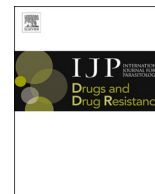Contents lists available at ScienceDirect

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Copper detoxification machinery of the brain-eating amoeba *Naegleria fowleri* involves copper-translocating ATPase and the antioxidant system

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ABSTRACT

Copper is a trace metal that is necessary for all organisms but toxic when present in excess. Different mechanisms to avoid copper toxicity have been reported to date in pathogenic organisms such as *Cryptococcus neoformans* and *Candida albicans*. However, little if anything is known about pathogenic protozoans despite their importance in human and veterinary medicine. *Naegleria fowleri* is a free-living amoeba that occurs naturally in warm fresh water and can cause a rapid and deadly brain infection called primary amoebic meningoencephalitis (PAM). Here, we describe the mechanisms employed by *N. fowleri* to tolerate high copper concentrations, which include various strategies such as copper efflux mediated by a copper-translocating ATPase and upregulation of the expression of antioxidant enzymes and obscure hemerythrin-like and protoglobin-like proteins. The combination of different mechanisms efficiently protects the cell and ensures its high copper tolerance, which can be advantageous both in the natural environment and in the host. Nevertheless, we demonstrate that copper ionophores are potent antiamoebic agents; thus, copper metabolism may be considered a therapeutic target.

1. Introduction

Copper is a trace element that is vital for all organisms from bacteria to higher eukaryotes. Based on its ability to cycle between reduced (Cu^+) and oxidized (Cu^{2+}) states, copper serves as a cofactor for different enzymes such as cytochrome c oxidase, Cu/Zn-SOD, tyrosinase, and hexose oxidase. High copper concentrations, however, are toxic. The main mechanism underlying copper toxicity is not well understood; it has generally been assumed that copper acts in a manner similar to iron, causing reactive oxygen species production via the Fenton and Haber-Weiss reactions with subsequent damage to macromolecules including nucleic acids, proteins and lipids (Rainsford et al., 1998). Indeed, cells that are exposed to high copper conditions demonstrate signs of oxidative stress (Gaetke and Chow, 2003). Recently, however, it was shown that enzymes containing iron-sulfur (Fe–S) clusters might be the primary targets of copper toxicity. Copper degrades Fe–S clusters by replacing iron, impairing the function of enzymes; additionally, the free iron concentration in the cell is increased, promoting iron toxicity via iron-based Fenton chemistry. Thus, excessive copper promotes iron toxicity and the subsequent oxidative stress (Macomber and Imlay, 2009). Moreover, copper might impair Fe–S clusters biogenesis (Brancaccio et al., 2017; Garcia-Santamarina et al., 2017).

Free copper is virtually nonexistent in living organisms, and there are different mechanisms to regulate cell copper concentrations. One is the downregulation of high-affinity copper transporters during copper excess, which has been shown, for example, in *Candida albicans* (Mackie et al., 2016) and *Aspergillus fumigatus* (Wiemann et al., 2017). Another mechanism is copper sequestration by metallothioneins or glutathione. Metallothioneins are small highly heterogeneous cysteine-rich proteins that bind copper with high stoichiometry; they are found in different organisms, including bacteria, fungi, plants and animals (Capdevila and Atrian, 2011). One of the most extensively characterized metallothioneins, Cup1 from *Saccharomyces cerevisiae*, binds 8 copper ions, while metallothioneins Cmt1 and Cmt2 from a well-studied human pathogen *Cryptococcus neoformans* bind up to 16 and 24 copper ions, respectively (Smith et al., 2017). Glutathione is another intracellular compound that is quite effective in buffering copper and effectively reducing its toxicity (Macomber and Imlay, 2009). Free amino acids such as methionine and histidine probably also participate in copper buffering (Fung et al., 2013; Pearce and Sherman, 1999). A third well-known mechanism of copper detoxification is its export by copper-translocating P-type ATPases. This mechanism is employed by *C. albicans* (Weissman et al., 2000). Although *C. albicans* possesses metallothionein CaCup1, it plays only a minor role in copper

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detoxification.

Less is known about copper detoxification in parasitic protists. *In silico* approaches have shown that at least some protists possess homologs of copper-transporting P-type ATPases and copper chaperones (Rasoloson et al., 2004). Most of the experimental evidence is available for *Plasmodium* as one of the most important human protozoan parasites. *Plasmodium falciparum* copper-transporting P-type ATPase (PfCu-P-ATPase), localizing to the plasma membrane of both the parasite and erythrocyte, is probably responsible for decreasing the copper content in the infected erythrocyte, suggesting that *P. falciparum* avoids copper toxicity by copper efflux (Rasoloson et al., 2004). P-type ATPase expression has also been demonstrated in *Trypanosoma brucei brucei,* where it is localized to subcellular vesicles and probably the cell membrane, although its detoxification function has not been confirmed (Isah et al., 2020). In *Trypanosoma cruzi,* a probable copper-transporting ATPase is upregulated in the intracellular stage in host cells compared with the extracellular stage in the insect vector (Meade, 2019). The intracellular stage has to survive in the phagosomal compartment with excessive copper levels, and the upregulation of copper translocating ATPase may indicate its role in detoxification.

Naegleria fowleri is a pathogenic free-living amoeba that is found in warm fresh water and sediments worldwide (Maciver et al., 2020; Mull et al., 2013; Sykora et al., 1983). It is often called a "brain-eating amoeba" because of its ability to infect humans, causing a rapid and deadly brain infection designated as primary amoebic meningoencephalitis (PAM). Infection occurs when water contaminated with *N. fowleri* enters the nose, for example, during swimming. The amoebae migrate via the olfactory nerve to the brain, where they multiply and destroy nerve cells, causing tissue damage and hemorrhagic necrosis. The disease has an abrupt onset, and most patients die within days after symptoms begin (Siddiqui et al., 2016). Treatment options are very limited: antibiotics and antifungals such as amphotericin B, fluconazole, rifampicin and miltefosine are usually used, but death rate is over 95%. Due to the rapid progression of the disease, the diagnosis is usually made after the patient's death (Bellini et al., 2018).

Although the genomes of *N. fowleri* as well as its nonpathogenic relative model amoeba *Naegleria gruberi* have been annotated, little is known about the physiology and biochemistry of this pathogen. Likewise, our understanding of the cellular processes that allow this free-living amoeba to survive and destroy the human host is very limited (Herman et al., 2020). We have recently described how *N. fowleri* respond to limited iron availability and how iron metabolism could potentially be exploited in antiamoebic interventions (Arbon et al., 2020). In the current study, we aimed to characterize the mechanisms employed by *N. fowleri* to deal with toxic copper levels. We show that a combination of antioxidant proteins with copper efflux mediated by copper-translocating ATPase is used to protect the cell and ensure high copper tolerance of *N. fowleri*. We also consider copper metabolism as a possible therapeutic target, since we demonstrated that copper ionophores are potent antiamoebic agents.

2. Materials and methods

2.1. Cell cultures

N. fowleri, strain HB-1, kindly provided by Dr. Hana Pecková (Institute of Parasitology, Biology Center CAS), was maintained in 2% Bactocasitone (Difco, USA), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) in 25-cm^2 aerobic cultivation flasks at 37 °C. When required, the culture was cultivated for 72 h with the addition of 25 μM, 100 μM or 1 mM CuSO$_4$ (Sigma-Aldrich, USA). *N. gruberi* strain NEG-M (kindly provided by Lillian Fritz-Laylin, University of Massachusetts Amherst, USA) was cultivated in M7 medium (Fulton, 1974) supplemented with streptomycin (100 μg/ml) in 25-cm^2 aerobic cultivation flasks at 27 °C.

2.2. Copper and ionophore toxicity

To determine the IC$_{50}$ values for copper, *N. fowleri* or *N. gruberi* cells were cultivated for 72 h in quadruplicate in black 96-well plates in 100 μl of medium per well with concentrations of copper up to 20 mM. To determine the IC$_{50}$ values for ionophores, *N. fowleri* cells were grown for 72 h in triplicate in black 96-well plates in 100 μl of medium per well with 1 μM–10 μM concentrations of 8-hydroxyquinoline, pyrithione or disulfiram with or without the addition of 100 μM copper. Dimethyl sulfoxide (DMSO) was added to control cells in the case of DSF and 8-HQ testing as they were diluted in DMSO. The maximum DMSO concentration did not exceed 1%. The cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) according to the manufacturer's protocol. Statistical analysis was performed using GraphPad Prism 8.3.1 (www.graphpad.com).

2.3. Copper content determination

N. fowleri cells supplemented with 25 μM, 100 μM or 1 mM copper or with 1 μM 8-HQ in the presence or absence of 100 μM copper were grown in triplicate, washed three times (1000 g, 15 min, 4 °C) in 10 mM Hepes buffer, pH 7.2, containing 140 mM NaCl and pelleted. The pellets were dried at 100 °C, digested in 65% HNO$_3$ overnight and then for 2 h at 130 °C in Savillex vials and diluted in deionized water (Millipore, USA) to final volume 10 ml. The copper concentration was determined by inductively coupled plasma mass-spectrometry (ICP-MS) using iCAP Q ICP-MS (Thermo Fisher Scientific, USA).

2.4. Whole-cell proteomic analysis

N. fowleri cells supplemented with 25 μM CuSO$_4$ were used as a control, and cells supplemented with 100 μM or 1 mM copper were used for the copper overload investigation. The cells (grown in triplicate) were washed three times with PBS and pelleted. Whole-cell proteomic analysis of the samples was carried out using the method described by Mach et al. (2018) employing nanoflow liquid chromatography coupled with mass spectrometry (MS). The resulting MS data were searched with MaxQuant software (Cox et al., 2014) against the AmoebaDB (Aurrecoechea et al., 2011) *N. fowleri* database downloaded on Aug 6, 2018. The carbamidomethylation of cysteine (Unimod #: 4) was set as a fixed modification, and methionine oxidation (Unimod #: 35) was allowed as a variable modification. Further processing of the data was performed with Perseus software (Tyanova et al., 2016). Normalized label-free quantitation values of intensities were used. We filtered out reverse hits, potential contaminants and proteins identified only by site. Then, we took the log of the intensities (binary logarithms) and filtered out proteins with insufficient numbers of valid quantification values (leaving only those with at least 2 values in at least one group). The Student's t-test with Benjamini-Hochberg correction was used to evaluate significantly changed proteins at the 5% false discovery rate level. Only the proteins that changed more than 1.5-fold or those found in just one condition and having a normalized intensity greater than 23 were considered. The selected proteins were manually annotated using HHpred (Söding et al., 2005) or by sequence alignment with homologous proteins from other organisms using BLAST (Altschul et al., 1990). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD018807. The additional information about proteomic experimental procedures is summarized in Table S2. Bioinformatics analysis was performed using Geneious Prime® 2019.2.3 (www.geneious.com).

2.5. Western blotting

N. gruberi cells were grown with the addition of 25 or 750 μM copper. The primary polyclonal antibody against *N. fowleri* protoglobin was

generated as described by Mach et al. (2018). The produced antibodies were used for protoglobin detection in the Western blot analyses of *N. gruberi* whole-cell lysates. For Western blot development, anti-rat IgG peroxidase secondary antibodies (Merck, Germany) and Luminata Forte Western horseradish peroxidase substrate (Merck, Germany) were used.

2.6. Real-time PCR

Total RNA was isolated from *N. fowleri* cultures supplemented with 25 μM or 1 mM copper (grown in quadruplicate) using the High Pure RNA Isolation Kit (Roche, Switzerland). RT-PCR was conducted using the KAPA SYBR® FAST One-Step universal kit (Sigma-Aldrich, USA) according to the manufacturer's protocol in the RotorGene 3000 PCR cycler (Corbett Research, Australia) with the following thermocycle conditions: 42 °C for 30 min (reverse transcription), 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 20 s; and melt-curve analysis from 55 °C to 95 °C with 1 °C step for 5 s per step. The transcript abundance was calculated after normalization to the endogenous reference gene β-actin. The primers are listed in Table 3.

2.7. RACE and cloning

The full 5′ sequence of Nf-CuATPase gene was determined by rapid amplification of 5′ cDNA ends using the FirstChoice™ RLM-RACE kit (Thermo Fischer Scientific, USA) according to the manufacturer's protocol. cDNA was synthesized with SuperScript™ III Reverse transcriptase (Thermo Fisher Scientific, USA). The Nf-CuATPase gene was amplified from cDNA using SapphireAmp® Fast PCR Master Mix (TakaraBio, Japan) and cloned into pCM189 plasmid with the tetracycline-regulatable promotor using FastDigest™ restriction enzymes and T4 DNA ligase (Thermo Fisher Scientific, USA).

2.8. Yeast procedures

Strains BY4741 (MATa; his3D1; leu2D0; met15D0; ura3D0), cup2Δ (BY4741; MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YGL166w::kanMX4) and ccc2Δ (BY4741; MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YDR270w::kanMX4) were obtained from Euroscarf (euroscarf.de). Yeast transformations were performed according to Gietz and Schiestl (2007). For phenotypic assays, yeast cultures were grown overnight in liquid synthetic complete medium without uracil (SC-ura); 5 μl of six serial ten-fold dilutions starting from OD$_{600}$ 0.2 were plated on agar SC-ura plates. For assays with the cup2Δ strain, plates were supplemented with 1 mM CuSO$_4$ with or without 1 μg/ml doxycycline. For assays with the ccc2Δ strain, plates were supplemented with 50 μM bathophenanthroline disulfonate (BPS) with or without doxycycline, or with 50 μM BPS and 50 μM (NH$_4$)$_2$Fe(SO$_4$)$_2$ · (H$_2$O)$_6$ (Sigma-Aldrich,

Table 1
Selected *N. fowleri* proteins that were significantly changed in 1 mM CuSO$_4$ compared with 25 μM CuSO$_4$. Arrows indicate upregulation (no downregulated proteins were selected).

Fold change	Gene number in AmoebaDB database	Annotation
↑ 11.2	NF0109980	Predicted protein
↑ 4.9	NF0013290	Probable methanethiol oxidase
↑ 4.3	NF0127030	Hemerythrin
↑ 3.6	NF0021200	Copper-transporting ATPase
↑ 3.5	NF0028890	Glutathione S-transferase
↑ 3.5	NF0117840	Protoglobin
↑ 2.1	NF0071710	Deferrochelatase/peroxidase
↑ 2.1	NF0042620	Glutathione S-transferase
↑ 1.9	NF0036980	Thioredoxin reductase
↑ 1.9	NF0112140	Sulfiredoxin
↑ 1.8	NF0061690	Protoglobin domain-containing protein
↑ 1.8	NF0108900	Glutathione S-transferase

Table 2
The changes in transcript abundancy of selected genes in 1 mM CuSO$_4$ compared with 25 μM CuSO$_4$ determined by real-time PCR. Arrows indicate downregulation or upregulation. Significantly changed genes (p-value < 0.05) are marked with a star. Cells were grown in quadruplicate.

	Fold change	p-value
Nf-CTR (1)	↓ 1.17	4.3914
Nf-CTR (2)	↓ 2.27	0.2666
Nf-CuATPase	↑ 4.21*	0.0491
Nf-Hemerythrin	↑ 4.71*	0.0057
Nf-Protoglobin	↑ 7.10*	0.0002

USA). For copper content analysis, we used ICP-AES. Yeast cells were grown overnight in triplicate in liquid SC-ura medium supplemented with 0, 0.05, 0.1 or 0.5 mM copper, washed three times (3000 g, 5 min, 4 °C) in ultrapure water and pelleted. All further manipulations were performed as described above for *N. fowleri*.

3. Results

3.1. Copper and copper ionophores are toxic to N. fowleri

Excess copper is toxic for *N. fowleri*, although it survives at relatively high copper concentrations: the half maximal inhibitory concentration (IC$_{50}$) of copper for *N. fowleri* is 1.62 mM (95% confidence interval from 1.48 to 1.70, Fig. 1). Copper is dramatically more toxic for *N. fowleri* when provided in the presence of lipid-soluble ionophores – substances that transport ions across the plasma membrane, such as pyrithione (PyS), disulfiram (DSF) or 8-hydroxyquinoline (8-HQ). We tested the toxicity of these compounds in the absence of copper and with the addition of 100 μM copper – a relatively low concentration that is well below the IC$_{50}$ and does not inhibit *N. fowleri* growth. Dimethyl sulfoxide (DMSO) was added to the control cells for the testing of DSF and 8-HQ, as they were diluted in DMSO. The maximum concentration of DMSO did not exceed 1%, and we did not observe any significant effect of DMSO on the growth of amoebae even at the highest concentration used. All three tested ionophores showed copper-dependent amoebicidal activity (Fig. 1). The IC$_{50}$ of DSF in the presence of copper was 2.09 μM, and the IC$_{50}$ of PyS was 1.05 μM and of 8-HQ was 2.09 μM. We believe that these results show a promising potential of ionophores as future potent antiamoebic agents.

3.2. Copper accumulation in N. fowleri

To determine how copper concentrations in the growth medium influence the amount of copper accumulated in cells, we used inductively coupled plasma mass spectrometry (ICP-MS). As a control, we used amoebae grown in 25 μM CuSO$_4$. The copper concentration in the growth medium before adding CuSO$_4$ was less than 1 μM. Copper accumulation in the amoebae was dependent on the concentration of free copper in the medium (Fig. 2): copper content in control cells was 84.10 ± 17.85 ng/mg dry mass, while the addition of 100 μM copper to the medium increased the copper content in the amoebae ~3.5-fold. The amount of copper in cells supplemented with 1 mM CuSO$_4$ was 369.21 ± 20.03 ng/mg dry mass which was slightly higher than in medium with 100 μM CuSO$_4$, however, the difference was not statistically significant.

Additionally, we determined the amount of copper in cells treated with the ionophore 8-HQ in the absence or presence of 100 μM CuSO$_4$ (Fig. 2). In the cells which were not supplemented with CuSO$_4$ the copper content was 8.64 ± 1.09 ng/mg, while supplementation with copper increased it to 259.08 ± 48.62 ng/mg. This number was close to the copper content in the cells grown with 100 μM CuSO$_4$ without the ionophore. Thus, the treatment of *N. fowleri* with the copper ionophore 8-HQ did not increase the amount of copper in the cells.

Table 3
Primers used for real-time PCR and product sizes.

|	Forward primer	Reverse primer	Product size
Nf β-actin	TTGGTATGGAAGCTGCTGGT	AACCTCCAATCCAGACCGAG	231
Nf-CTR (1)	TGGTGAAGAGAAAGGTGACCA	ATCCGACAGTGTTACCAGCA	225
Nf-CTR (2)	GTGAAACTACAGAGCATGAGGA	CTCCTGACTGAAGAGCTCGT	220
Nf-CuATPase	ATTGGTGGAACCGTCAATGT	GCAAGATGACAGCAGGAACA	178
Nf-Hemerythrin	CCAACAGATCCAGTGCTTCA	TGTGATTCACCAACCATTGC	210
Nf-Protoglobin	CGAGGAACAACGTGTCAAGA	TTGTTGAGCAAAGCAGCATC	175
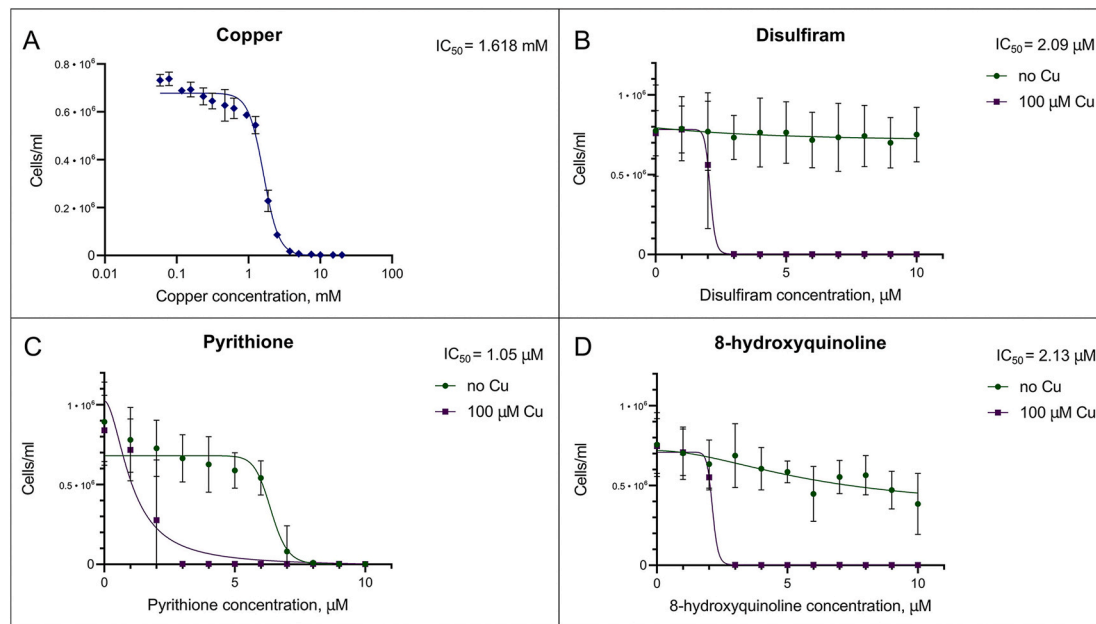
Fig. 1. Copper and copper ionophore toxicity to *N. fowleri*. **A.** Copper toxicity to *N. fowleri* cells after 72 h in the presence of different concentrations of $CuSO_4$. Cells were grown in quadruplicate. **B-D**. Determination of the IC_{50} of ionophores on *N. fowleri* in the presence or absence of 100 μM $CuSO_4$. Cells were grown in triplicate.
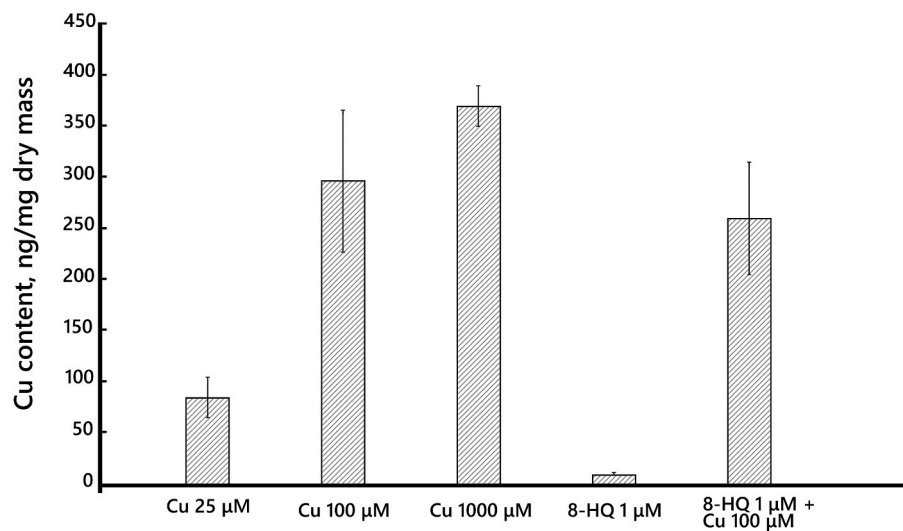

Fig. 2. Copper content in *N. fowleri* cells grown in media supplemented with 25 μM, 100 μM or 1 mM $CuSO_4$, or with 1 μM 8-HQ in absence or presence of 100 μM $CuSO_4$. Data represent the mean with 95% confidence intervals of three replicates.

3.3. Proteomic analysis of the N. fowleri response to high copper concentrations identified a set of detoxification proteins

To identify the proteins responsible for copper detoxification in *N. fowleri*, we analyzed the whole-cell proteomes (including soluble and membrane proteins) of amoebae grown in medium supplemented with 25 μM $CuSO_4$ as a control and either 100 μM or 1 mM $CuSO_4$, with the latter being considered a toxic copper concentration. We identified 57 proteins that were significantly changed when the cells were grown in 1 mM copper: 36 upregulated and 21 downregulated (Supplementary

Table 1). When 100 μM copper was added to the medium, there were no statistically significant changes in protein expression levels compared with the control conditions. The proteins most relevant for this study are summarized in Table 1.

The most upregulated protein (NF0109980 in AmoebaDB Database (Aurrecoechea et al., 2011)) did not show any significant homology to known proteins. It contained 133 amino acid residues, including 4 cysteine-proline motifs and 6 histidines, which might indicate copper- or iron-binding properties (Kung et al., 2006; Wu et al., 2010; Zhang and Guarente, 1995). The second most upregulated protein (NF0013290) showed homology to methanethiol oxygenases and to a family of selenium-binding proteins.

Importantly, we observed a 3.6-fold upregulation of a protein homologous to copper-translocating P-type ATPase (which we named Nf-CuATPase). Copper transporting ATPases have been shown to mediate copper tolerance and detoxification in *C. albicans* (Weissman et al., 2000) and *P. falciparum* (Rasoloson et al., 2004), suggesting a similar role in *N. fowleri*.

Moreover, among the 36 upregulated proteins, at least 9 are known or proposed to be oxidative stress related, namely, three glutathione S-transferases (GSTs), thioredoxin reductase, sulfiredoxin, deferrochelatase/peroxidase (dye-decolorizing peroxidase), a hemerythrin-like and two protoglobin-like proteins. Since a homolog of protoglobin is also present in the genome of the nonpathogenic amoeba *N. gruberi*, we were curious to determine whether its expression was affected by copper toxicity similarly to in *N. fowleri*. Western blot analysis using a specific antibody against *N. gruberi* protoglobin confirmed the induction of protein expression under conditions of copper toxicity (Supplementary Fig. 1), suggesting a common detoxification mechanism in *Naegleria*.

3.4. Bioinformatics analysis of Nf-CuATPase confirms its structural similarity to copper-translocating ATPases

Since the annotation of Nf-CuATPase in the AmoebaDB database (Aurrecoechea et al., 2011) is incomplete at the N-terminal end of the protein, we performed a rapid amplification of cDNA ends (RACE) to obtain the full mRNA sequence. The correct Nf-CuATPase gene had 1330 amino acids with no introns. Topology prediction programs, including CCTop (Dobson et al., 2015), Phobius (Käll et al., 2004), Scampi (Bernsel et al., 2008) and others, proposed 8 transmembrane domains and 3 cytosolic segments, including a long N-terminal part (Fig. 3) containing 5 CXXC motifs, which are considered to be involved in binding heavy metals such as copper and cadmium (Solioz and Vulpe,

1996). The overall topology with the first small and second big cytosolic loop was characteristic of P-type ATPases. The number of transmembrane domains indicated a heavy metal ATPase: it had four transmembrane domains between the N-terminus and small cytosolic loop (while nonheavy metal ATPases only have two) and, in contrast, only two transmembrane domains preceding the C-terminus instead of four or six characteristic of nonheavy metal ATPases (Solioz and Vulpe, 1996). The second cytoplasmic loop of Nf-CuATPase encompassed the highly conserved D^{1013}-K-T-G-T^{1017} phosphorylation motif containing the aspartate residue, which is phosphorylated by ATP, and the G^{1217}-D-G-I-N-D-A-P^{1224} site presumably involved in ATP binding (Møller et al., 1996). The first cytoplasmic domain contained the invariant T^{851}-G-E^{853} motif, which is probably necessary for stabilization of phosphorylated aspartate (Kühlbrandt, 2004). There was a proline residue 43 amino acids upstream of the phosphorylation site in the predicted transmembrane domain, which is conserved in all P-type ATPases and considered to be involved in ion transduction. Together with two surrounding cysteines, it forms the C^{969}–P–C^{971} motif, which is believed to be necessary for the translocation of heavy metals. Downstream from the phosphorylation site, a histidine-proline dipeptide was present. This is a peculiar feature for heavy metal ATPases, and although its function is unknown, it is not found in other ATPases (Solioz and Vulpe, 1996). Alignments with other copper-transporting ATPases with confirmed function are presented on Fig. S3.

3.5. RT-PCR of selected copper-regulated genes

The genome of *N. fowleri* contains two homologs of yeast and human high-affinity copper transporters (CTRs) that presumably mediate copper influx. It would be rational to expect copper influx to be downregulated at high copper concentrations, as shown in *C. albicans* (Mackie et al., 2016) and *C. neoformans* (Ding et al., 2013). We did not identify any CTRs in our proteomic data, which was not unexpected considering that membrane transporters might be lost during sample preparation for proteomic analyses (Harwood et al., 2014). Thus, to investigate the regulation of putative *N. fowleri* copper transporters at high copper concentrations, we conducted RT-PCR using cells grown with 25 μM or 1 mM $CuSO_4$. Additionally, we investigated relevant genes encoding proteins that were upregulated by toxic copper levels based on the proteomic analysis (Nf-CuATPase, hemerythrin and protoglobin) to determine whether they were regulated at the transcriptional or translational level.

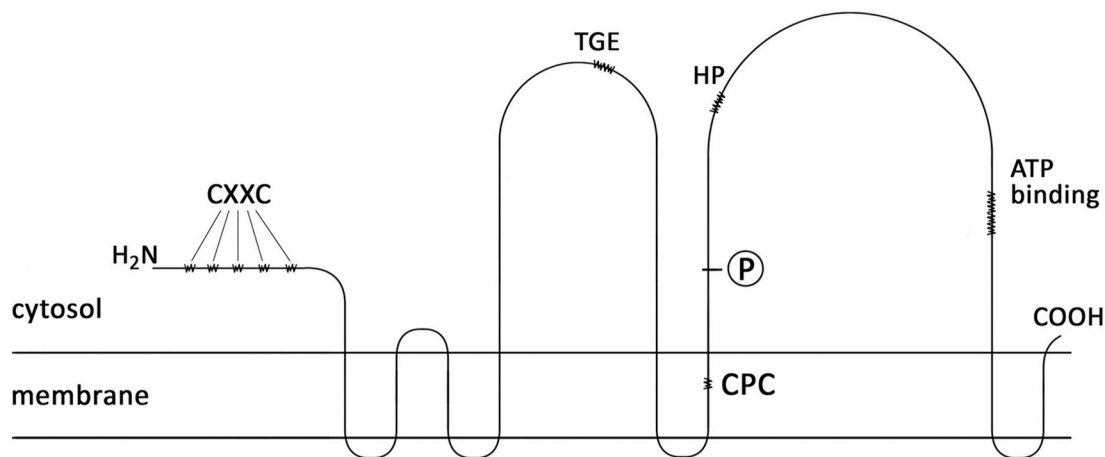Our results showed that, while there was no statistically significant

Fig. 3. Schematic representation of the Nf-CuATPase membrane topology and of its key features: 5 CXXC motifs near the N-terminus are supposed to be involved in copper binding; the TGE motif is needed for stabilization of phosphorylated aspartate; the CPC motif in the transmembrane domain is necessary for copper translocation; P indicates conserved aspartate, which is phosphorylated and dephosphorylated in the enzymatic cycle; the HP motif with unknown function is typical for heavy metal ATPases; the ATP binding site participates in ATP binding.

change in the CTR1 and CTR2 transcription levels between control and copper-loaded cells, Nf-ATPase was indeed upregulated at the transcriptional level and increased 4.21 ± 1.57 times under copper overload (Table 2). Nf-protoglobin and Nf-hemerythrin were also increased 7.10 ± 2.28 and 4.71 ± 1.07 times, respectively. Thus, the response of amoebae to copper stress appeared to occur at the transcriptional level, in contrast to the iron-starvation-induced changes that were almost exclusively post-transcriptional in *N. fowleri* (Arbon et al., 2020).

3.6. *N. fowleri Cu-ATPase complements the S. cerevisiae cup2Δ mutant*

To validate a role for Nf-CuATPase in copper detoxification, we cloned the gene into a yeast expression plasmid pCM189 with the tetracycline-regulatable promotor and transformed two *S. cerevisiae* strains impaired in copper metabolism: the cup2Δ strain lacks a copper metallothionein transcription activator and fails to grow at high copper concentrations; the ccc2Δ strain is deficient in trans-Golgi copper-translocating ATPase and is unable to grow on media containing iron chelators due to inefficient copper incorporation into the multicopper iron oxidase involved in iron acquisition. In agreement with the proposed role in copper detoxification, Nf-CuATPase enables the cup2Δ strain to grow at high copper concentrations, and the effect is lost in the presence of doxycycline, which blocks transcription from the plasmid (Fig. 4). Similar to the copper transporting ATPase of *C. albicans* CaCrp1 (Weissman et al., 2000), Nf-CuATPase was unable to functionally complement the ccc2Δ mutant and restore its defective growth on medium containing the iron chelator bathophenanthroline disulfonate (BPS) (Fig. 4), indicating that it does not translocate copper into trans-Golgi vesicles. To localize Nf-CuATPase, we overexpressed the Nf-CuATPase-GFP fusion as well as Nf-CuATPase fused to the hemagglutinin (HA) epitope tag in the cup2Δ mutant; however, neither of the fusion proteins complemented the mutant copper sensitivity (data not shown). Thus, we decided to establish the localization of the protein indirectly to determine whether Nf-CuATPase exports copper from the cell or compartmentalizes it within the cell.

3.7. *Analysis of copper content in transformed yeast*

To determine whether copper was transported outside or accumulated within the cell, we carried out ICP-AES analysis of copper content in wild type yeast cells (BY4741) containing empty pCM189, cup2Δ mutant cells with empty plasmid and cup2Δ cells expressing Nf-CuATPase (Fig. 5). From the obtained data, it was evident that copper content in wild type and cup2Δ yeasts was significantly higher than in Nf-CuATPase-expressing cup2Δ cells. The amount of copper in wild type grown in 0.05 and 0.1 mM copper was approximately twice as high compared with the Nf-CuATPase-expressing mutant, which strongly suggested that Nf-CuATPase exports copper.

4. Discussion

Copper is an essential element for life. There are at least thirty enzymes containing copper cofactors catalyzing redox reactions or transporting dioxygen (Flemming and Trevors, 1989). However, copper is toxic when present in excess as it displaces iron in Fe–S clusters and promotes oxidative stress, possibly via the Fenton reaction caused by released iron (Macomber and Imlay, 2009). Copper toxicity is employed by innate immune cells in host-parasite interactions: macrophages actively accumulate copper and use it against pathogens (Sheldon and Skaar, 2019; White et al., 2009). High copper resistance is crucial for the
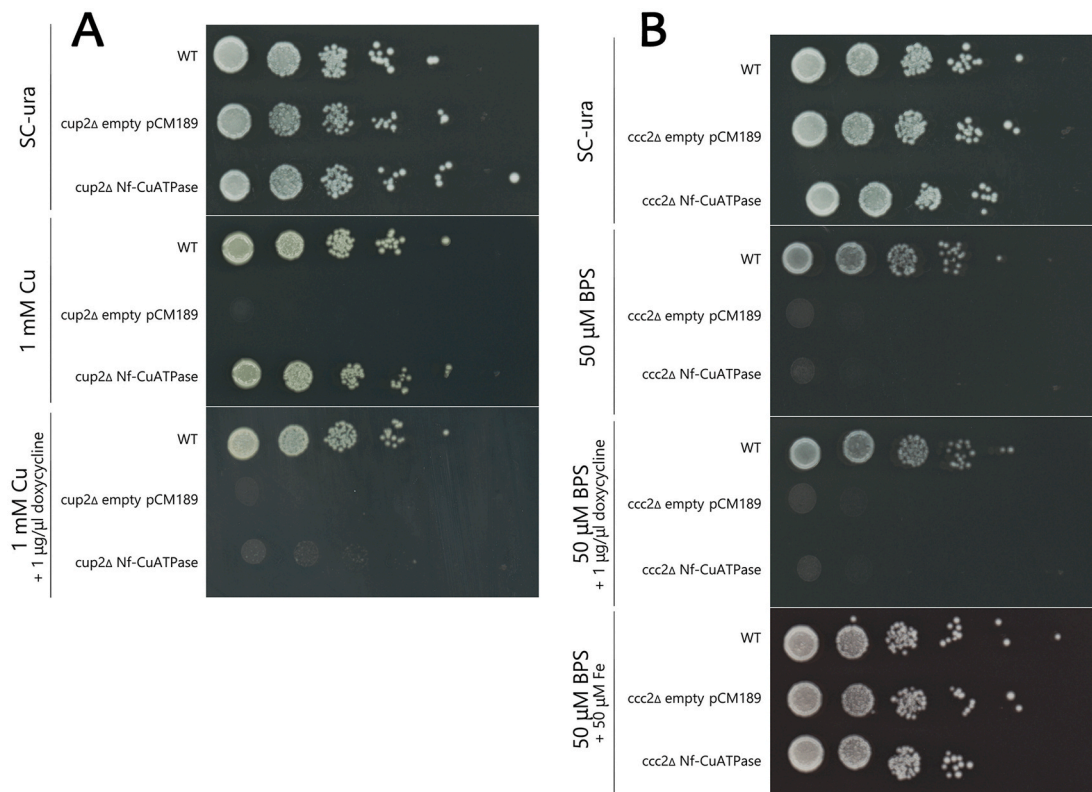

Fig. 4. Nf-CuATPase functional complementation of the *S. cerevisiae* cup2Δ copper sensitivity and inability to complement ccc2Δ defective growth on medium containing the iron chelator. Wild type strain BY4741 (WT) transformed with empty tetracycline-regulatable plasmid, cup2Δ and ccc2Δ mutant strains transformed with empty plasmid or plasmid carrying Nf-CuATPase were spotted on synthetic complete medium without uracil (SC-ura) and grown for 3 days at 30 °C. **A.** The cup2Δ mutant with empty vector failed to grow on SC-ura supplemented with 1 mM CuSO₄, while cup2Δ-expressing Nf-CuATPase grew normally. This effect was eliminated by doxycycline, which blocks transcription from the vector. **B.** Nf-CuATPase was unable to restore ccc2Δ mutant defective growth on medium containing the 50 μM iron chelator bathophenanthroline disulfonate (BPS). Iron supplementation restored the growth of the mutant.
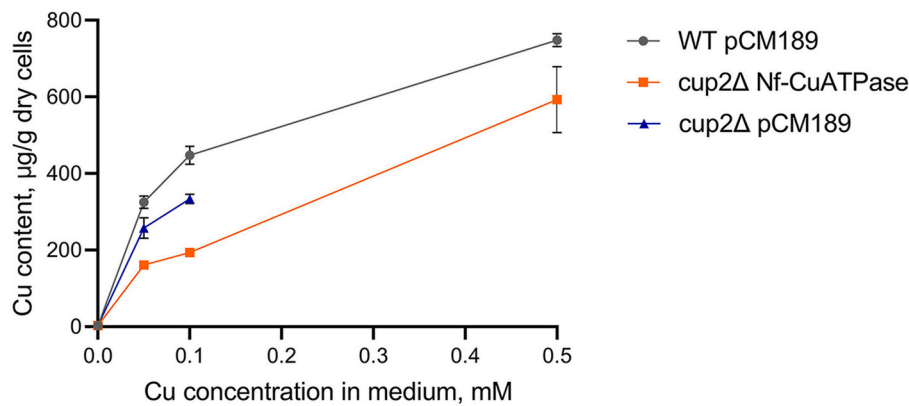
Figure 5. Copper content in *S. cerevisiae* cells grown under different copper concentrations. Wild type strain BY4741 (WT) transformed with empty plasmid (pCM189) and cup2Δ mutant strain transformed with the empty plasmid or the plasmid carrying Nf-CuATPase were grown overnight in liquid SC-ura medium supplemented with 0, 0.05, 0.1 or 0.5 mM CuSO$_4$ and analyzed for copper content. The amount of copper in wild type and mutant cells with empty plasmid was higher than in Nf-CuATPase-expressing mutant cells, suggesting that Nf-CuATPase exports copper. Cup2Δ with empty plasmid failed to grow at copper concentrations higher than 0.1 mM. Cells were grown in triplicate; data represent the mean with 95% confidence intervals.

virulence of many pathogenic species, and deletions of copper resistance-related genes result in severe virulence reduction (Hodgkinson and Petris, 2012). Resistance to copper is higher in pathogenic yeasts *C. albicans* and *C. neoformans* compared with nonpathogenic *S. cerevisiae*, suggesting that efficient copper detoxification is essential for occupation of niches within a host (Ding et al., 2013; Weissman et al., 2000).

In this study, we explored copper resistance of the human pathogenic protist *N. fowleri*. The half maximal inhibitory concentration (IC$_{50}$) of copper on *N. fowleri* was 1.62 mM (Fig. 1), which was lower than that obtained for *C. neoformans* but higher than that estimated for *S. cerevisiae* (although comparisons are difficult because the chemistry of metals may differ in different media even for the same species). The IC$_{50}$ of copper on *C. neoformans* was shown to be 2.3 mM (Ding et al., 2013), on *C. albicans* approximately 10 mM (Weissman et al., 2000), on *S. cerevisiae* approximately 1.1 mM and on *N. gruberi* (a close nonpathogenic relative of *N. fowleri*) approximately 1.05 mM (this work, Supplementary Fig. 2). Unlike *C. neoformans*, *N. fowleri* infection progresses from the primary site directly to the brain, a niche that is rather depleted of copper bioavailable to pathogens: experimental evidence showing the upregulation of high-affinity copper importers in *C. neoformans* during brain infection is in agreement with the reduced copper availability in the brain (Smith et al., 2017). Thus, it is questionable whether such a high copper tolerance plays a role in the pathogenicity of *N. fowleri*. Conversely, we cannot exclude the possibility that the parasite faces high copper concentrations during immune response in later stages of the disease.

Nevertheless, high copper tolerance may be beneficial for *N. fowleri* in its natural habitats. The levels of biologically available copper in the environment are rising as a result of anthropogenic activities (Flemming and Trevors, 1989). Elevated copper levels have been widely documented in surface waters and especially sediments which are the natural habitat of *N. fowleri* (Mull et al., 2013; Sykora et al., 1983). The addition of copper changes sediment microbial communities structure and modifies the levels of biological processes (Ahmed et al., 2018). Organisms that are highly tolerant to elevated copper, including *N. fowleri*, may thrive in such environments and become more prevalent.

Copper toxicity is exploited not only by the immune system but also in chemotherapeutic interventions. The lipophilic substances that reversibly bind ions and transfer them across the cell membrane, called ionophores, show anticancer and antimicrobial activity (Ding and Lind, 2009; Helsel et al., 2017). Several ionophores, such as 8-hydroxyquinoline (8-HQ), pyrithione (PyS), thiosemicarbazones and others, are known to increase the copper content in cells (Helsel et al., 2017; Reeder et al., 2011). The mechanism of ionophore action is not completely understood.

Ionophores 8-HQ, PyS, disulfiram (DSF) and thiomaltol have been shown to have strong fungicidal activity against *C. neoformans* in the

presence of copper (Helsel et al., 2017). Also, derivatives of 8-HQ have been tested as potential drugs for Alzheimer disease: *in vitro* assays, experiments with transgenic mice and pilot phase clinical trials demonstrated their neuroprotective effects and high blood brain barrier permeability (Adlard et al., 2008; Song et al., 2015; Yang et al., 2018). Since *N. fowleri* is a parasite occupying brain, we proposed that some of the copper ionophores may be repurposed as potential agents against primary amoebic meningoencephalitis if they show strong antiamoebic properties, although investigation of mechanism of action and cellular targets is needed.

In our study, we tested the effect of 8-HQ, PyS and DSF on *N. fowleri* in the absence and presence of copper (Fig. 1). All three compounds demonstrated copper-dependent amoebicidal activity with the lowest IC$_{50}$ for PyS (1.05 μM) and slightly higher IC$_{50}$ for 8-HQ and DSF (~2.1 μM), representing ionophore antimicrobial efficiency similar to that observed against *C. neoformans* (Helsel et al., 2017). We believe that these results are promising, especially considering the possibility of employing more effective ionophore derivatives. A remarkable approach is the use of inactive prochelator which is converted to 8-HQ via reactive oxygen species produced by the immune system, a strategy shown to be effective *in vivo* against *C. neoformans* (Festa et al., 2014). Nevertheless, it is important to understand the mechanism of action and cellular targets of ionophores in *N. fowleri*. Since the antiamoebic properties of these ionophores are copper-mediated, we studied the mechanisms generally exploited by *N. fowleri* for copper detoxification.

First, we studied the copper content in *N. fowleri* cells grown in different copper concentrations. The amount of copper in the amoebae under normal conditions was 84.10 ± 17.85 ng per mg dry mass. The addition of copper to growth medium up to 1 mM concentrations did not affect the growth of amoebae, although the amount of copper inside the cells significantly increased: 100 μM and 1 mM copper in the medium increased the copper content in the amoebae to 295.90 ± 61.09 and 369.21 ± 17.70 ng/mg, respectively (Fig. 2). Further, we determined the copper content in the cells grown with the addition of the ionophore 8-HQ in the absence or presence of 100 μM CuSO$_4$ (Fig. 2). The amount of copper in the cells grown in 100 μM CuSO$_4$ with 8-HQ was the same as in the cells grown in the same copper concentration without the ionophore. Thus, the addition of 8-HQ did not increase the amount of copper in the amoebae. Presumably, this ionophore rather promoted copper toxicity against the amoebae than mediated killing of the cells by increasing intracellular copper concentration.

To inspect the proteins involved in copper detoxification, we analyzed the whole-cell proteomes of amoebae grown in media containing different concentrations of CuSO$_4$. The most upregulated protein NF0109980 (11.2-fold change in 1 mM CuSO4) showed homology to an unknown protein from another species of the same genus, *N. gruberi,* but not to any other known proteins. The protein is rather short: it contains

101 amino acid residues, containing 6 histidines, including H-(X)7-H and H-(X)12-H motifs, and 4 cysteine-proline motifs, which might indicate that it binds copper (Kung et al., 2006; Wu et al., 2010) or interacts with heme (Ogawa et al., 2001; Shimizu, 2012; Zhang and Guarente, 1995).

The second most upregulated protein (NF0013290) was a probable methanethiol oxidase: this enzyme oxidizes methanethiol to formaldehyde and hydrogen sulfide. Its bacterial homolog was recently shown to be copper-dependent, and the enzyme is widely distributed among bacteria from different environmental samples and participates in global marine carbon and sulfur cycling (Eyice et al., 2018). However, the role of methanethiol oxidase in *N. fowleri* metabolism is obscure. Upregulation of this enzyme may demonstrate an increased turnover of cellular sulfur-containing compounds and mobilization of available sulfur sources for the synthesis of protective compounds such as glutathione. Glutathione balance is highly important for copper homeostasis. It effectively buffers copper and reduces its toxicity.

The importance of glutathione and thiol groups for *N. fowleri* subjected to high copper concentrations is emphasized by upregulation of three probable GSTs, thioredoxin reductase and sulfiredoxin. *i/*GSTs are known to participate in detoxification processes conjugating glutathione to different compounds (Vuilleumier and Pagni, 2002). The data regarding the influence of copper on GSTs are controversial; however, there is evidence that GSTs may be inhibited by increased copper concentrations (Cunha et al., 2007; Letelier et al., 2006; Salazar-Medina et al., 2010). Additionally, certain GSTs are upregulated at the transcriptional level under copper toxicity (Rhee et al., 2007). Thus, upregulation of GST expression may compensate for its reduced enzymatic activity. *ii/*Thioredoxin reductase and thioredoxin comprise the major cellular disulfide reducing system. Thioredoxins are multifunctional dithiol-containing proteins: they are used as a substrate for reductive enzymes, or function as protein disulfide oxidoreductases, or regulate the enzymes or receptors (Holmgren, 1985). Thioredoxin reductase is an enzyme that specifically reduces the disulfide bond in the active site of oxidized thioredoxin (Arnér and Holmgren, 2000). The thioredoxin reductase/thioredoxin system regenerates the proteins inactivated by oxidative stress (Fernando et al., 1992; Holmgren and Lu, 2010). *iii/*-Sulfiredoxin is an antioxidant protein that reduces sulfinic acid to cysteine residues (Biteau et al., 2003). Sulfiredoxin and subsequent thioredoxin action regenerate thiol groups in various proteins. Another function of sulfiredoxin is to deglutathionylate proteins. Glutathionylation occurs under oxidative stress conditions when a glutathione thiol radical reacts with a thiol group of protein forming a disulfide bond, and sulfiredoxin reverses this process (Findlay et al., 2006).

The upregulation of these proteins indicates that the thiol-reducing system is of particular importance under copper overload and assumes that copper causes oxidative stress, either directly or indirectly.

Interestingly, a hemerythrin-like protein was highly upregulated (4.3-fold) under copper overload. Hemerythrins are ancient iron-containing oxygen-binding respiratory proteins of some marine invertebrates (Bailly et al., 2008). Hemerythrin-like proteins are also found in bacteria, archaea and some eukaryotic groups including humans; however, their functions are not clear. They are proposed to be oxygen or iron sensors, oxygen carriers, or to act as protectors under oxidative stress conditions (Alvarez-Carreño et al., 2018). Also, among the proteins that were considerably upregulated under excessive copper we found two homologs of a heme-containing protein, protoglobin. Protoglobins are ancient archaeal and bacterial proteins that can bind O_2, CO and NO. Their functions remain unknown, although they have been proposed to participate in CO metabolism for methanogenesis or in scavenging reactive nitrogen and oxygen species, or to act as oxygen sensors (Pesce et al., 2013). The upregulation of hemerythrin and protoglobin in copper-overloaded *N. fowleri* cells is intriguing and may indicate their protective role against oxidative stress. Macomber and Imlay (2009) proposed that free iron is released from Fe–S clusters due

to the action of copper. We believe that hemerythrin may participate in iron buffering to prevent the Fenton reaction. This hypothesis is supported by the finding that hemerythrin is dramatically regulated by iron availability in both *N. fowleri* and *N. gruberi* (Arbon et al., 2020; Mach et al., 2018). However, both hemerythrin and protoglobin of *N. fowleri* failed to functionally complement the cup2Δ yeast mutant (data not shown), probably due to the lack of some redox partner or other interacting protein in the yeast cell. Thus, the exact function of hemerythrin and protoglobin in *Naegleria* metal homeostasis and/or oxidative stress response remains to be elucidated.

Importantly, the proteomic analysis revealed the upregulation of a P-type copper translocating ATPase (Nf-CuATPase), a protein homologous to CaCrp1 of *C. albicans* as well as human ATP7A and ATP7B and *S. cerevisiae* CCC2. CaCrp1 is localized to the plasma membrane and extrudes copper into the environment, thus functioning as the main copper detoxification system in *C. albicans* (Weissman et al., 2000). Detoxification of copper by the efflux pump was also demonstrated for *A. fumigatus* (Wiemann et al., 2017) and *P. falciparum* (Rasoloson et al., 2004). However, *S. cerevisiae* CCC2 is localized to the Golgi apparatus and is not employed for copper detoxification (Yuan et al., 1997). Human ATP7A and ATP7B are normally localized to the Golgi network and participate in copper trafficking but not detoxification (Lutsenko et al., 2007). Additionally, a copper translocating ATPase was recently demonstrated to be expressed in *T. brucei brucei*, although its detoxification or trafficking function has not been confirmed (Isah et al., 2020).

Bioinformatics analysis of the Nf-CuATPase sequence predicted a topology typical of copper-translocating ATPases and indicated the presence of all the sites required for functional ATP-dependent copper transport, including a cysteine-containing binding site, conserved phosphorylation and ATP-binding sites, among others (Fig. 3, Fig. S3). Due to the lack of established protocols for *N. fowleri* transfection or knockout, we employed a yeast model to investigate the mechanism of Nf-CuATPase copper detoxification. To test whether Nf-CuATPase extrudes copper from the cytosol to the environment or actions in the trans-Golgi compartment, we cloned Nf-CuATPase into a yeast expression vector and tested its functional complementation in two *S. cerevisiae* mutants, cup2Δ and ccc2Δ. The first one failed to express metallothioneins for copper detoxification, and the second one lacked Golgi-localized ATPase CCC2. Nf-CuATPase allowed the cup2Δ strain to grow in high copper concentrations, but it was unable to functionally complement ccc2Δ (similarly to copper-transporting ATPase of *C. albicans* CaCrp1 (Weissman et al., 2000),), indicating that it did not translocate copper into trans-Golgi vesicles but rather functioned in a copper toxicity resistance mechanism. Thus, it might be localized to the plasma membrane, as observed for *C. albicans* CaCrp1, or to some subcellular compartment such as the vacuole, shown to play a role in maintaining copper homeostasis in *S. cerevisiae* (Szczypka et al., 1997). We were not able to establish the localization of Nf-CuATPase using the GFP fluorescent tag or hemagglutinin epitope tag since the fusion proteins did not complement the cup2Δ mutant copper sensitivity, possibly due to mislocalization or loss of function caused by the fusions. However, we found that Nf-CuATPase-expressing cup2Δ yeasts grown in media with copper overload accumulated less copper than wild type cells and cup2Δ cells with an empty plasmid. These results indicated that Nf-CuATPase transported copper outside the cell rather than compartmentalizing it within the cell. Thus, it is more probable that Nf-CuATPase localizes to the plasma membrane and mediates copper efflux, akin to *C. albicans* CaCrp1, although more studies will be needed to verify the localization of this protein once appropriate antibodies or effective transfection system for *N. fowleri* are available.

We observed no significant increase of the copper content in the cells grown with 1 mM extracellular copper compared to the cells grown with 100 μM copper, which could be the effect of either upregulation of copper export or the restriction of copper import. It is known that *C. albicans*, *C. neoformans* and *A. fumigatus* downregulate copper importers under copper overload (Ding et al., 2011; Mackie et al., 2016;

Wiemann et al., 2017). Although the *N. fowleri* genome contains two CTR homologs that might function in copper influx, RT-qPCR data demonstrated that their expression was not significantly changed at the transcriptional level. Apparently, *N. fowleri* relies more on the removal of excessive copper by Nf-CuATPase than on copper influx restriction.

In conclusion, our data demonstrate that *N. fowleri* employs a range of different strategies to ensure its high copper tolerance, including copper efflux and production of antioxidant enzymes and obscure hemerythrin-like and protoglobin-like proteins. This capability can be advantageous for amoebae both in the natural environment and in the host. Nevertheless, copper metabolism may be considered a therapeutic target since copper ionophores have been demonstrated to be potent antiamoebic agents.

Declaration of competing interest

The authors declare that they have no conflicts of interests.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2020.10.001.

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