Research Square

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Yhk8 as a genomic modifier of copper susceptibility in yeast

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Research Article

Keywords: copper, modifier genes, QTL mapping, yeast, Yhk8, toxicity

Posted Date: July 29th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10451293/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BioMetals on November 6th, 2026. See the published version at https://doi.org/10.1007/s10534-026-00905-8.

Abstract

Copper is essential for life as an enzymatic cofactor and antioxidant participant, but in excess, it is toxic to all living organisms, including yeast. Because this toxicity varies by genetic background, we investigated the underlying modifier genes using a panel of four genetically diverse strains—Sake (SA), North American (NA), West African (WA), and Wine/European (WE)—and their segregants. Copper sulfate ($CuSO_4$) susceptibility, quantified by the maximum growth rate (μMax), was strain-dependent, with resistance decreasing in the order SA > WE > WA > NA. Furthermore, cellular growth was inversely correlated with intracellular superoxide levels measured via dihydroethidium staining. By analyzing μMax in 96 segregants from an SA x NA cross exposed to $CuSO_4$, we performed quantitative trait locus (QTL) mapping and prioritized 17 candidate modifiers. Functional validation in deletion strains identified Yhk8—a putative drug/proton antiporter functionally similar to the human *SLC47A1* gene—as a novel copper susceptibility gene, with its deletion resulting in impaired growth and elevated superoxide levels. Together, these findings establish Yhk8 as a key genetic modifier of copper response in yeast.

Introduction

Copper is a fundamental transition metal essential for diverse biological processes, serving as a critical cofactor for enzymes involved in mitochondrial respiration, iron mobilization, and antioxidant defense (Tsang et al. 2021). Despite its utility, the redox-active nature of copper poses a significant challenge to cellular homeostasis; in excess, it generates reactive oxygen species (ROS) that induce deleterious oxidative damage to proteins, lipids, and DNA (Kahlson and Dixon 2022). Consequently, all organisms have evolved elaborate regulatory networks to maintain copper concentrations within a physiological range, yet susceptibility to copper-induced stress varies markedly across populations, suggesting that genetic factors play a decisive role in defining this tolerance (Fay et al. 2004; Stern et al. 2007; Everman et al. 2023; Wang et al. 2024).

The genomic basis of copper susceptibility is complex. In *Saccharomyces cerevisiae*, this trait is polygenic, involving modifier genes—*loci* that enhance or suppress the effects of the primary disease-causing variants (Szenfeld et al. 2026a)— that regulate copper sequestration, trafficking, and transcriptional responses. For instance, the metallothioneins Cup1-1 and Cup1-2 (Fogel et al. 1983; Welch et al. 1989; Buchman et al. 1989) act as essential copper chelators, while human conserved proteins such as the P-type ATPase ATP7B play critical roles in cellular efflux and compartmentalization to prevent toxic accumulation (Iida et al. 1998). Dysregulation of these pathways is linked to metabolic and neurological pathologies, such as Alzheimer's (Brewer 2012), Wilson's disease (Dusek et al. 2015), among others, underscoring the tight control required for copper homeostasis (Tsang et al. 2021).

Model organisms serve as powerful tools for dissecting the complex genetic architecture of traits for human translational research (Olguín et al. 2022). Studies in *Drosophila* and *Saccharomyces cerevisiae* have identified both known and novel genes contributing to these traits (Fay et al. 2004; Everman et al. 2021). Here, we utilized a genetically diverse panel of *S. cerevisiae* isolates—representing Sake (SA),

North American (NA), West African (WA), and Wine/European (WE) lineages, alongside their segregants (Cubillos et al. 2013) —to dissect phenotypic variance and map quantitative trait loci (QTLs) associated with copper susceptibility. Our analysis identified a significant genomic locus, and subsequent functional validation revealed that Yhk8 deletion confers increased copper susceptibility, establishing it as a novel genetic modifier of the copper response in yeast.

Results

Copper susceptibility is strain-dependent and associated with intracellular ROS levels

To optimize the $CuSO_4$ concentration for parental strain (SA, NA, WA, and WE) experiments, we performed dose-response assays (5–30 µM). We identified a strain- and concentration-dependent response, with SA exhibiting the highest resistance, WE an intermediate phenotype, and WA and NA showing greater growth impairment at higher concentrations (Figure S1). We selected 20 µM $CuSO_4$ for downstream analyses, as it elicited a reproducible, differential response without inhibiting growth across all backgrounds. While growth kinetics were similar among untreated strains, $CuSO_4$ exposure caused strain-specific defects (Fig. 1A–B). Comparison of the maximum growth rate (µMax) confirmed these differences: SA was largely unaffected, WE and WA showed moderate growth reduction, and NA exhibited a pronounced defect (Fig. 1C). Normalizing µMax to untreated controls confirmed that the copper response is background-dependent (Fig. 1D).

Given that copper is a known inducer of reactive oxygen species (ROS), we hypothesized that the observed growth variations reflect divergent oxidative stress responses. To test this, we measured intracellular ROS using dihydroethidium (DHE) fluorescence in parental strains exposed to 20 µM CuSO4 relative to untreated controls. Normalized ROS levels remained low in the tolerant strains SA and WE (1.3 ± 0.1 and 1.8 ± 0.6 A.U., respectively), whereas the sensitive strains WA and NA exhibited markedly higher accumulation (8.6 ± 1.8 and 9.4 ± 0.8 A.U., respectively; Fig. 2A). This corresponds to a 6.8-fold and 7.5-fold increase over SA (one-way ANOVA, $p = 0.00011$). Moreover, ROS accumulation strongly correlated with growth performance under copper stress ($r = -0.994$, $p = 0.00601$; Fig. 2B), supporting the role of oxidative damage in copper susceptibility. Together, these results demonstrate that differential ROS management is a key component of the background-dependent response to copper.

Identification and validation of Yhk8 as a copper-response modifier

To identify genetic loci underlying copper susceptibility, QTL mapping was performed using the normalized µMax phenotype of 96 segregants derived from the SA x NA cross exposed to 20 µM $CuSO_4$ (Fig. 3A). This analysis revealed a significant linkage peak on chromosome VIII, indicating that genetic variation within this region contributes to growth differences under copper stress (Fig. 3B). We examined 62 genes within a ± 40 kb window flanking the INM1 marker and prioritized candidates harboring non-

synonymous variants (relative to the S288C reference) that distinguished the susceptible NA strain from the other parental backgrounds. This included variants present exclusively in NA or, conversely, those shared by other parental strains but absent in NA. Although the *CUP1* locus (comprising *CUP1-1* and *CUP1-2*), a known determinant of copper resistance (Fogel et al. 1983), also falls within this interval, it was not captured by our prioritization criteria. Consequently, our filtering yielded 16 candidate modifier genes: Myo1, Pih1, Brl1, Rpn1, Dap2, Rrm3, Put2, Bcd1, Aap1, Yhk8, Rsc30, Gic1, Ssz1, Rrp3, Ssf1, and Rrp4 (Table 1). SIFT scores were used as complementary information to estimate the potential functional impact of amino acid substitutions. As a control, QTL mapping was also performed using µMax values obtained under mock conditions (Figure S3). No significant linkage peaks were detected in this analysis, confirming that the genomic region associated with the copper-normalized phenotype was not primarily linked to basal growth differences among the segregants.

To validate the candidate genes identified within the chromosome VIII QTL interval, we evaluated deletion mutants under control and 1.1 mM CuSO4 conditions. We utilized a higher copper concentration for these experiments because the BY4742, BY4741, and BY4743 reference backgrounds used for validation displayed greater copper tolerance than the parental strains analyzed in our initial screen (data not shown); consequently, a stringent challenge was required to reveal differences in susceptibility. Among the candidates tested (Supplementary Figure S3), Yhk8 was the only gene that exhibited a copper-specific growth defect (Fig. 4A–B). Under control conditions, the *Δyhk8* mutant showed a µMax comparable to that of the isogenic BY4742 reference strain (0.21 ± 0.01 vs. 0.23 ± 0.01 OD/h, respectively; adjusted P = 0.545), indicating that loss of YHK8 does not markedly affect basal growth. In contrast, under $CuSO_4$ exposure, *Δyhk8* displayed a strong reduction in µMax compared with BY4742 (0.06 ± 0.02 vs. 0.16 ± 0.01 OD/h, respectively; $P = 3.87 \times 10^{-6}$), corresponding to an approximately 58.5% lower maximum growth rate. These results confirm that loss of *YHK8* significantly impairs growth performance specifically under copper stress, reinforcing its role as a modifier of copper susceptibility (Fig. 4B).

Given that we observed an association between µMax and superoxide levels measured as DHE in the four parental strains upon copper exposure (Fig. 2), we evaluated whether the increased copper susceptibility of *Δyhk8* was accompanied by changes in ROS accumulation. DHE fluorescence analysis showed that *Δyhk8* accumulated significantly higher ROS levels than the BY4742 reference strain under copper exposure, measured as DHE fluorescence normalized by optical density (UFA/OD) (Fig. 4C). Under 1.1 mM $CuSO_4$, *Δyhk8* showed a marked increase in ROS levels compared with BY4742 (10.0 ± 1.4 vs. 4.3 ± 3 UFA/OD, respectively; $P = 9.81 \times 10^{-7}$), corresponding to an approximately 2.3-fold increase. These results indicate that loss of *YHK8* not only impairs growth under copper stress, but also enhances copper-associated oxidative stress, highlighting it as a new determinant of copper homeostasis in yeast.

Discussion

In this study, we explored the genetic architecture underlying copper tolerance in *Saccharomyces cerevisiae* using four parental strains and one F1 cross. Our results demonstrate that copper

susceptibility is highly strain-dependent and associated with intracellular ROS levels. Through QTL mapping and functional validations, we uncovered Yhk8 as a novel modifier of copper toxicity, providing a new piece to the complex puzzle of how yeast modulate their response to metal-induced stress.

Copper homeostasis in yeast is a tightly regulated process essential for survival, relying on a sophisticated network of metallothioneins and transporters. Established mechanisms include the Cup1-1 and Cup1-2 metallothioneins (Thiele et al. 1987; Welch et al. 1989), which sequester excess copper, with their expression orchestrated by the ACE1 transcription factor (Thiele 1988). Copy number variants (CNV) in the *CUP1* locus regulates copper susceptibility (Fogel et al. 1983). Interestingly, there is CNV variation in this locus across the four parental yeast strains that may contribute to the differences observed in our study (Yue et al. 2017). Furthermore, copper uptake and trafficking are fine-tuned by proteins like MAC1 (Jungmann et al. 1993) and efflux systems such as the P-type ATPase ATP7B (Forbes et al. 1999), which collectively prevent the accumulation of toxic levels. Despite this well-characterized framework, our study highlights that genetic background significantly influences the efficacy of these mechanisms, suggesting that modifier genes—such as Yhk8—play a critical, supplementary role in fine-tuning the copper tolerance landscape.

Yhk8 encodes a putative drug/proton antiporter from the DHA1-family (Gbelska et al. 2006), a class of proteins often involved in cellular detoxification and homeostasis. While the specific mechanism by which YHK8 contributes to copper resistance remains to be fully elucidated, transcriptional profiling provides key insights into its functional role. Notably, previous studies have shown that under certain stress conditions, such as itraconazole exposure, Yhk8 expression is regulated in coordination with the Cup1-2 metallothionein genes (Barker et al. 2003). This transcriptional parallelism hints that YHK8 may function as an essential auxiliary component of the cellular detoxifying arsenal. As a candidate antiporter, YHK8 could potentially maintain the electrochemical gradients or pH balance required for efficient metallothionein-mediated copper sequestration or limit the accumulation of toxic metal intermediates within cellular compartments.

Beyond its fundamental biological implications, the identification of Yhk8 underscores the translational and biotechnological value of using yeast as a model system for metal homeostasis. Given that copper dyshomeostasis is a recognized hallmark of several human metabolic and neurodegenerative disorders —conditions associated with mitochondrial and lysosomal dysfunction—deciphering the regulatory network involving YHK8 provides a potential framework for understanding how eukaryotic cells mitigate copper-induced oxidative stress, mirroring the protective role of the yeast Cup-1 metallothionein overexpression observed in human cell models (Xie et al. 2015). While humans lack a direct evolutionary orthologue to the yeast DHA1-family protein YHK8, *SLC47A1* (also known as *MATE1*) represents its closest human functional analogue, utilizing an identical proton-coupled antiport mechanism to mediate multidrug extrusion (Otsuka et al. 2005; He et al. 2010). Notably, *SLC47A1* has been proposed as a cuproptosis-related gene in human uveal melanomas (Huang et al. 2023).

Furthermore, our findings offer direct biotechnological applications. In industrial microbiology, where environmental copper stress often impedes fermentation efficiency (Sun et al. 2015), YHK8 represents a tangible target for the rational engineering of robust microbial strains. This is of particular relevance in agriculture; for example, in organic viticulture, copper is the primary antifungal agent used to control parasites such as *Plasmopara viticola* (García-Esparza et al. 2006). Analogously, as the human immune system utilizes copper as a weapon to restrict fungal growth, our findings shed light on the genetic modifiers that allow fungi to overcome this stress, potentially informing the development of novel antifungal strategies designed to sensitize pathogens to host-mediated defenses (Festa et al. 2014).

While our findings provide compelling evidence for the role of YHK8 as a copper modifier, this study has inherent limitations. Our QTL analysis was restricted to the SA × NA cross, which, while highly informative, may not capture the full spectrum of genetic variation present in natural isolates (Peter et al. 2018). Furthermore, the functional validation was performed under controlled laboratory conditions using YNB medium; therefore, the impact of YHK8 under more complex environmental scenarios or distinct nutrient conditions requires further investigation. Future studies should focus on the biochemical characterization of the YHK8 protein and its physical interaction with other copper-related pathways to delineate its precise regulatory mechanism.

In conclusion, our work expands the understanding of the polygenic nature of copper susceptibility in yeast. By successfully mapping and validating Yhk8, we have identified a new genetic determinant that contributes to the differential tolerance observed across diverse yeast lineages. These insights not only advance our knowledge of yeast metal homeostasis but also underscore the value of using natural genetic diversity to uncover non-canonical modifier genes. Future research into the YHK8 pathway will likely shed further light on the evolution of stress resistance mechanisms in fungi and provide a template for studying similar modifiers in other model organisms.

Materials and Methods

Yeast strains

We used four genetically diverse *Saccharomyces cerevisiae* strains:: Y12 [Sake, (“SA”): MATα *ho*::NatMX, *ura3*::KanMX], YPS128 [North American (“NA”): MATα *ho*::NatMX, *ura3*::KanMX], DBVPG6044 [West African, (“WA”): MATα *ho*::NatMX, *ura3*::KanMX] and DBVPG6765 [Wine/European, (“WE”): MATα *ho*::NatMX, *ura3*::KanMX]. These strains were provided by Dr. Francisco Cubillos (Cubillos et al. 2013). Validation studies were performed using the BY4742 (Y10000), BY4741 (Y00000) and BY4743 (Y20000) background strains. The following deletant strains were on the BY4742 background: *△aap1*; Y11874, *△gic1;* Y11889, *△ssz1;* Y16414, *△ssf1;* Y11894, *△yhr049c-a;* Y11877, *△dap2;* Y10991; *△rrm3;* Y10994; *△put2;* Y11000; and *△yhk8*; Y11875. The following on the BY4741 background *△rpn1;* Y40789, *△brl1;* Y41370, and the following on the BY4743 background: *△myo1*; Y26607; *△rrp3*; Y21893, *△rrp4*; Y21897, and *△bcd1*; Y21003. All strains were obtained from Euroscarf (http://www.euroscarf.de).

Cell growth

Yeast strains cells were inoculated in 200 µL of YNB medium (0.67% YNB without amino acids supplemented with 2% glucose, 0.2% uracil, and with 0.0875% yeast synthetic drop-out medium without uracil) for 48 h at 28°C as previously described (Las Heras et al. 2025). Subsequently, the yeast cells were inoculated to an optical density (OD) of 0.03–0.1 (wavelength 620 nm) in 200 µL of medium (with and without $CuSO_4$ 20µM and incubated without agitation at 25°C for 36–48 h in a Tecan Sunrise absorbance microplate reader. The OD was measured every 30 minutes using a 620 nm filter. Each experiment was performed in triplicate. Maximum specific growth rate (µMax) was calculated using BIOSZEN (v1.0.0), a custom R-based Shiny application, as previously described (Szenfeld et al. 2026b). The application processes optical density (OD) readings by using a sliding-window linear regression algorithm implemented in the stats package.

Measurement of levels of Reactive Oxygen Species (ROS)

Yeast cultures were incubated for 48 hours at 28°C in 3 ml of YNB medium and centrifuged at 1700 x g at 4°C for 4 min. The pellet was incubated with the DHE (Thermofisher Scientific, NºD1168, U.S.A) probe at a concentration of 10 µg/mL in 500 µL of PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl) for 30 min and washed with PBS as previously reported (Rubilar et al. 2025). Fluorescence intensity was visualized using a Cytation3 microplate reader (BioTek Instruments, Santa Clara, CA, U.S.A.) at 518/605 nm.

Genetic linkage mapping of quantitative trait loci

Quantitative trait locus (QTL) mapping was performed as previously described (Szenfeld et al. 2026b) using the following phenotypes derived from growth curves of 96 segregants from the SA × NA cross: µMax under $CuSO_4$ 20µM was normalized to mock. Mapping was conducted using R/qtl (Broman et al. 2003). LOD scores were calculated using a nonparametric model, and significance thresholds were determined by permutation testing. Phenotype values were permuted 1,000 times and the maximum LOD score was recorded for each permutation. QTL were considered significant when the LOD score exceeded the 0.05 significance threshold. The percentage of phenotypic variance explained by each QTL was calculated as: % phenotypic variance = 100 × (1 − 10^((− 2 × LOD)/n)), where n represents the sample size (Cubillos et al. 2009).

SIFT and GenomeMUSter for variant effect prediction

Variants identified in the top-ranked genes at each linkage peak were further evaluated using *in silico* pathogenicity prediction tools, including Sorting Intolerant From Tolerant (SIFT)(Kumar et al. 2009). SIFT predictions were performed using SIFT 4G, on *S. cerevisiae* VCF files generated after aligning each parental strain genome (WA, SA, NA, and WE) to the reference strain S288C. SIFT assesses amino acid substitutions based on sequence homology and physicochemical properties; variants were classified as “tolerated” or “deleterious,” with SIFT scores ≤ 0.05 considered deleterious (Ng and Henikoff 2003).

Statistics

Statistical analyses were performed using BIOSZEN (v1.0.0) (Szenfeld),with standard R workflows (rstatix, stats) as previously performed (Las Heras et al. 2025). At least three biological replicates were analyzed, and data are presented as mean ± standard error of the mean (SEM). Normality was assessed using the Shapiro-Wilk test ($p > 0.05$). For normally distributed data, one-way ANOVA was performed followed by Tukey's post hoc test, and paired t-tests were used for two-group comparisons, as appropriate. For non-normally distributed data, the Kruskal-Wallis test was applied followed by Dunn's multiple-comparisons test. A p-value < 0.05 was considered statistically significant. The specific statistical tests used are indicated in the figure legends.

Declarations

Acknowledgements: This work was supported by ANID-CHILE: Fondecyt grant No 1230317 (A.D.K). F.A.C. is supported by Programa Iniciativa Científica Milenio ICN17_022 and NCN2024_040.

Competing Interests: The authors declare that there are no competing interests (financial and non-financial).

CRediT author statement

VO: Methodology, Investigation, Data Analysis, Data Curation, Writing-Original draft, and Editing. S.C., F.A, J.S, B.S: Investigation. FAC: Conceptualization, Methodology, Writing- Editing. ADK: Conceptualization, Methodology, Data curation, Writing-Original draft, Supervision, Writing-Reviewing, Editing, and Funding the Project.

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

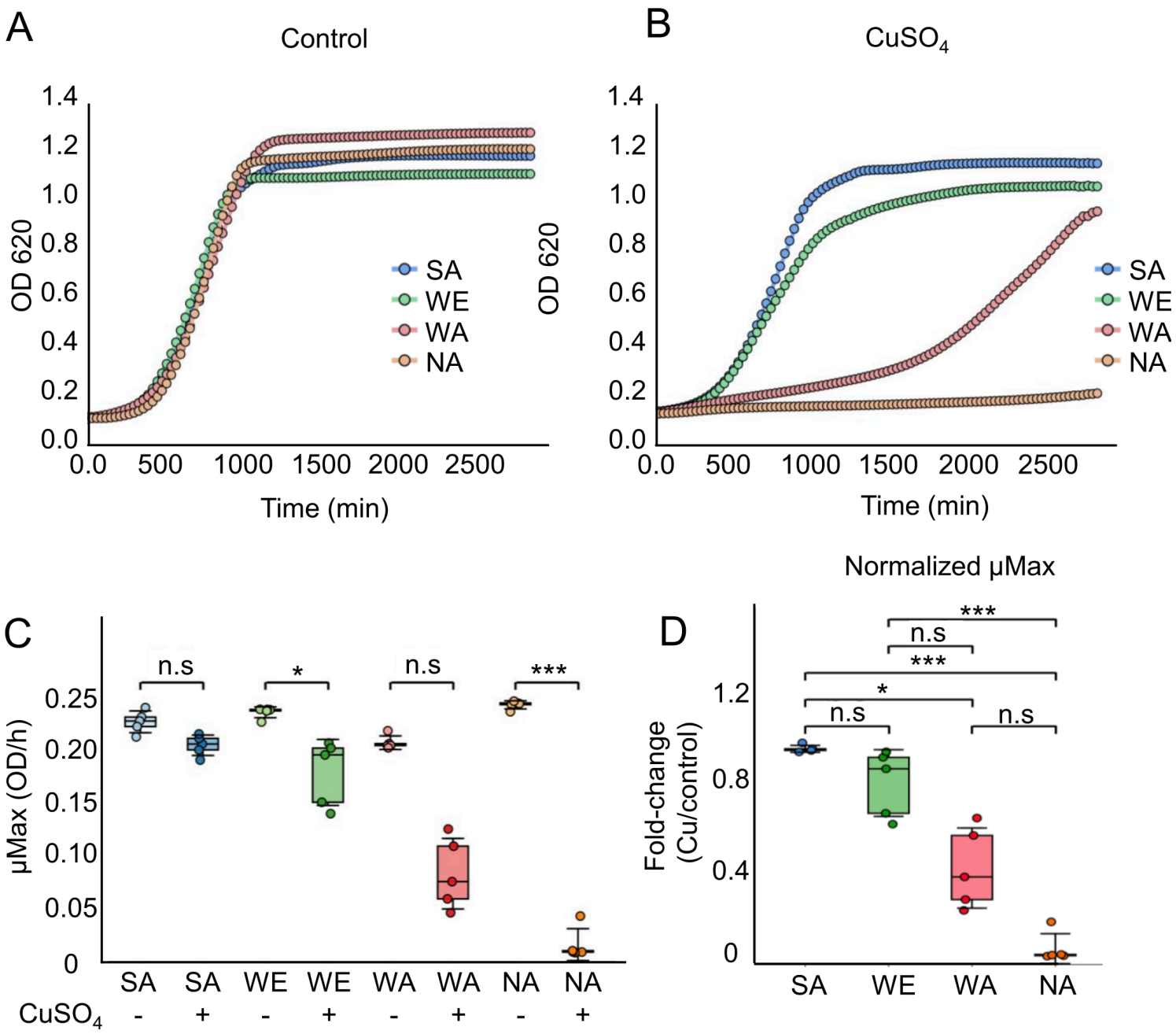


Figure 1

Copper-mediated toxicity is genetic background dependent. A) Growth curves in four yeast strains in control condition B) Growth curves in four yeast strains exposed to 20 µM $CuSO_4$ .C) Comparison of OD/h parameters derived from the growth curve between control condition and conditions treated with 20 µM $CuSO_4$ in parental strains (SA, WE, WA, NA). D) Comparison of OD/h parameter under 20 µM $CuSO_4$ conditions between parental strains, normalized to the control condition. The experiment was performed in 3 technical replicates in YNB medium with supplements. The Shapiro–Wilk test was used to assess normality and the Kruskal–Wallis test for multiple comparisons (* = P < 0.05; = P < 0.01; * = P < 0.001). Subsequently, Dunn's test was used as post-hoc.

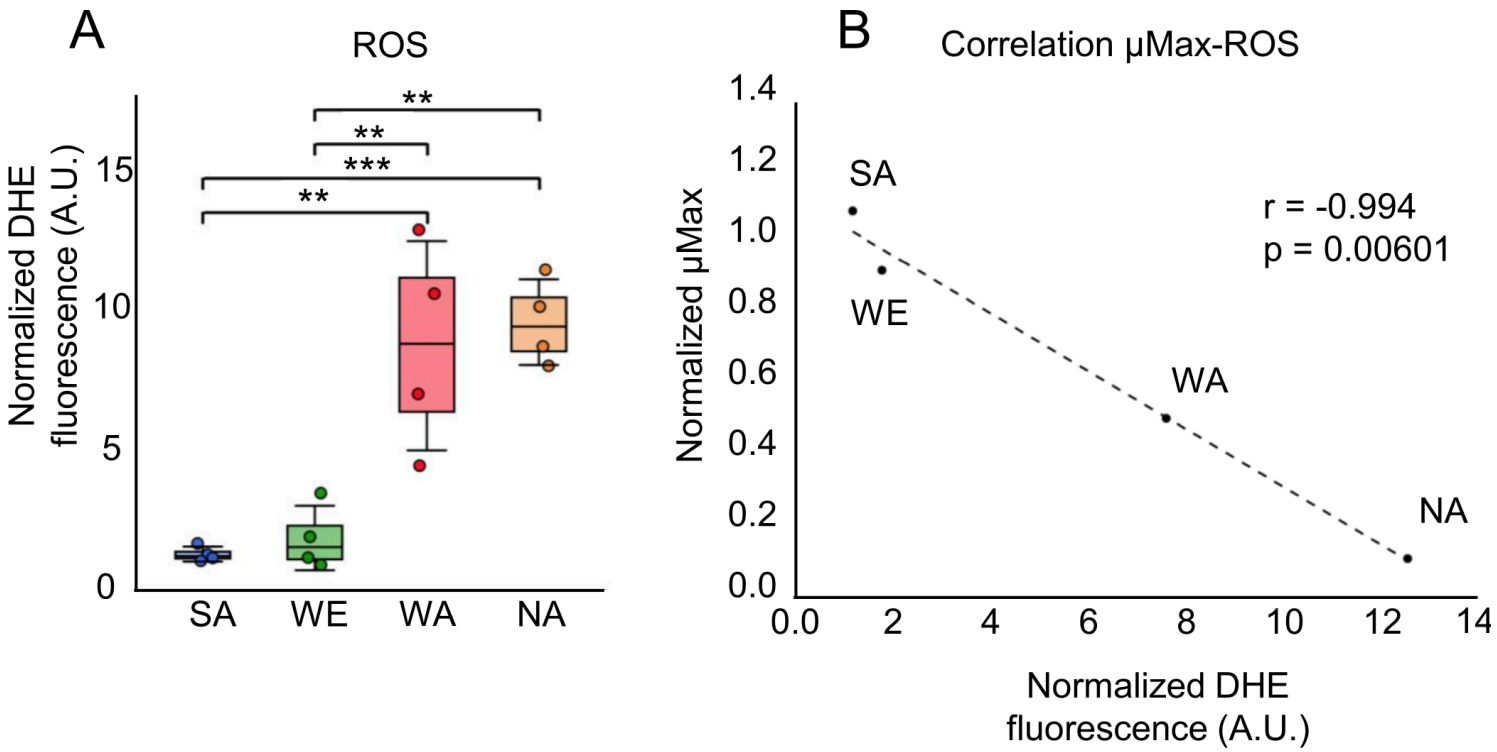


Figure 2

Copper-mediated toxicity correlates with ROS levels. A) Comparison of ROS levels among the four parental strains. UFA/OD values in 20 µM $CuSO_4$ condition, normalized by the values in the control condition for each strain. n=4. (* = P < 0.05; ** = P < 0.01; *** = P < 0.001). The Shapiro–Wilk test was used to assess normality, followed by ANOVA for multiple comparisons, and subsequently Tukey's post hoc test to identify which pairs of groups have significantly different means. B) Linear regression between the normalized µmax and normalized UFA/OD parameters. A strong negative correlation of r = -0.994 was obtained (p = 0.00601).

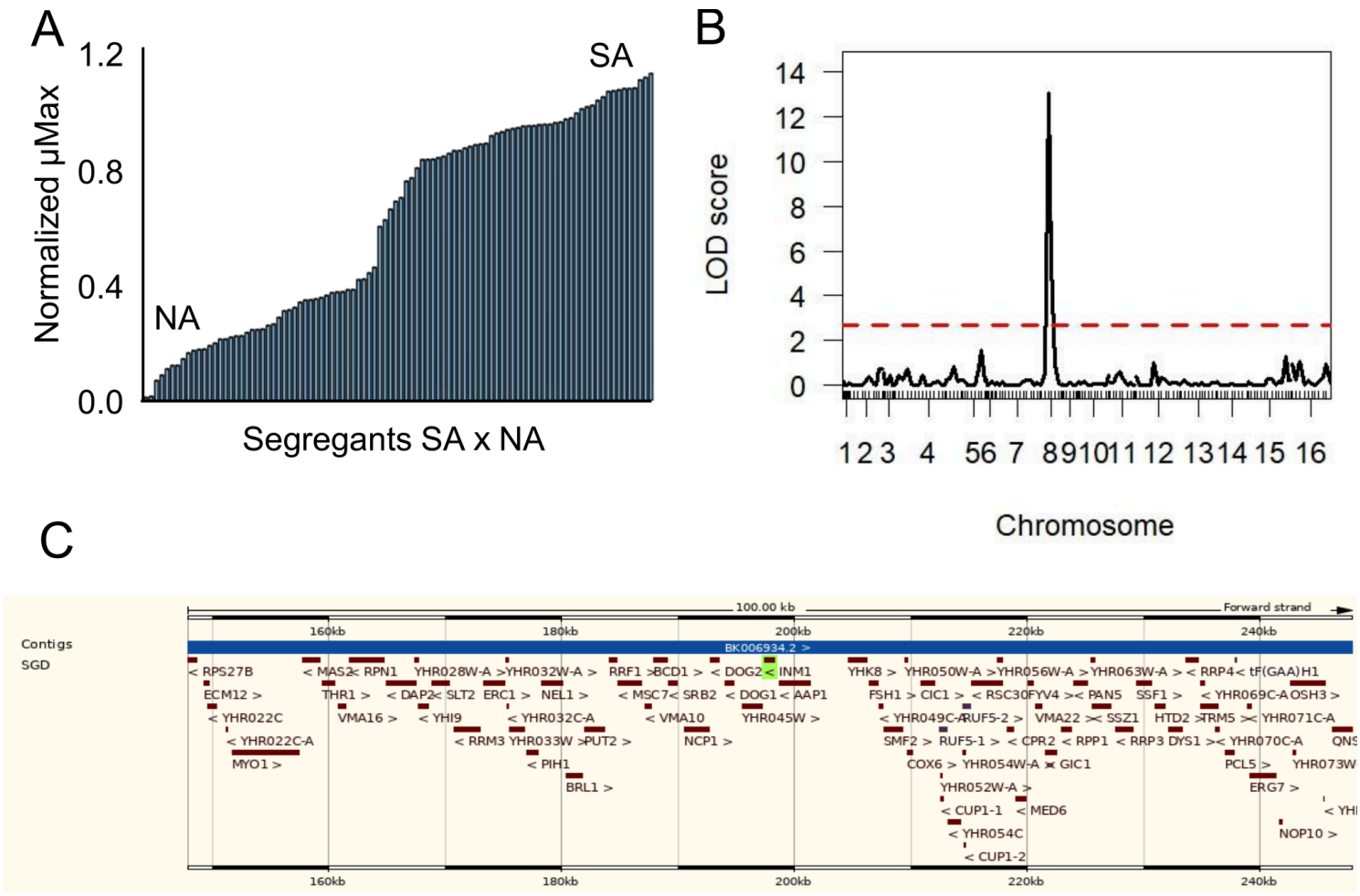


Figure 3

Identification of modifier genes underlying copper responses in yeast. A) Distribution of µmax $CuSO_4$/µMax control across 96 segregants from the SA × NA cross (n = 3). B) QTL mapping based on the relative maximum growth rate (µmax in $CuSO_4$/µmax in control medium) of 96 segregants derived from the SA × NA cross. The LOD threshold after 1,000 permutations was of 2.661. The analysis identified a significant locus on chromosome VIII with a LOD of 13.027 (peak centered at the INM1 marker). C) Genomic interval surrounding the QTL peak, defined as ±40 kb from INM1 and visualized using Ensembl. A total of 62 genes were located within this region and considered for subsequent candidate-gene prioritization.

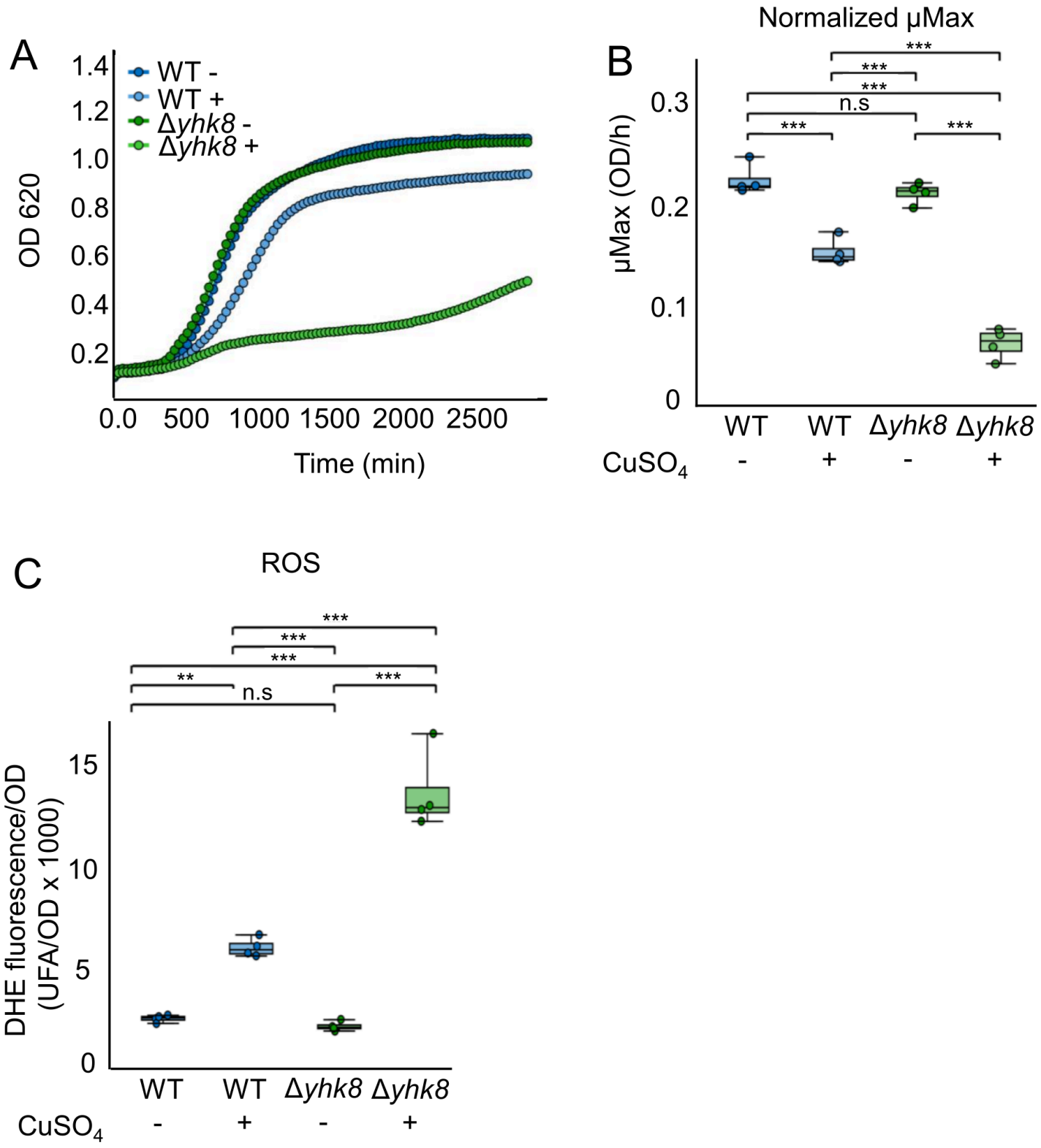


Figure 4

YHK8 as a modifier of the copper response in yeast. A) Growth curves of the BY4742 wild-type strain and the *Δyhk8* mutant under control conditions and in the presence of $CuSO_4$. B)) ROS levels were quantified as DHE fluorescence normalized by optical density, expressed as UFA/OD x 1000. For panels A and B, normality was assessed using the Shapiro–Wilk test, followed by ANOVA and Tukey's post hoc test for multiple comparisons. For panel C, normality was assessed using the Shapiro–Wilk test, and

statistical significance was determined using an unpaired t-test. n = 4 for ROS measurements. *P < 0.05; **P < 0.01; ***P < 0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1legend.docx
- Table1.xlsx
- figuresS13.pdf