green*) reveals electrostatic interactions mediating protein-protein interactions (*top*). Atx1 contains several positively charged residues (*blue space filling*) that interact with several negatively charged residues (*red space filling*) of the MBD. This drives proper orientation of the copper (*purple spheres*)-coordinating cysteine residues (*yellow sticks*) to facilitate copper transfer (*bottom*). Modified from PDB 2GGP. (*b*) Structure of Ccs (*blue*) bound to Sod1 (*magenta*) reveals hydrophobic interactions (*space filling*) that mediate the docking interactions (*top*). Copper is transferred from the cysteine residues of Ccs to the active site pocket (*red*) of Sod1 (*bottom*). The three domains of Ccs are indicated. Modified from PDB 1JK9.

Figure 4.
Mitochondrial copper handling. Pic2 imports copper from the intermembrane space (IMS) to the lumen, where it is bound to a ligand (CuL). This copper is thought to be exported by an unidentified transporter from the matrix into the IMS, where it is acquired by Cox17 and transferred to Sco1/2 or Cox11 for insertion into COX subunits, or acquired by Ccs for insertion into Sod1. Coa6 plays a critical role in COX metalation and assembly through currently unknown mechanisms.

Figure 5.
Copper-responsive transcription in fungi. (*a*) Two-component copper-sensing transcriptional regulation in *Saccharomyces cerevisiae*. Mac1 induces expression of the high-affinity copper acquisition machinery during copper limitation to facilitate copper uptake, through recognition of promoter CuREs. As intracellular copper equilibrates to homeostatic levels, copper cluster formation in Mac1 results in its inactivation, and Mac1 is degraded at higher copper concentrations. Under conditions of copper excess, activated Ace1 induces expression of the copper detoxification machinery through recognition of target gene

promoter MREs. The stronger copper-binding affinity of Mac1 ensures no overlap in activation with the weaker copper-binding affinity of Ace1, effectively establishing a homeostatic copper window (163). (*b*) Single-component copper-sensing transcriptional regulation in *Schizosaccharomyces pombe*. During copper limitation, an exposed amino-terminal nuclear localization sequence (NLS) of Cuf1 directs its nuclear translocation, via an unknown importer, where Cuf1 induces expression of the high-affinity copper-acquisition machinery through recognition of target gene promoter CuSEs. As copper levels exceed a homeostatic threshold, copper cluster formation inactivates Cuf1 DNA binding, and the subsequent unmasking of a carboxyl-terminal nuclear export signal (NES) targets Cuf1 for nuclear export via the Crm1 exportin. Abbreviations: CuRE, copper-responsive element; CuSE, copper-signaling element; MRE, metal response element.
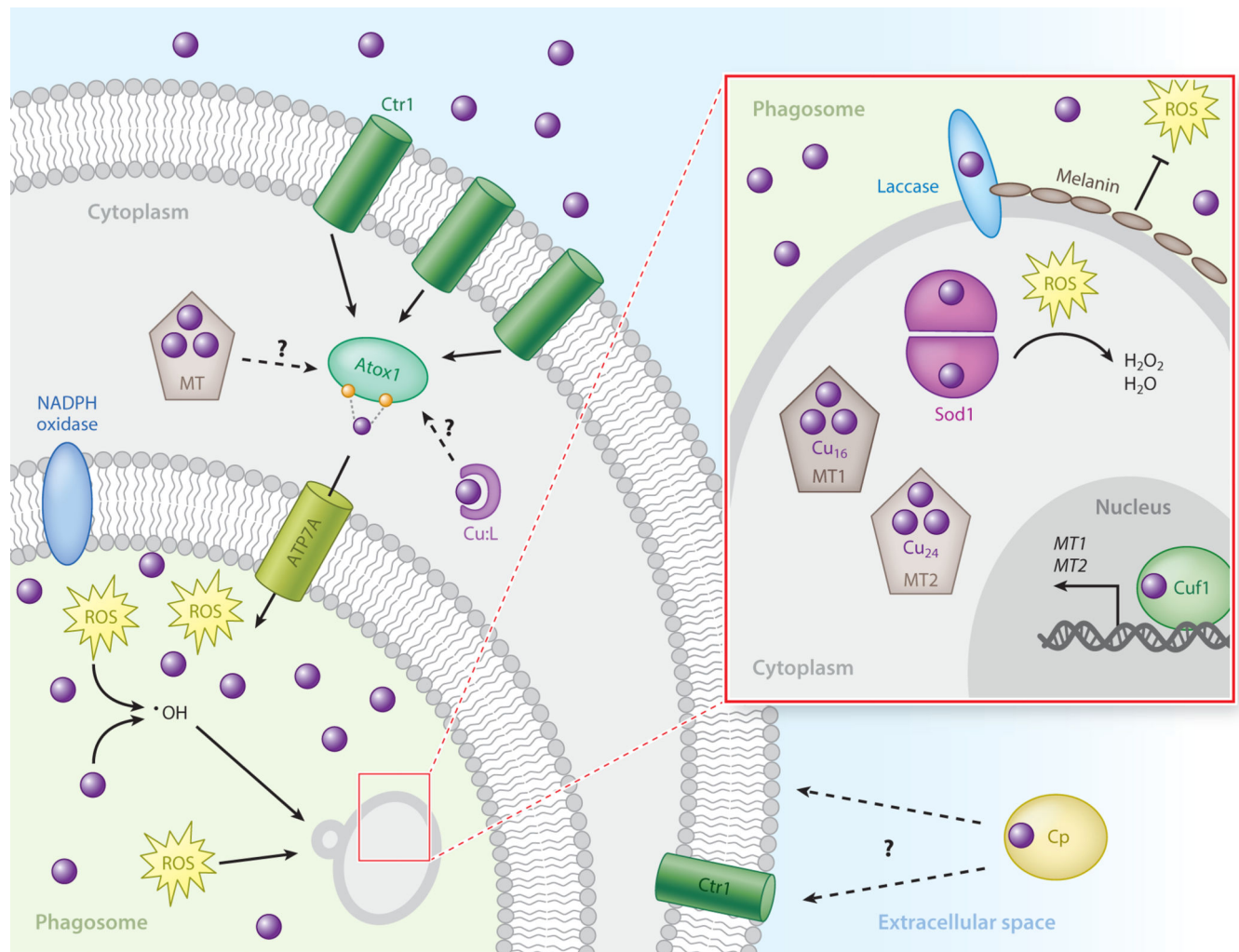
Figure 6.
Model of innate immune cell–directed copper bombardment of the fungal pathogen
Cryptococcus neoformans. Activated macrophages induce the expression of the mammalian
Ctr1 transporter at the plasma membrane to facilitate copper uptake. Additionally, ATP7A
expression is elevated, and the protein localizes to the phagosomal membrane. The copper
chaperone Atox1 directs the channeling of copper from Ctr1 at the plasma membrane to
ATP7A. The source of extracellular antifungal copper is currently unknown and could
involve the acute phase-responsive multi-copper oxidase Cp that has been shown to function
in copper import in Ctr1-dependent and -independent mechanisms. Alternatively,
intracellular mobilization of copper stores, such as from endosomal compartments, MTs,
copper-glutathione, or other copper-ligand complexes in the cytoplasm could supply copper.
In response to host-imposed copper toxicity, *C. neoformans* counters the copper
bombardment strategy by inducing transcription of the MT genes *MT1* and *MT2* in a Cuf1-
dependent manner (*inset*). Additionally, expression of Cu,Zn Sod1 neutralizes host-derived
ROS in the cytoplasm. The expression of copper-requiring laccase produces a protective
layer of melanin across the cell surface that has the ability to absorb and neutralize host-

derived ROS extracellularly. Abbreviations: Cp, ceruloplasmin; MT, metallothionein; ROS, reactive oxygen species.