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A functional screen for copper homeostasis genes identifies a pharmacologically tractable cellular system

Ulrich Schlecht, Sundari Suresh, Weihong Xu, Ana Maria Aparicio, Angela Chu, Michael J Proctor, Ronald W Davis, Curt Scharfe and Robert P St.Onge*

Abstract

Background: Copper is essential for the survival of aerobic organisms. If copper is not properly regulated in the body however, it can be extremely cytotoxic and genetic mutations that compromise copper homeostasis result in severe clinical phenotypes. Understanding how cells maintain optimal copper levels is therefore highly relevant to human health.

Results: We found that addition of copper (Cu) to culture medium leads to increased respiratory growth of yeast, a phenotype which we then systematically and quantitatively measured in 5050 homozygous diploid deletion strains. Cu's positive effect on respiratory growth was quantitatively reduced in deletion strains representing 73 different genes, the function of which identify increased iron uptake as a cause of the increase in growth rate. Conversely, these effects were enhanced in strains representing 93 genes. Many of these strains exhibited respiratory defects that were specifically rescued by supplementing the growth medium with Cu. Among the genes identified are known and direct regulators of copper homeostasis, genes required to maintain low vacuolar pH, and genes where evidence supporting a functional link with Cu has been heretofore lacking. Roughly half of the genes are conserved in man, and several of these are associated with Mendelian disorders, including the Cu-imbalance syndromes Menkes and Wilson's disease. We additionally demonstrate that pharmacological agents, including the approved drug disulfiram, can rescue Cu-deficiencies of both environmental and genetic origin.

Conclusions: A functional screen in yeast has expanded the list of genes required for Cu-dependent fitness, revealing a complex cellular system with implications for human health. Respiratory fitness defects arising from perturbations in this system can be corrected with pharmacological agents that increase intracellular copper concentrations.

Keywords: Copper, Iron, Yeast, Functional Genomics, Disulfiram, Elesclomol, Menkes, Wilson's, Vacuole

Background

Copper is an essential element for most living organisms. Its incorporation into specific enzymes is required for catalysis of several vital, and highly conserved cellular processes [1]. For example, copper binding to specific sites in cytochrome c oxidase (*i.e.* complex IV of the electron transport chain) is required for complex assembly and stability, electron transfer activity, and ultimately respiratory metabolism [2]. Copper metallation also has important roles in responding to oxidative stress, by activating superoxide dismutase (SOD1) [3,4], and in regulating iron import by activating ferroxidases, which oxidize substrates for iron transporters [5-7].

The unique redox chemistry of copper ions, which allows it to exist in an oxidized (Cu2+) or reduced (Cu1+) state, is key to its catalytic properties but also contributes to its production of reactive oxygen species (ROS). To keep this deleterious capacity in check, the cell has evolved mechanisms to maintain tight regulatory control of copper storage and transport [1]. Metallochaperones protect the cell from copper toxicity by binding to free copper and facilitating its transport to, and incorporation into, important target proteins [8]. The yeast cytosolic

* Correspondence: bstonge@stanford.edu
Stanford Genome Technology Center, Department of Biochemistry, Stanford University, 855 S California Avenue, Palo Alto, CA 94304, USA

chaperone Atx1 for example, delivers copper to the Ccc2 ATPase, which transports copper into the Golgi where it activates the Fet3 ferrooxidase, ultimately leading to iron uptake.

The importance of maintaining proper copper homeostasis is underscored by several rare, yet severe, genetic diseases. Wilson's disease is an autosomal recessive disorder resulting from mutations in the copper transporter ATP7B, the human ortholog of yeast *CCC2*. Patients express a variety of neurological, psychiatric, and hepatic problems arising from abnormal copper accumulation, primarily in the brain and liver [9]. Mutations in the related gene ATP7A cause the X-linked recessive disorder Menkes disease [10]. Notably, the clinical symptoms of this disease arise from copper deficiency, and include neurological defects (mental retardation, seizures), growth retardation, hypothermia and "kinky" or "steely" hair [11]. Mitochondrial complex IV disorders, including Leigh syndrome, have also been linked to genes with important roles in copper metabolism, including SCO1, SCO2, and SURF-1 [12-19]. Abnormal copper concentrations have also been observed in Alzheimer's and Huntington's disease [20-22], and copper has been shown to promote aggregation of the α-synuclein protein, a hallmark of Parkinson's disease [23].

Yeast has been an exceptionally useful model organism for deciphering the cellular mechanisms regulating copper homeostasis [24,25], and indeed yeast orthologs exist for most of the disease genes described above. Here, we leverage the dependency between respiratory growth rate and copper availability to conduct a systematic screen for genes associated with copper homeostasis. This screen identified genes with known roles in copper regulation, and those with no previously identified functional association with copper. Consistent with the vacuole being a cellular store for copper, many of the genes identified are required for vacuole acidification, and we go on to show that copper is in fact limiting under conditions of vacuole stress. Several genes were also orthologs of human disease genes that have not been previously linked to copper imbalance. Finally, we demonstrate that the approved drug disulfiram (DSF), and the experimental drug elesclomol (ES), can rescue respiratory growth defects arising from copper deficiency.

Results and discussion

Respiratory fitness and copper

Though yeast preferentially ferment glucose for energy, they are able to switch to oxidative phosphorylation in the absence of a fermentable carbon source [26,27]. This attribute, in addition to its simple growth requirements, makes yeast a powerful model system for identifying chemical probes targeting energy metabolism [28]. To identify chemical compounds that 'boost' respiratory growth, and potentially serve as leads for human mitochondrial diseases, we conducted a phenotypic screen of ~3000 natural product derivatives (TimTec) for compounds that accelerated growth of respiring yeast (grown in Yeast Extract, Peptone, Ethanol, Glycerol medium; YPEG), but not fermenting yeast (grown in Yeast Extract, Peptone, Dextrose medium; YPD). This screen identified a series of structurally related compounds with the desired effect, but we determined that this effect was attributed to the $CuCl_2$ salt with which each compound was supplied (Additional file 1: Figure S1A and S1B).

To further examine the effect of copper ions on respiratory growth, yeast (BY4743) was grown in YP media containing the non-fermentable carbon source ethanol (YPE), at various concentrations of copper sulfate ($CuSO_4$). Growth of 100 μl cultures was monitored in 96well plates by measuring the increase in absorbance over time (see Methods). Supplemental copper concentrations as low as 10 nM were found to boost growth (Figure 1A and B). This boost was dose-dependent, but plateaued at 2 μM, indicating a saturation of copper above this concentration. In contrast, no such boost was observed in dextrose-containing media (YPD) (Figure 1B). This positive effect of Cu was further confirmed to be specific to respiratory growth conditions by comparing growth in other fermentable (galactose) and non-fermentable (glycerol and lactate) carbon sources (Additional file 1: Figure S1C). Similar results were obtained using spot assays on agar plates. When respiring yeast were pre-grown in the presence of copper-supplemented media, no significant increase in the number of colony forming units was observed, however, the size of each colony was substantially increased (Additional file 1: Figure S1D). From this we conclude that copper (at the concentration tested) does not dramatically influence yeast viability, but rather, increases growth under respiratory conditions.

We also tested the effect of copper depletion on respiratory growth, by culturing yeast in increasing concentrations (1 – 1000 μM) of the copper chelator, bathocuproine disulphonic acid (BCS). A significant dose-dependent reduction in growth was observed at BCS concentrations as low as 1 μM, and growth was strongly inhibited at BCS concentrations higher than 100 μM (Figure 1A and C). This inhibitory effect, however, was attenuated by addition of $CuSO_4$ to the growth medium (Figure 1A). For example, the addition of 500 μM $CuSO_4$ completely suppressed the inhibitory effects of BCS. Similar experiments using YPD media showed that addition of BCS does not alter fermentative growth (Figure 1C). Collectively, these results demonstrate a critical dependency between respiratory growth and copper ion concentrations in the growth medium.

A screen for copper homeostasis genes

To better understand the underlying cellular mechanisms controlling copper-dependent respiratory growth, we used

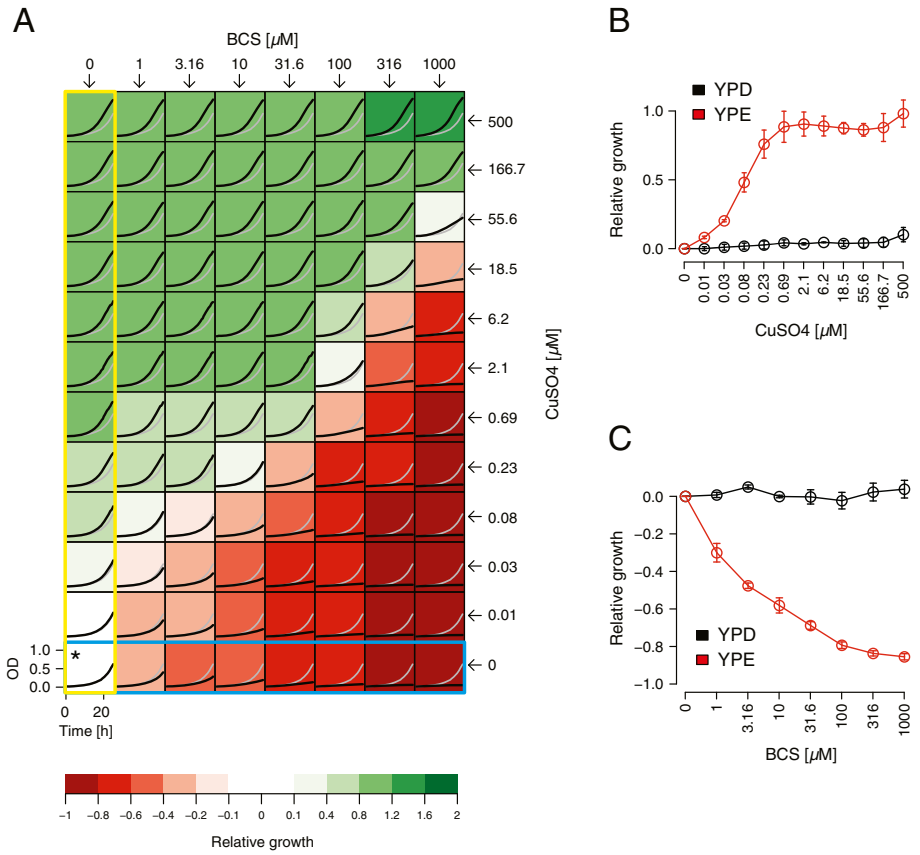

Figure 1 Media copper concentrations influence respiratory, but not fermentative, growth in yeast. (A) Dose–response matrix measuring the combinatorial effects of copper sulfate ($CuSO_4$) and bathocuproine disulphonic acid (BCS, a copper chelator) on respiratory growth. Yeast (BY4743) was grown under respiring conditions (YPE media) in increasing concentrations of $CuSO_4$ (see right) and BCS (see top). Optical density (*i.e.* OD_{600}, y-axis) of each culture is plotted over time (x-axis). Each condition is colored based on growth relative to the untreated condition (*i.e.* no BCS and no $CuSO_4$ added; marked with an asterisk and represented by the grey curve). Green and red indicate increased and decreased growth relative to this reference, respectively (see legend below). Growth data highlighted in yellow and blue were used in **(B)** and **(C)**, respectively. **(B)** Dose–response curves illustrating the effect of $CuSO_4$ (x-axis) on yeast growth. Growth relative to that in the absence of $CuSO_4$ is plotted on the y-axis, and was measured in both fermenting (YPD) and respiring (YPE) conditions (indicated in black and red, respectively). The mean of three replicates is plotted; error bars represent the standard deviation. **(C)** Similar to **(B)** but measuring the effects of BCS (x-axis).

the yeast homozygous gene deletion collection, in which individual diploid strains have both copies of a non-essential gene deleted. This collection has been a valuable resource for genome-scale analysis of conditions affecting growth [29-31]. Each strain contains unique 20-bp "barcode" sequences flanked by common priming sites, allowing the relative abundance of individual strains to be quantitatively monitored within a pool of competitively-grown strains [32,33]. Previous work has used this resource to identify genes that protect fermenting yeast from toxic concentrations of copper [34]. To identify genes that are important for Cu-dependent respiratory growth, we measured the fitness of 5050 homozygous deletion strains (Additional file 2: Table S1) in parallel in rich media (*i.e.* Yeast Extract, Peptone) using dextrose, ethanol, glycerol, or lactate as a carbon source, in the presence or absence of 500 μM $CuSO_4$ (a concentration that increases respiratory growth rate of the parental strain). Each condition was tested in triplicate. CEL files are available via the NCBI's Gene Expression Omnibus [35] under accession number GSE47175. Though it was not a goal of this study, examination of this dataset reveals genes that are specifically required for metabolizing different non-fermentable carbon sources. For example, the *fsh1Δ/fsh1Δ* deletion strain showed an ethanol-specific growth defect (Additional file 1: Figure S2). This suggests a role for *FSH1*, a putative serine hydrolase that has sequence-similarity to the human candidate tumor suppressor OVCA2 [36,37], in ethanol metabolism.

Importantly, these experiments identified a group of 313 respiratory-deficient strains (see Methods and Additional file 2: Table S1) that grew in YPD but were undetectable following competitive growth in media using non-fermentable carbon sources. As expected, the majority

(259; ~83%) of these strains were previously identified in a screen for mutants with impaired mitochondrial respiration [31]. Genes deleted in these strains were also enriched for the biological processes *mitochondrion organization* (*p-value*: 1.44e-99) and *cellular respiration* (*p-value*: 2.03e-22). We reasoned that the inability of these strains to compete with respiratory-proficient strains in our competitive assay may preclude accurate measurement of their response to Cu. Therefore, to better assay these strains, we assembled a smaller pool consisting only of these 313 strains, plus an additional 18 strains of interest (see Material and Methods), and interrogated this pool in YPE, in the presence or absence of 500 μM $CuSO_4$.

The effect of $CuSO_4$ on the collective growth of the pools was similar to that observed for BY4743 in Figure 1. To identify individual strains where this effect was altered, we compared the abundance of each barcode in the copper-treated samples to untreated samples, for both deletion pools, and for each non-fermentable carbon source (see Figure 2A). Using a stringent cutoff (log2 fold change > 1.5 and q-value < 0.05; see Methods), we identified 79 deletion strains in which Cu's positive effect on respiratory growth was quantitatively reduced, and 105 strains in which Cu's positive effects were substantively greater (compared to other strains in the pool) in at least one non-fermentable carbon source (Additional file 2: Table S1 and Additional file 1: Figure S3 and S4). The latter set was largely comprised of strains with respiratory growth defects that were specifically rescued by the addition of $CuSO_4$. However, the majority of strains with respiratory growth defects were not substantively aided by the addition of $CuSO_4$ (Additional file 1: Figure S5), underscoring that the copper-rescue effect was specific to the 105 strains identified.

We applied hierarchical clustering to the Cu-induced fitness changes observed in each of the four carbon sources for these 184 strains (Figure 2B). An examination of the clusters across the experiment axis showed that all replicate experiments correlated as nearest neighbors, confirming that the genome-wide data were highly reproducible. Samples grown in YPD clustered separately from the non-fermentable (YPE, YPG, and YPL) carbon-sources and in YPD, the fitness of most of these strains was not greatly affected by the addition of $CuSO_4$. This is consistent with copper affecting respiratory, but not fermentative growth at the concentration tested. While the results were generally consistent between the non-fermentable carbon sources, carbon source-specific effects were apparent as well. Most notably, growth in lactate identified many more strains exhibiting an increased Cu boost than did growth in ethanol or glycerol. This difference may be attributed to the higher respiratory fitness of many strains in YPL (due to the lower pH of this media; discussed further below) which facilitates their detection in our competitive assay. While this explanation attributes carbon-source specific results to technical limitations of the competitive growth assay (*i.e.* slow growing strains dropping out of the pool in YPE and YPG, but not YPL), it is also possible that mechanistic differences in the catabolism of ethanol, glycerol, and lactate result in discrete genetic dependencies for the copper response. Further experimentation will be needed to determine whether such cases do indeed exist.

To search for functional enrichment among the genes that were deleted in the 105 and 79 strains identified above, we used the Biological Networks Gene Ontology plugin (BiNGO) for Cytoscape (see Methods). The two gene lists contained 11 and 6 'dubious' open-reading frames, respectively, that partially overlapped with a verified gene. In several instances these strains confirmed the results for verified genes (such as the dubious ORFs *YDR203W* and *YDR455C* which overlap with *RAV2* and *NHX1*, respectively). One gene, *ADA2,* was represented twice in the deletion pool and both strains were identified in our screen. For our Gene Ontology (GO) analysis we used the non-redundant set of genes. The 73 genes that were required for Cu-dependent growth (*i.e.* whose deletion diminished the Cu-dependent growth boost), were significantly enriched for several GO categories, including *2-oxoglutarate metabolic process*, and *iron ion homeostasis* (Additional file 1: Figure S6 and Additional file 2: Table S2). Among the genes belonging to the latter term is *FET3*, which encodes the high-affinity iron uptake protein that links iron and copper homeostasis [39]. The dependence of copper's effects on Fet3 was verified in isogenic cultures, and in fact, the *fet3Δ*/*fet3Δ* strain exhibited reduced (not enhanced) growth in 500 μM $CuSO_4$ (Additional file 1: Figure S7A). Copper-limiting conditions are known to result in cellular iron deficiency [24,40], and we found that addition of high concentrations of $FeSO_4$ was itself sufficient to enhance respiratory growth of yeast (Additional file 1: Figure S7B). Therefore, to further explore the role of iron, we repeated the experiment described in Figure 1A in the presence of excess $FeSO_4$. Addition of 1 mM $FeSO_4$ was found to confer resistance to low concentrations (≤10 μM) of BCS, and to effectively mask the growth advantages imparted by $CuSO_4$ under these conditions (Additional file 1: Figure S7C). Collectively, these data suggest that increased iron uptake contributes to the respiratory growth increase arising from copper supplementation.

In contrast, the 93 genes in the enhanced growth group were significantly enriched for the GO processes *Golgi to vacuole transport* and *vacuolar acidification* (Figure 2C, Additional file 1: Figure S6 and Additional file 2: Table S3). Among this list of 93 genes are three genes that are directly linked to copper transport in yeast (*CTR1, ATX1* and *CCC2*) and in higher eukaryotes (*hCTR1/2, Atox1* and *Atp7A/B*). Deletion of these genes was previously shown to

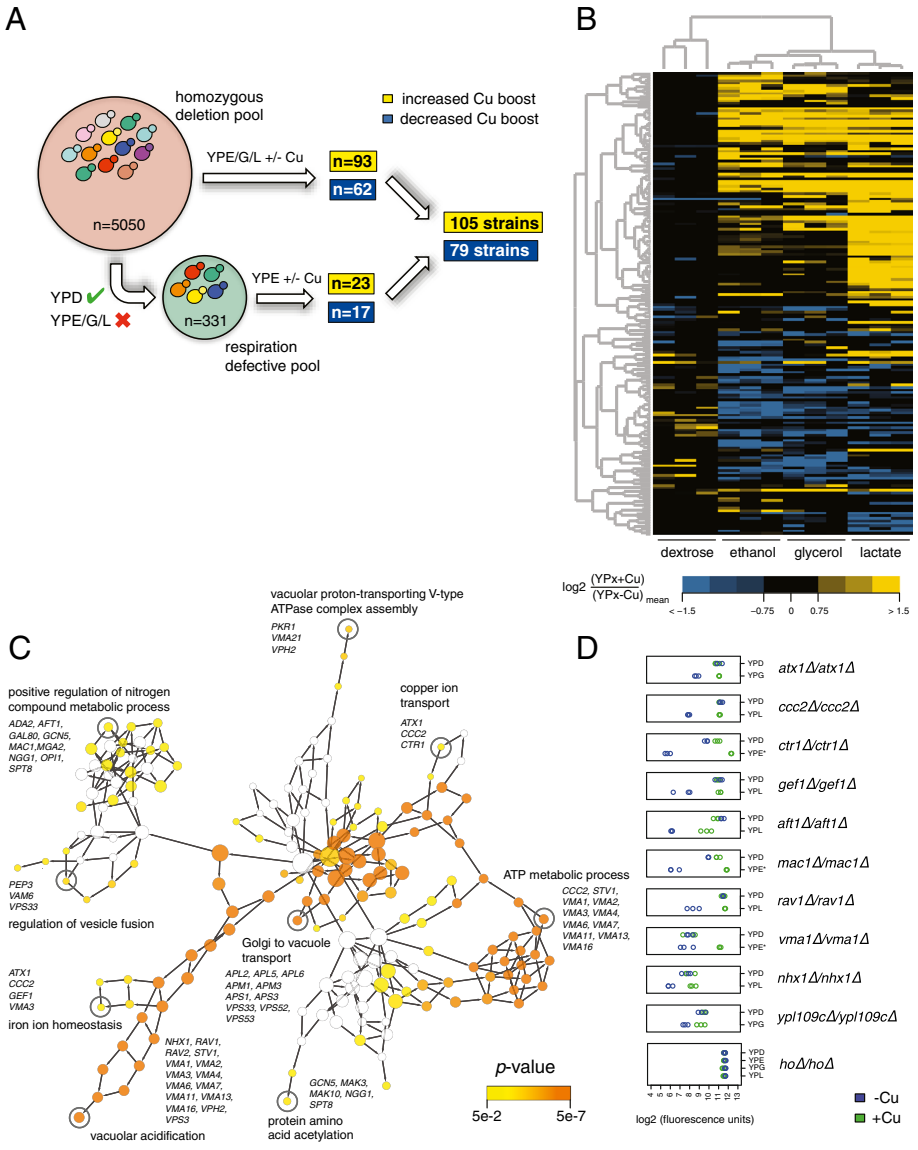

Figure 2 A systematic screen for genes involved in Cu-dependent growth. (A) Schematic of the experimental workflow. A complete pool of homozygous deletion mutants and a smaller pool of respiration deficient strains (see Methods) were grown under respiratory conditions in the presence and absence of $CuSO_4$. Comparison of $CuSO_4$-treated and -untreated samples, identified 105 and 79 strains where Cu's effect on growth was increased or decreased, respectively (see Methods). **(B)** Heatmap depicting log2 ratios of $CuSO_4$-treated and -untreated samples (see legend below) for the 184 strains identified in **(A)**. Yellow indicates greater Cu-dependent growth and blue indicates reduced Cu-dependent growth. Dendrograms depict the results from hierarchical clustering of genes (y-axis) and experiments (x-axis). **(C)** Network illustrating overrepresented Gene Ontology categories (nodes) among the 105 strains with an enhanced response to copper. This gene list was analyzed, and the network image created, using the BiNGO plugin for Cytoscape [38] (see Methods). Representative nodes are circled and labelled with those genes identified in the screen. **(D)** Dotplots of log2-transformed microarray fluorescence intensities for a select set of deletion strains. Triplicate data points for YPD and the non-fermentable carbon source in which the greatest copper response was observed are shown for copper-treated (green) and -untreated (blue) conditions. YPE, YPG, and YPL refer to data from the complete homozygous deletion pool experiment; YPE* refers to the respiration defective pool.

result in growth defects on low copper media [25]. The cytosolic copper metallochaperone Atx1 transports Cu ions from the high-affinity copper transporter Ctr1 in the plasma membrane to Ccc2 on post-Golgi vesicles to eventually target it to Fet3 on the cell surface [6,7,41,42]. Our screen also identified *GEF1*, a chloride channel involved in cation homeostasis. The Gef1 protein co-localizes with Ccc2 at late- and post-Golgi vesicles and is required for loading Cu ions onto Fet3 [43]. For other genes identified by our screen, evidence supporting a functional role in cellular copper homeostasis is either limited or lacking entirely. These include *YPL109C*, which encodes a protein of

unknown function that was detected in highly purified mitochondria [44,45]. Figure 2D shows quantile-normalized fluorescence values for several of the deletion strains specifically discussed herein. The complete results for all strains identified in our screen are shown in Additional file 1: Figure S3 and S4. Collectively, these data will be a rich source of information for future characterization of genes involved in copper homeostasis.

Previous work in yeast has demonstrated that mitochondria maintain a non-proteinaceous copper pool that exceeds CcO-associated copper concentrations [46]. It is thought that this pool may serve as a source of copper ions for chaperones that mediate metallation of CcO, which occurs at two CcO subunits encoded by the mitochondrial genome, Cox1 and Cox2 [8]. The metallochaperone Cox17 transfers copper to two mitochondrial inner membrane proteins Cox11 and Sco1 [47], which then mediate metallation of Cox1 and Cox2, respectively [8]. It is notable that our genomic screen did not identify the *cox17*, *cox11*, or *sco1* deletion strains as being rescued by copper. These results are consistent with previous analysis of *sco1* null strains [48], and of *cox17* null strains, whose respiratory growth defect was rescued by copper, but only at concentrations much higher than those used in the present study [49].

Vacuole acidity and copper homeostasis

The yeast vacuole, generally regarded as the functional equivalent of the mammalian lysosome, is an acidic organelle with a variety of important functions including: protein degradation, ion and small-molecule storage, and pH homeostasis [50,51]. Consistent with previous work demonstrating an important role for the vacuole in storage and mobilization of Cu ions [1,52-54], several genes that maintain vacuolar pH, when deleted, were found to produce respiratory growth defects that were rescued by copper. These included numerous subunits of the vacuolar H^+–ATPase (such as *VMA1, VMA2, VMA3, VMA4, VMA6, VMA7, VMA11, VMA13, VMA16,* and *STV1*) and 2 subunits of the RAVE complex (*RAV1* and *RAV2*) which promotes assembly of the V-ATPase holoenzyme. Mobilization of vacuolar copper is thought to involve the Ctr2 protein, a copper transporter that localizes to the vacuolar membranes of budding yeast [52,53], fission yeast [55], and mammalian cells [56]. Though a *ctr2Δ* mutant was not included in our deletion collection and thus not identified in our screen, we did identify the *CTR2*-regulating transcription factors *AFT1* and *MAC1* [57].

These results suggest that the pH gradient across the vacuolar membrane plays a critical role in maintaining optimal copper homeostasis. We therefore examined the effects of increasing media pH on the cellular response to copper (Figure 3A). Because alkaline growth conditions increase vacuolar pH more than cytosolic pH [58,59], they effectively reduce the pH gradient between the two cellular compartments. Consistent with previous findings on dextrose-containing media [60], we find that respiratory growth impairment by alkaline stress, is largely alleviated by supplementing the growth medium with $CuSO_4$ (Figure 3A and B). To more directly assess the role of vacuole acidity on copper homeostasis, we applied the small molecule Bafilomycin A1 (BafA), a natural product derived from *Streptomyces griseus* that specifically inhibits vacuolar H^+–ATPase [61]. BafA strongly inhibited growth of respiring yeast at sub-micromolar concentrations however this inhibition could be suppressed by supplementing the media with $CuSO_4$ (Figure 3C and D). These data further establish an important role for the vacuole in controlling cellular copper homeostasis, and moreover identify copper as a major limiting factor under vacuolar stress conditions. Notably, addition of high concentrations of $FeSO_4$ also partially suppressed growth inhibition by BafA and masked the effects of $CuSO_4$ under these conditions (Additional file 1: Figure S7D). This is consistent with Cu-limitation arising from vacuole deacidification causing a cellular iron deficiency that negatively impacts growth.

Human disease genes

Our functional screen was highly sensitive in detecting small Cu-dependent changes in cell fitness. Mutations in non-essential genes that yield subtle phenotypes may be more likely to cause disease, compared to for example, genes required for viability. Therefore, we next examined the genes we identified for sequence homology to human genes associated with disease (see Additional file 2: Table S4 for complete results). Table 1 lists those human disease genes with yeast orthologs, that when deleted, yield respiratory fitness-defects that are rescued by copper supplementation. The diseases associated with these genes include Menkes and Wilson's disease, both of which are caused by mutations in genes orthologous to yeast *CCC2*, and both of which are well established Cu-related disorders [9,11]. The other diseases in Table 1, typically present with neurologic, musculoskeletal and hematologic features, which show remarkable similarities to clinical phenotypes seen in patients with Menkes' copper deficiency [11]. Many of these disease genes have a role in intracellular trafficking, including several members of the AP-1 adaptor complex, an important component of clathrin-coated vesicles [62]. Interestingly, a combined zebrafish/yeast chemical-genetic screen has also identified this complex as having a conserved role in buffering the effects of copper limitation [63]. Knock-down experiments in human cell lines will determine whether these genes fulfill a similar function in man, and may prompt a closer examination of whether copper plays a role in the disorders listed in Table 1.

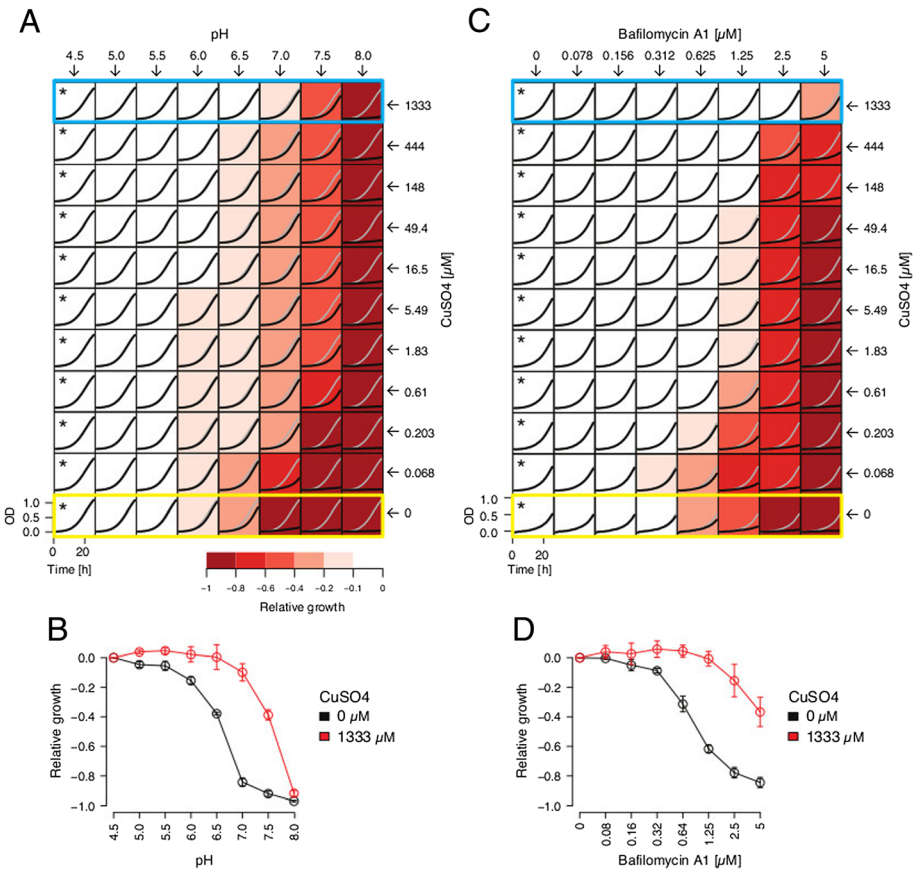

Figure 3 Vacuolar pH plays a critical role in maintaining copper homeostasis. (A) Dose–response matrix measuring the combinatorial effects of $CuSO_4$ and media pH on respiratory growth. BY4743 was grown in buffered YPE media of increasing pH (see top), in multiple concentrations of $CuSO_4$ (see right). Optical density (*i.e.* OD_{600}, y-axis) of each culture is plotted over time (x-axis). Samples are color-coded according to growth relative to that in pH 4.5 for each copper concentration (see asterisks and grey curves). Data in blue and yellow boxes were used for the dose–response curve in B. **(B)** Dose–response curves measuring the effect of increasing media pH (indicated on x-axis) on respiratory growth in the presence (red) or absence (black) of 1333 μM $CuSO_4$. Growth relative to that in pH 4.5 is plotted on the y-axis. The mean of three replicates is plotted; error bars represent the standard deviation. **(C and D)** as in **(A and B)**, only examining the combinatorial effects of $CuSO_4$ and bafilomycin A1 (a specific inhibitor of vacuolar H^+–ATPase) on growth in unbuffered YPE media.

Pharmacological rescue of copper deficiencies

Pharmacological modulation of Cu homeostasis is an attractive therapeutic strategy for a variety of clinical applications. Copper chelating agents are currently the standard treatment to ameliorate copper overloading of affected tissues in Wilson's disease [11], whereas early treatment of Menkes disease with copper has shown some promise in newborns having mutations that do not completely abrogate ATP7A [65]. Recent work in model systems has also demonstrated that host cell copper metabolism influences RNA virus replication [66], and leveraging copper's cytotoxic properties is also being explored for cancer therapy, and may be effective in targeted killing of tumor cells [67-72].

The ability of several compounds to increase copper concentrations in different cellular compartments was recently characterized [67]. We examined the effects of two of these compounds (disulfiram and elesclomol; Figure 4A), on respiratory growth in yeast. Consistent with our results in Figure 1, and with their ability to increase intracellular copper concentrations, both DSF and ES conferred significant resistance to the copper chelating agent BCS (Figure 4B). At the equivalent concentration (0.228 μM), ES was more effective than DSF in rescuing BCS-induced growth defects, an observation that is consistent with ES having a stronger effect on intracellular copper concentrations in human cell lines [67].

We next assessed the ability of DSF and ES to rescue the effects of genetically-induced copper deprivation. Our genomic screen identified the *ctr1Δ/ctr1Δ* strain, which lacks the primary copper transporter in yeast [42], as being most aided by supplemental copper among the >5000 strains tested (Figure 2D and Additional file 2: Table S1). We found that DSF rescued the severe respiratory defect of this strain in a dose-dependent manner, with the strongest effect observed at 0.468 μM DSF (Figure 4C).

Table 1 Human disease genes putatively associated with copper imbalance

Yeast gene	Human gene	% ident.	Subcellular localization	Biological function	OMIM	Clinical phenotype
CCC2	ATP7A	23	Golgi apparatus, Plasma membrane	Cu ion transport across membranes	309400	Menkes disease (copper deficiency)
	ATP7B	24	Golgi apparatus, Mitochondria		277900	Wilson disease (copper overload)
ADK1	AK2	54	Mitochondrial inter-membrane space	Energy and nucleotide metabolism	267500	Immunodeficiency, sensorineural deafness
COX12	COX6B1	42		Energy metabolism, respiratory chain complex	220110	Encephalopathy, growth retardation, vision loss
VMA2	ATP6V1B1	73	Endomembrane, plasma membrane	Vacuolar proton-translocating ATPase	267300	Renal tubular acidosis, sensorineural deafness
HFA1	ACACA	38	Mitochondria, cytoplasm	Fatty acid biosynthesis	613933	Encephalopathy, growth retardation, myopathy
GEF1	CLCN5	30	Endosome membrane, lysosomal membrane	Chloride channels and ion transporter	300009	Renal tubular disease,kidney stones
	CLCN7	20			166600	Osteosclerosis, multiple fractures, vision loss
VPS33	VPS33B	23		Protein transport, membrane fusion	208085	Arthrogryposis, renal dysfunction, cholestasis
NHX1	SLC9A9	27	Endosome membrane	pH regulation, ion transport	613410	Autism, seizures
	SLC9A6	26			300243	Mental retardation, seizures, ataxia
APS1	AP1S2	51	Golgi apparatus	AP-1 adaptor complex, protein transport, vesicular trafficking	300630	Mental retardation, cerebral calcifications
	AP4S1	26			614067	Spastic paraplegia, mental retardation
APL2	AP4B1	22			614066	
APM1	AP4M1	24			612936	
ARL1	ARL6	39		Protein transport, metal ion binding, membrane trafficking	209900	Mental retardation, obesity, retinopathy
ARL3	ARL13B	13			612291	Cerebral malformation, mental retardation
COG6	COG6	19		Oligomeric Golgi complex, vesicular transport	606977	Vitamin K deficiency, intracranial bleedings
APL6	AP3B1	20		AP-3 adaptor complex, protein transport	608233	Platelet defect, albinism, immunodeficiency
ERG24	DHCR7	27	Endoplasmatic reticulum	Cholesterol biosynthesis, sterol metabolism	270400	Mental retardation, congenital malformation
	LBR	27	Nuclear membrane		215140	Skeletal dysplasia, leukocyte disorder

Twenty-one human disease genes which have a yeast orthologue (% identity of human protein in column 3) that, when deleted, produced a respiratory fitness defect that was specifically rescued by copper supplementation. The gene products of the 21 disease genes have distinct functions and subcellular localizations [64], while mutations in these genes are typically associated with neurologic, musculoskeletal and hematologic disease phenotypes resembling those of copper deficiency (http://omim.org/).

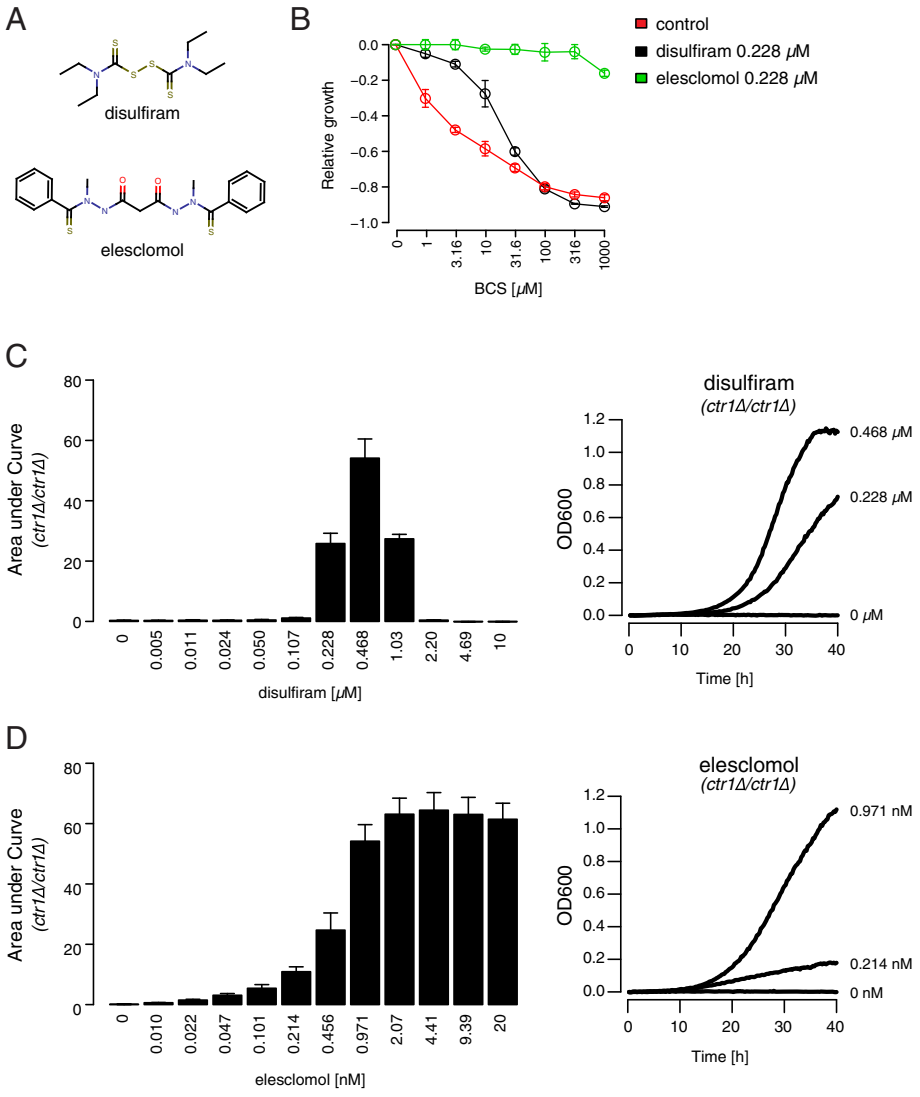

Figure 4 Pharmacological rescue of Cu-deficiency phenotypes. (A) Chemical structure of disulfiram (DSF) and elesclomol (ES). **(B)** Dose–response curves measuring the sensitivity of respiring yeast (BY4743 grown in YPE) to increasing concentrations of BCS (indicated on x-axis), in the presence of 0.228 μM DSF (black) or 0.228 μM ES (green). Growth relative to that in the absence of BCS is indicated by the y-axis. The mean of three replicates are plotted; error bars indicate the standard deviation. **(C)** Respiratory growth of the *ctr1*Δ/ctr1Δ strain in response to various concentrations of DSF (indicated on the x-axis). Growth after 40 hours in YPE was measured by area under the curve (y-axis). The mean of 3 replicates is plotted. Error bars indicate the standard deviation. Representative growth curves are shown on the right. **(D)** Similar to **(C)** only involving ES.

ES rescued growth of the *ctr1Δ*/*ctr1Δ* strain as well, and did so over a much broader concentration range, and at sub-nanomolar concentrations (Figure 4D). Comparable to the effects of Cu, both drugs also increased respiratory growth of the parental BY4743 strain (Additional file 1: Figure S8) suggesting that either drug could be used to correct a range of Cu-deficiencies. It is noteworthy that while DSF inhibited growth at higher concentrations, ES-induced growth inhibition was dependent on supplementing the media with Cu (Additional file 1: Figure S8), an observation that is consistent with previous findings [72]. When tested against several strains listed in Table 1, most strains were rescued by both ES and DSF, but interestingly, neither drug rescued the respiratory growth defects of the *gef1Δ*/*gef1Δ* or *ccc2Δ*/*ccc2Δ* strains (Additional file 1: Figure S9), which lack genes specifically required for loading Cu into Fet3. These data suggest that, unlike $CuSO_4$ added to the growth media, DSF- and ES-delivered copper require Gef1- and Ccc2-dependent Cu loading into Fet3 to confer enhanced growth.

ES is a promising anti-cancer agent that induces oxidative stress in a copper-dependent manner [67,72]. DSF has been used for the treatment of alcoholism for decades [73] and confers therapeutic value by inhibiting aldehyde

dehydrogenase (ALDH), which decreases the alcohol tolerance in patients [74]. DSF has also been shown to perturb vacuolar pH in yeast, and inhibit V-ATPase activity in cell-free assays [75]. Though our results clearly identify copper homeostasis as a rate-limiting effect of these drugs on respiratory growth, further experiments will be required to determine their precise mechanism-of-action. Nevertheless, our data highlight the ability of copper ionophores to potently and precisely tune cellular fitness under copper-limiting conditions. Leveraging the pharmacological tractability of copper homeostasis, though confronted with inherent cytoxicity obstacles, may warrant exploration as a therapeutic strategy for copper- or iron-deficiency in man.

Conclusions

A systematic screen of the yeast deletion collection was conducted to identify genes that have a role in Cu-dependent respiratory fitness. The screen revealed a complex cellular system involving many genes that were not previously associated with copper homeostasis. The results are consistent with copper limitation producing a secondary iron deficiency that negatively impacts respiratory growth, and with the vacuole playing a vital role in regulating intracellular copper levels. Roughly half of the genes identified in our screen are conserved in man, and many of these are associated with Mendelian diseases, underscoring the potential translation of our findings to human health. Pharmacological agents that increase intracellular copper concentrations can be effective in compensating for perturbations in copper homeostasis.

Methods

Reagents

Copper (II) sulfate, tetraethylthiuram disulfide (disulfiram), and bathocuproine disulfonic acid disodium salt (BCS) were purchased from Sigma-Aldrich. Elesclomol was purchased from Selleckchem and bafilomycin A1 was purchased from Enzo Life Sciences. Copper (II) sulfate was dissolved in water and stored at room temperature. BCS, elesclomol, bafilomycin A1, and disulfiram were dissolved in DMSO, aliquoted, and stored at −20°C until use. We used a HP D300 Liquid Dispenser (Tecan) for all experiments involving DMSO-dissolved compounds.

Media and growth rate analysis

All media was prepared using deionized water. Rich media (YP) consisted of 10 g yeast extract, 10 g bactopeptone per liter. For Figure 3 A and B the YP media was buffered with 25 mM HEPES and pH-balanced as indicated using 1 M NaOH or 12.1 M HCl, to raise or lower the pH, respectively. 25 mM HEPES was also included in the media used for Additional file 1: Figure S1, and we note that addition of the HEPES buffer reduced the respiratory growth rate of yeast. All other experiments were performed using YP media without HEPES. Pre-cultures of the isogenic strains were grown overnight to saturation in rich media supplemented with 2% dextrose. These cultures were then used to inoculate YP media supplemented with carbon sources at 2% as indicated in the Figure legends. Isogenic cultures (100 μl) were inoculated at a concentration of 0.03 OD_{600}/ml and grown in 96well microtiter plates at 30°C. The plates were sealed with adhesive plate seals (Thermo Scientific) and holes were poked in the seal for every well using a 21 gauge needle to allow sufficient aeration (cultures using non-fermentable carbon sources only). Optical density was measured every 15 min over the course of several hours (as indicated in graphs) using a GENios microplate reader (Tecan). The growth rate of a strain was calculated as follows: 1) the first 10 OD readings were averaged and subtracted from all OD readings of the corresponding curve in order to set the baseline of the growth curve to zero, 2) the area under the curve (AUC) was then calculated as the sum of all OD readings. In general, 50 and 100 reads (corresponding to 12.5 and 25 hours, respectively) were used for strains grown in fermentative conditions (dextrose and galactose) and in respiratory conditions (ethanol, glycerol, and lactate), respectively. 160 reads (40 hours) was used for Figures 4C and D (*ctr1Δ/ctr1Δ*). A "relative growth" value was calculated as previously described [76], as follows: $(AUC_{condition} - AUC_{control})/AUC_{control}$; where $AUC_{control}$ represents the growth rate of the reference condition (marked with an asterisk in all Figures) that was assayed on the same microtiter plate.

Deletion pool construction and growth conditions

The homozygous deletion pool was constructed as described [30] and stored in aliquots at −80°C. For all screening experiments, aliquots of the pool were thawed and diluted in YP media plus a carbon source (2% of dextrose, ethanol, glycerol or lactate) to a concentration of 0.04 OD_{600}/ml. 700 μl of this cell suspension was then aliquoted into every well of a 48well plate and copper sulfate was then added (from an 500 mM stock solution in water) to a final concentration of 500 μM. The plates were sealed with adhesive plate seals and holes were poked for every well to allow sufficient aeration. The plates were incubated at 30°C with vigorous shaking and optical density was measured every 15 min over the course of the experiment. Each sample was grown for precisely 9 generations. Cells were maintained in logarithmic phase by robotically diluting cultures every three doublings using a Packard Multiprobe II four-probe liquid-handling system (PerkinElmer, Wellesley, California, United States). Experiments were performed in triplicate. After reaching 9 pool generations 600 μl of the cultures were harvested, pelleted by centrifugation and snap frozen in liquid nitrogen. The pellets were kept

at −80°C until further processing (genomic DNA preparation, PCR, and microarray hybridization, see below).

To construct the pool of respiration deficient mutants we first analyzed samples from the YPD, YPE, YPG, and YPL experiments where no additional copper was added to the media. Strains with a median of log2-transformed raw fluorescence units < 7 in the combined YPE, YPG, and YPL samples and a median of log2-transformed raw fluorescence units > 8 in the YPD samples were selected. This identified 313 strains, to which we added an additional 18 strains of interest (Additional file 2: Table S1). The 331 strains were streaked from frozen stocks onto YPD agar plates, grown for 3 days at 30°C, and then pooled and stored in aliquots at −80°C. This pool was assayed as described above for the complete homozygous deletion pool.

Genomic DNA preparation, PCR, and microarray hybridization

Genomic DNA preparation, PCR amplification of molecular tags, and microarray hybridization were as described [77]. Genomic DNA was extracted from cell pellets using the YeaStar Genomic DNA kit from Zymo Research (D2002). The relative abundance of each strain was then assessed by amplifying and hybridizing their molecular barcodes to a Genflex Tag 16 k array (Affymetrix).

Identification of strains exhibiting increased or decreased Cu-dependent growth

To quantify Cu-dependent growth of each deletion strain, we calculated a "Copper Response Score" (CRS) for each strain in each of the four respiratory pool assays; *i.e.* the homozygous diploid (HD) deletion pool in ethanol-, glycerol-, or lactate-containing media (YPE, YPG, and YPL), and the respiratory-deficient (RD) deletion pool in ethanol-containing medium (YPE*). The majority of strains carry two tags that hybridize to the array, an "uptag" and a "downtag" [30]. From each array, we extracted the fluorescence intensity values for every uptag and downtag associated with the 5050 strains in the HD pool, or in the case of the RD pool, the up- and downtags associated with the 331 strains in that pool (Additional file 2: Table S1). In total, this amounted to 9937, and 675 tags, respectively. The number of tags is not exactly twice the number of strains because some strains contain only one tag. These raw fluorescence values were then log2-transformed, and quantile-normalized using the normalize.quantiles function of the preprocessCore package in R. Quantile normalization was performed on four separate groups of tags; uptags from the HD pool samples, downtags from the HD pool samples, uptags from the RD pool samples, and downtags from the RD pool samples. Eight experimental conditions (*i.e.* the four respiratory pool assays +/− $CuSO_4$) were performed in triplicate, and from this set of triplicate measurements, we calculated the mean of the normalized fluorescence value for every tag. For each of the four respiratory pool assays, the mean values from $CuSO_4$-untreated samples were then subtracted from the mean values of the $CuSO_4$-treated samples. This value is the CRS. In cases where a strain contained two tags (*i.e.* the vast majority of strains), the CRS was derived from that tag having the greatest difference between Cu-untreated and -treated samples (*i.e.* the maximum absolute value of the Δmeans for the uptag and downtag). A moderated t-statistic was used to define differential effects of Cu-treatment in the four respiratory pools using the R package LIMMA [78,79], and the derived p-values were further converted to q-values using Benjamini & Hochberg False Discovery Rate (FDR) correction [80]. 105 strains that exhibited a significant (q-value < 0.05) and large (CRS > 1.5) response to copper in at least one of the four respiratory pool assays (YPG, YPL, YPE, YPE*) were defined as having an increased Cu-boost, and 79 strains that had a significant (q-value < 0.05) and small (CRS < −1.5) response to copper in at least one of these assays were defined as having a decreased Cu-boost. All instances where these criteria are met are illustrated by the filled circles in Additional file 1: Figure S3 and S4. The complete list of CRSs is provided in Additional file 2: Table S1. CEL files are available via the NCBI's Gene Expression Omnibus [35] under accession number GSE47175.

Hierarchical clustering

For the cluster analysis in Figure 2B, we calculated the Cu-induced fold change of the 184 (79 + 105) strains identified above, in each of the 12 individual replicate experiments involving the HD pool (YPE, YPG, YPL, and YPD), and applied hierarchical clustering to these data. Cu-induced fold change was calculated as (log2 (YPx + Cu)/$(YPx\text{-}Cu)_{mean}$), where x is one of the 4 carbon sources (ethanol, glycerol, lactate, dextrose) tested. Fold changes of uptag and downtag were averaged. Clustering along both experiment and gene axes was performed on these values using the TIBCO Spotfire software platform.

GO term enrichment analysis

To identify enriched Gene Ontology terms among the genes that were identified in this study as enhancing or diminishing the response to copper we used the Biological Networks Gene Ontology plugin (BiNGO, version 2.4.4) for Cytoscape (version 2.8.3). Both gene lists (consisting of 93 and 73 open-reading frames, respectively), were analyzed separately using a Hypergeometric test with Benjamini & Hochberg False Discovery Rate (FDR) correction and a significance level of < 0.05. As a reference set we used the list of 4913 unique open-reading frames represented in the complete pool of 5050 homozygous deletion strains. We applied a force-directed layout to minimize the number of crossing edges and to

enhance readability of node labels. Nodes were colored based on the degree of significance of overrepresentation. Uncolored nodes are not overrepresented, but they are the parents of overrepresented categories further down. The yellow and orange nodes represent terms with significant enrichment, with darker orange representing a higher degree of significance, as shown by the color legend panel in Figures 2C and Additional file 1: Figure S6. The size of each node is proportional to the number of genes with that term in the query set.

Availability of supporting data

The array data supporting the results of this article have been deposited in NCBI's Gene Expression Omnibus [35] and are accessible through GEO Series accession number GSE47175 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47175).

Additional files

Additional file 1: Figures S1. Effect of copper in media containing different carbon sources, **Figures S2.** Deletion pool results for the *fsh1*Δ/*fsh1*Δ deletion strain in different carbon sources, **Figures S3.** Deletion pool results for 105 strains in which the beneficial effects of $CuSO_4$ were greater compared to other strains in the pool, **Figures S4.** Deletion pool results for 79 strains in which the beneficial effects of $CuSO_4$ were smaller than other strains in the pool. **Figures S5.** Relationship between Copper Response Score and respiratory fitness, **Figures S6.** Network representation of Gene Ontology (GO) categories that are significantly over-represented among strains exhibiting diminished or enhanced Cu-dependent growth, **Figures S7.** Effects of $FeSO_4$ on Cu-dependent growth, **Figures S8.** Growth kinetics of elesclomol and disulfiram-treated cultures in the presence and absence of high concentrations of $CuSO_4$, **Figures S9.** Respiratory growth of eleven deletion strains listed in Table 1, in $CuSO_4$, disulfiram, or elesclomol.

Additional file 2: Tables S1. A summary of the strains and their Copper Response Scores, **Tables S2.** Significantly enriched Gene Ontology categories among the 79 strains in which Cu's positive effect on respiratory growth was quantitatively reduced, **Tables S3.** Significantly enriched Gene Ontology categories among the 105 strains in which Cu's positive effects were substantively greater than other strains in the pool, **Tables S4.** 151 human orthologs of 81 yeast genes identified in this study.

Abbreviations

DSF: Disulfiram; ES: Elesclomol; BCS: Bathocuproine disulphonic acid; Cu: Copper; YPD: Yeast extract, peptone, dextrose; YPE: Yeast extract, peptone, ethanol; YPG: Yeast extract, peptone, glycerol; YPL: Yeast extract, peptone, lactate; AUC: Area under curve; BafA: Bafilomycin A; CRS: Copper response score.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

US, CS, and RPS conceived and designed the experiments, analyzed the data, and wrote the paper; WX analyzed the data; RWD provided insight and advice; MJP developed the robotics for deletion pool screening; US, SS, AMA, AC, and RPS performed experiments. All authors read and approved the final manuscript.

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