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**Dealing with copper toxicity: New insights into copper
detoxification in yeast**

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“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained.”

Marie Curie

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Abstract

Copper, an essential metal, is a double-edged sword, as its vital nature is counterbalanced by the toxic effect that it can exert on cells when not properly controlled. As such, organisms have evolved defence mechanisms against copper toxicity and, in the yeast *Saccharomyces cerevisiae*, the transcription factor Ace1 orchestrates several of those mechanisms, by activating copper-detoxification genes.

In *Saccharomyces cerevisiae* iron uptake is partially dependent on copper, as the membrane multicopper-oxidase Fet3, which is part of the high-affinity iron uptake system, requires copper as a cofactor. Aft1, the low iron-sensing transcription factor in yeast, is known to regulate the expression of *FET3* gene. However, we found that a strain lacking *FET3* is more sensitive to copper surplus conditions than a strain carrying the *AFT1* gene disrupted. This finding suggests that under such conditions another regulator came into play and controls *FET3* gene expression.

We next evaluated whether Ace1 could be the alternative regulator of *FET3* under copper excess. To test this hypothesis, the expression of *FET3* gene in cells lacking *ACE1*, *AFT1* or both was monitored, using yeast-one hybrid and qRT-PCR approaches. A decrease of *FET3* transcripts is observed, under copper loading, when *ACE1* is deleted, suggesting that Ace1 is involved in *FET3* regulation. Remarkably, in the absence of *AFT1* we observed a copper-dependent induction of *FET3* gene that was, however, completely abrogated when *ACE1* was deleted from the *aft1* mutant background. Our data clearly indicates that *FET3* expression is dependent on Ace1 when copper is overly abundant.

In this study, the link between iron and copper homeostasis was further established. Indeed, we found that *ace1* cells display marked upregulation of Aft1's targets, such as *CTH2*, and accumulate higher amounts of iron and copper, in a mechanism dependent on Aft1.

Altogether, this work unveiled a novel protection mechanism against copper toxicity mediated by Ace1, which relies in the activations of *FET3* and results in the restriction of Aft1 activity as to prevent excessive copper accumulation.

Key-words: *Saccharomyces cerevisiae*, copper, Ace1, Fet3, copper-detoxification

Resumo

O cobre é um metal essencial para a vasta maioria dos organismos, maioritariamente devido às suas propriedades de oxidação-redução, que o tornam ideal para ser cofactor de inúmeras proteínas. Nos humanos, o cobre é essencial para inúmeras funções celulares, nomeadamente para o metabolismo energético, produção de melanina e catecolaminas, destoxificação de espécies reactivas de oxigénio e homeostase de ferro. No entanto, a sua natureza essencial é contrabalançada pelos efeitos tóxicos causados quando este metal está presente em níveis acima dos considerados fisiológicos. De facto, sabe-se que o excesso de cobre potencia a produção de espécies reactivas de oxigénio (ROS), através da reacção de Fenton, e que este metal pode mesmo danificar os centros metálicos de enzimas e proteínas, levando à perda de função. Assim, os organismos desenvolveram inúmeros mecanismos de defesa contra a toxicidade provocada pelo cobre. Na levedura *Saccharomyces cerevisiae*, o factor de transcrição Ace1 é o responsável pela regulação destes mecanismos, ao activar genes de destoxificação do cobre, tais como genes codificantes de metalotioneínas *CUP1* e *CRS5*, que actuam como quelantes de iões de cobre, e o gene *SOD1*, que codifica o superóxido dismutase, enzima que além de conter cobre no seu centro activo, é essencial na resposta ao *stress* oxidativo causado por este metal.

Em *Saccharomyces cerevisiae* a entrada de ferro (Fe) na célula é parcialmente dependente do cobre, uma vez que o Fet3, uma ferroxidase de membrana que faz parte do complexo de transporte de alta afinidade de ferro, requer cobre como cofactor. O Aft1, o principal factor de transcrição envolvido na resposta à deficiência de ferro em levedura, é o conhecido regulador da expressão do gene *FET3*. No entanto, diversos resultados apresentados neste trabalho demonstram claramente que uma estirpe sem o gene *FET3* é mais sensível ao excesso de cobre do que uma estirpe mutada no gene *AFT1*. Esta evidência sugere que, em condições de excesso de cobre, um outro regulador controla a expressão do gene *FET3*. Deste modo, foi avaliada a possibilidade de o factor de transcrição Ace1 ser o regulador alternativo do gene *FET3* em condições de excesso de cobre. De facto, a região promotora do gene *FET3* contém uma possível sequência de ligação do Ace1, localizada a -578 bp a montante do codão de iniciação ATG (+1). Através da monitorização dos níveis de expressão do *FET3* na estirpe-selvagem e na estirpe mutada no gene *ACE1* (mutante *ace1*), foi possível concluir que o factor de

transcrição Ace1 está envolvido na regulação do gene *FET3*. Foi ainda observado que mesmo na ausência do regulador Aft1, ocorre uma indução da expressão do *FET3*, dependente do cobre que, no entanto, é totalmente suprimida quando se elimina o gene *ACE1*.

Através da monitorização da expressão de um dos genes alvo do factor de transcrição Aft1, *CTH2*, na estirpe selvagem e na estirpe *ace1*, em condições de excesso de cobre, através da técnica de qRT-PCR, observou-se que, na ausência do Ace1, há um aumento drástico dos níveis dos transcritos de *CTH2*. Estes dados sugerem que, na estirpe mutante *ace1*, a homeostase de ferro encontra-se desregulada, pelo que ocorrerá uma super-activação do factor Aft1. De acordo com esta hipótese, foi observado que a estirpe *ace1* contém níveis de ferro e de cobre intracelulares marcadamente elevados quando comparados com os da estirpe-selvagem. Esta acumulação é dependente da actividade do regulador Aft1, uma vez que a sua eliminação (na estirpe mutante *aft1ace1*) repõe os níveis dos dois metais para valores semelhantes aos da estirpe-selvagem.

O Fet3 é um enzima importante para a resposta celular ao excesso de cobre. Foi descrito que a estirpe mutante *fet3* acumula grandes quantidades de cobre. Neste estudo, observou-se que esta acumulação ocorre através de um mecanismo dependente do factor de transcrição Aft1, dado que o duplo mutante *fet3aft1* reverte os níveis de cobre intracelulares para níveis semelhantes aos da estirpe selvagem. Através da análise dos genes alvo do Aft1, foram seleccionados possíveis proteínas envolvidas neste mecanismo. A deleção do genes *FET4*, que codifica um transportador de ferro de baixa afinidade, que também tem a capacidade de transportar cobre, e do *ARN4*, que codifica um transportador de sideróforos de ferro, cujo substrato é a enterobactina, levou a um ganho de resistência ao cobre quando deletados de uma estirpe *fet3*. Resultados semelhantes foram registados após a deleção do gene *FRE1*, que codifica uma metaloreductase de membrana, envolvida no transporte de ferro e cobre. Estes resultados evidenciaram que a acção combinada de várias proteínas envolvidas no transporte de ferro contribuem para a acumulação de cobre observada numa estirpe mutante *fet3*. Assim a deficiência de ferro observada na estirpe mutante *fet3*, conduzirá à activação do factor de transcrição Aft1 que induzirá a expressão dos genes dessas proteínas, numa tentativa de colmatar a falta de ferro consequente da perda do sistema de transporte de ferro de alta afinidade.

Neste estudo, foi ainda possível observar que a estirpe mutante *aft1*, que apresenta um fenótipo de sensibilidade em condições normais crescimento, recupera o seu défice

de crescimento quando cobre é adicionado ao meio de cultura. Contrariamente ao esperado, a atenuação observada não é devida a um aumento do conteúdo intracelular de ferro, quando as células são crescidas na presença de cobre, uma vez que os níveis de ferro da estirpe *aft1* são inferiores aos níveis registados quando cultivada em condições normais de crescimento. Com o objectivo de compreender o mecanismo subjacente a este fenómeno, testaram-se outras vias dependentes de cobre poderiam estar afectadas em condições normais de crescimento no mutante *aft1*, nomeadamente a respiração mitocondrial, (dado que o cobre é um cofactor do citocromo c oxidase), e a resposta ao *stress* oxidativo (uma vez que o cobre faz parte do Cu,Zn-Superóxido dismutase). Através da análise da sensibilidade do mutante *aft1* em meio YPDGE (meio de cultura rico, contendo quase exclusivamente fontes de carbono não fermentáveis), concluiu-se que a adição de cobre melhora a capacidade respiratória da estirpe *aft1*, dado que foi observado uma significativa recuperação da sensibilidade deste mutante. Além disso, a análise fenotípica do mutante *aft1* cultivado em meio contendo paraquat (um composto gerador de anião superóxido), suplementado ou não com cobre, permite concluir que a adição de cobre alivia também a sensibilidade desta estirpe ao *stress* oxidativo provocado pelo paraquat. Assim, os resultados obtidos sugerem que numa estirpe *aft1* a adição de cobre promove o metabolismo respiratório e fortalece a resposta ao *stress* oxidativo, contribuindo desta forma para o aliviar do défice de crescimento exibido por esta estirpe.

Assim, no seu conjunto, os dados apresentados nesta dissertação permitem propor uma nova via de destoxificação de cobre mediada pelo factor de transcrição Ace1. Em condições de excesso de cobre, as células de levedura activam este regulador, que por sua vez irá induzir a expressão do gene *FET3*. Sendo uma proteína que contém cobre no seu centro activo, o enzima Fet3 contribui não só para o tamponamento dos iões de cobre livres presentes no citosol, como também para a entrada de ferro na célula. A entrada de ferro irá prevenir a activação excessiva do Aft1, e conseqüente expressão dos seus genes alvo, limitando assim a entrada de mais cobre através de transportadores de baixa afinidade para ferro, o que eventualmente, levaria à morte celular. Assim, neste estudo foi pela primeira vez demonstrado que além do seu papel essencial na destoxificação do cobre intracelular, o Ace1 possui também um papel fundamental na prevenção da entrada de cobre extracelular.

Palavras-Chave: *Saccharomyces cerevisiae*, cobre, Ace1, Fet3, destoxificação de cobre

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List of Acronyms

APS	Ammonium persulphate
ATP	Adenosine triphosphate
CuSO₄	Copper sulphate
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
FeSO₄	Iron(II) Sulphate
LiAc	Lithium Acetate
M	Molar
mM	Mili Molar
Na₂HPO₄	Sodium phosphate dibasic
NaH₂PO₄	Sodium phosphate monobasic
nm	Nanometres
OD₆₀₀	Optical Density at 600 nanometres
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Phenylmethilsulfonyl fluoride
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpm	Revolutions <i>per</i> minute
RT	Reverse Transcriptase
SC	Synthetic Complete Medium
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS- Polyacrylamide gel electrophoresis
SOD	Superoxide Dismutase
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine\
TGN	Trans-Golgi network
μM	Micro molar
V	Volt
YPD	Yeast Peptone Dextrose growth medium
ZnSO₄	Zin Sulphate

I. Introduction

1.1 Copper in biological systems

Copper is a transition metal that is present in nature predominantly in two oxidation states, Cu (I) and Cu (II). Given its ability to bind organic ligands and its electron transfer properties, this metal has a high potential to be utilized by organisms, mainly as a cofactor for proteins. In humans, copper is essential for oxidative phosphorylation, melanin and catecholamine production (through tyrosinase and dopamine- β -hydroxylase, respectively), superoxide dismutase activity and iron homeostasis [1].

1.1.1 Copper and disease

Wilson and Menkes diseases are rare genetic conditions caused by mutations in Cu-ATP-Binding-Cassette transporters, ATP7B and ATP7A, respectively [2, 3]. Within the cell, both proteins co-localize to the trans-Golgi network (TGN), where they import copper into TGN's lumen, to be then incorporated in copper-containing proteins. However, both proteins appear to translocate to intracellular vesicles and to the plasma membrane in response to copper overload [4, 5]. These proteins have different localization patterns, with ATP7B being present mainly in the liver, and ATP7A distributed across the whole organism, with the exception of the liver. The phenotype of Wilson disease, due to a deficient biliary excretion of copper that causes an accumulation of copper in tissues, is a failure of liver function and neurological impairment. Menkes disease is characterized by systemic copper deficiency, as copper fails to reach circulation, which leads to the loss of activity of copper-containing proteins, and has as consequence a delay in neuronal development and a reduction of muscle tone [2, 3, 6].

There is now increasing evidence suggesting that neurodegenerative disorders, such as Alzheimer, Parkinson and Creutzfeldt-Jakob disease, are associated with an imbalance of copper homeostasis [7]. Copper is known to promote protein aggregation and oxidative stress, which are determinant factors for disease progression [7, 8]. Although copper levels above the homeostatic threshold have been observed in these and

other diseases, such as cancer, the mechanisms underlying this phenomenon are not yet clearly understood [7, 9] .

Copper-chelation therapies are currently used to treat Wilson disease and are considered an attractive strategy to prevent the onset of neurodegenerative diseases [9].

The treatment of Menkes disease relies on copper-supplementation therapy, through copper-histidine complex formulations, that can increase copper in ceruloplasmin and in organs, such as the liver [9–11].

1.1.2 Copper at the host pathogen interface

In addition to their major role as protein cofactors, metals, such as Cu, Fe and Zn play active functions in infection biology. The use of copper as an antimicrobial compound dates from the early 19th century, when it began to be incorporated in the Bourdeaux and Burgundy mixtures, the vineyard fungicides [12, 13]. In fact copper is now thought to have a protective role in infection, as Menkes disease carriers, that have systemic copper deficiency, are more susceptible to infections [10]. Also, mice fed on a low-copper diet have higher mortality rates upon infection with *C. albicans* [14].

One of the host mechanisms to fight microbial pathogens is the withdrawal of essential metals, such as iron and calcium, from the pathogen's vicinity, as to limit its growth [15, 16]. In contrast, upon infection, host copper-stores are mobilized, and innate immunity cells accumulate copper ions next to the pathogen in an attempt to increase the efficacy of pathogen killing (Figure 1.1) [17]. Macrophages constitute the first line of defence against microbial pathogens, such as fungi. These innate immunity cells have the ability to phagocytose the pathogen. In the phagolysosome environment, copper toxicity is maximized, as the low pH and the high concentrations of ROS potentiate copper-driven Fenton chemistry [13]. This was further corroborated by the observation that activated macrophages show marked upregulation of the copper-transporter, hCtr1, and of ATP7A at the phagolysosome membrane, which suggest that copper is being compartmentalized into the phagolysosome, after entering the macrophage (Figure 1.1) [18]. Interestingly, the macrophage-activating cytokine interferon- γ (IFN- γ) and lipopolysaccharide (LPS) also mediate the accumulation of copper inside the phagosome [18]. This regulated mechanism of copper compartmentalization ensures copper-damage only to the pathogen. Although accumulation of copper inside activated macrophages occur, the source of this

metal is unknown. Copper is thought to be mobilised from circulating ceruloplasmin, which contains over 90% of Cu in the plasma and, indeed, ceruloplasmin is increased during the acute phase of infection. However there are no direct evidences that ceruloplasmin is the copper-donor to activated macrophages [13, 19].

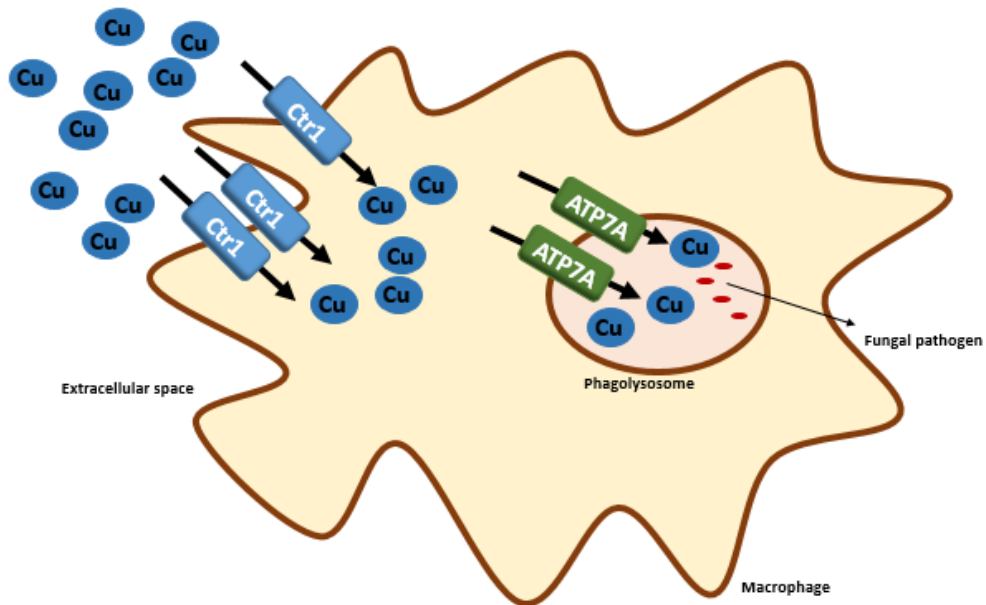


Figure 1.1 – Copper role in pathogen killing by macrophages. Activated macrophages exploit copper toxic properties to promote pathogen killing by concentrating copper (Cu) inside the phagolysosome. Upregulation of copper transporters Ctr1 and ATP7A is observed upon infection

1.2 Yeast as a model organism for metal homeostasis studies

In 1996, the first complete sequence of a eukaryote genome, the *Saccharomyces cerevisiae* genome, was published [20, 21]. The yeast's 12.8 Mb genome, composed of 16 chromosomes, was revealed to contain more than 5.800 protein-coding genes, 85% of which are presently assigned to a biological function. Although twenty years have passed since the release of the genome, the baker's yeast is still one of the most widely used eukaryotic model organism. This is due to the fact that it is genetically amenable, allowing genes to be deleted, altered or inserted in the genome, it has a short generation time and, importantly, it has many genes which are conserved among species, including plants, fungal pathogens and mammals [22]. A few years after the sequencing of *S. cerevisiae* genome, a library with almost each open-reading frame (ORF) mutated was generated [23]. The study of the effect of each deletion has allowed to uncover the biological function of many genes and to shed light on the molecular mechanisms of several human diseases [24–27].

Toxic metals (Cd, As, Hg, *etc.*) are major environmental and human health-threatening hazards. On the other hand, essential metals (Zn, Fe and Cu), present in virtually all biological systems, are a double-edge sword, as its essential nature is counterbalanced by the toxic effect that it can exert on both eukaryotic and prokaryotic cells when not properly controlled (i.e., when scarce or in excess). To maintain adequate cellular metal levels and avoid metal-loading, cells have developed sophisticated systems to assure the balanced cellular metal homeostasis. The single celled yeast *S. cerevisiae* has proven to be an invaluable model organism to study metal homeostasis in higher eukaryotes, as a consequence of conservations across species of several of the key players involved in those processes [26].

1.3 Copper homeostasis in the yeast cell

In the budding yeast *Saccharomyces cerevisiae* copper is involved in three major biological processes: mitochondrial oxidative phosphorylation, superoxide anion detoxification and iron homeostasis [28, 29]. However, the same redox properties that make it ideal for electron transfer reactions also render it susceptible to generate reactive oxygen species (ROS), causing oxidative damage to cells [30].

To counteract sudden fluctuations in copper bioavailability, *Saccharomyces cerevisiae* cells have evolved mechanisms to maintain a tight control of copper homeostasis. These mechanisms are involved on the one hand in the upregulation of copper-uptake and mobilization systems, which enable copper to be utilized in crucial cellular processes, and on the other hand in the activation of copper detoxification systems, in order to prevent toxicity and repair Cu-induced damages [31–33].

1.3.1 Copper depletion

When the intracellular copper content is low, yeast cells import copper mainly via the high affinity copper transport system, composed of two functionally redundant proteins: Ctr1 and Ctr3 [34] (Figure 1.2). Yeast cells deleted for *CTR1* gene (*ctr1* mutant strains) grow poorly in copper-depleted medium, have low Cu,Zn-SOD1 activity (and consequently higher oxidative stress), cannot grow in non-fermentable carbon sources and exhibit impaired high-affinity iron uptake [35]. *CTR3* was identified during a screen for mutations allowing growth in low copper conditions, in the absence of *CTR1* [34]. In most yeast laboratory strains, however, *CTR3* gene is disrupted by a Ty2 transposable element and high-affinity copper transport is only conveyed by Ctr1 [36, 37]. The high-affinity copper uptake system transports copper in the reduced, cuprous, state. As such, copper uptake is dependent on the activity of membrane metalloreductases, such as Fre1 and Fre2, which reduce copper that, in the extracellular environment, is mainly in the oxidised, cupric, state (Figure 1.2) [38]. Fre1/2 are also involved in the reduction of extracellular iron prior to its uptake [39].

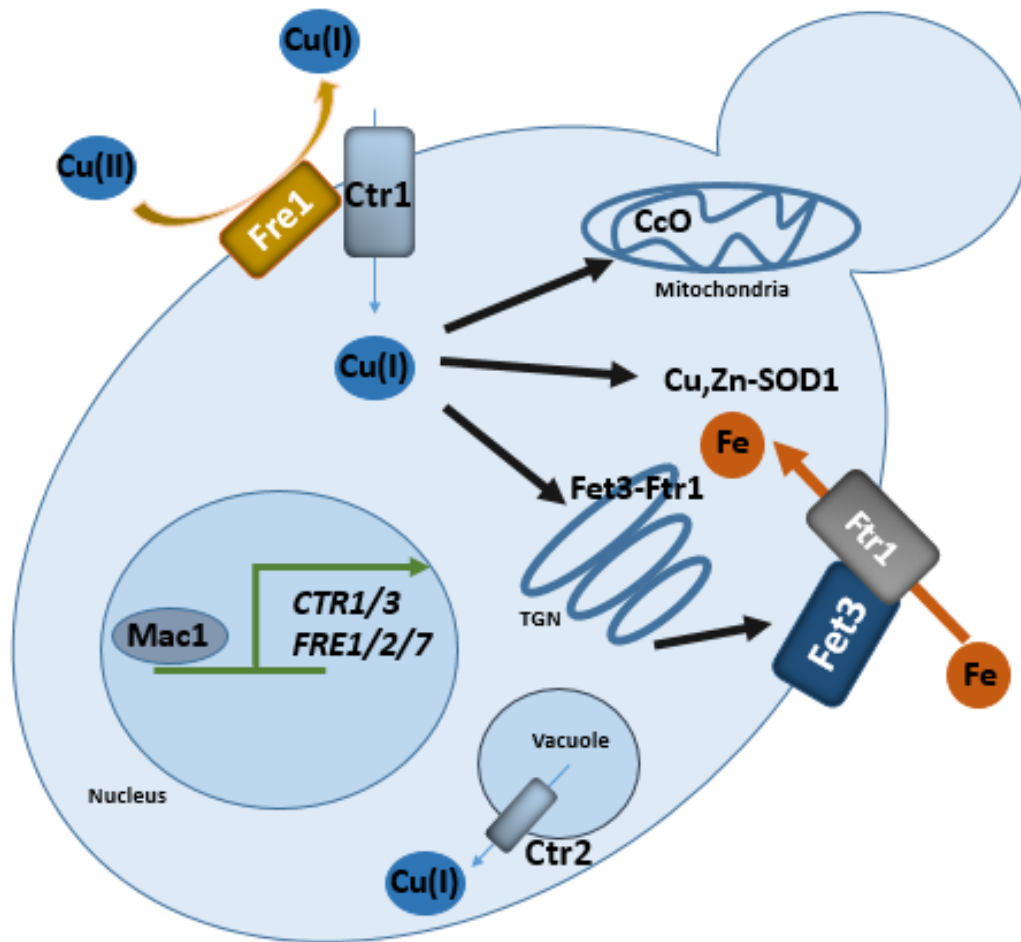


Figure 1.2 - Key player in the yeast response to copper depletion. In low-copper conditions, Mac1, a copper-responsive transcription factor, activates the expression of copper-uptake genes, such *CTR1* and *CTR3*, but also of metalloreductases, as *FRE1/2/7*. Vacuole copper stores can also be mobilised, in this conditions, through Ctr2. In the cell, through the action of metallochaperones, copper can be incorporated in several enzymes, such as superoxide dismutase (SOD1), Fet3 and cytochrome c oxidase (CcO).

Besides the high-affinity transporters, copper can enter cells through low-affinity metal transporters, such as Fet4, Smf1 and Pho84 [40]. Fet4 is a cell surface low affinity iron transporter also involved in the uptake of other metals such as cobalt, cadmium, and copper [41]. Other transporters such as Smf1 and Pho84 are also thought to be able to transport copper to cells, however their significance is low, since Smf1, a manganese transporter, and Pho84, a phosphate transporter, are only active in manganese- and phosphate-starvation conditions, respectively [42, 43]. Furthermore, it was demonstrated that copper transported by Smf1 and Pho84 is not bioavailable, since *ctr1ctr3fet4* mutants are defective in acquiring copper for SOD1 [44].

In the cytosol, free copper has to be minimized to avoid inadequate interactions that could lead to generation of reactive oxygen species (ROS). For this reason, under normal growth conditions, the yeast cell is thought to have less than one free copper atom, suggesting the existence of mechanisms that effectively bind and distribute intercellular copper [45]. This observation led to the discovery of a class of proteins, named metallochaperones, that bind and deliver copper to organelles and proteins [46]. In yeast several classes of metallochaperones have been identified. CCS (Cu Chaperone for SOD1) delivers copper to the Cu,Zn-SOD1 [47]. Cox17 binds and delivers copper to Sco1 and Cox11, that promote the insertion of copper into the active sites of CcO, CuA and CuB, respectively [48]. Atx1 delivers copper to Ccc2 a Cu-P-type-ATPase, orthologue of the human proteins ATP7A and ATP7B [26, 49, 50], that pumps copper into the TGN's lumen, where apoFet3 is loaded with copper [51, 52]

In conditions of copper deficiency, yeast cells can also mobilize intracellular copper stores. Copper is predominantly stored in the vacuole through a mechanism yet to be investigated, as no vacuole copper importer has been identified so far. Copper mobilization from the vacuole is known to be mediated by Ctr2 [31].

1.3.2 Copper overload

Copper accumulation beyond homeostatic levels is highly toxic. Copper cycling between reduced and oxidised state allow it to undergo Fenton reactions, generating hydroxyl radical, an extremely labile molecule that reacts with biomolecules [30]. Additionally, metals, when in excess, can bind metal-sites in metalloproteins causing mismetallation, and consequently protein loss of function.

In 2009, another possible mechanism of copper-mediated cellular toxicity was suggested by Imlay and Macomber [53]. Studies conducted in *E.coli* unravelled that copper is able to interfere with the [4Fe-4S] iron sulphur clusters present in dehydratases. Later, in 2014 Tan *et al.* [54] expanded this concept by showing that copper can also interact with [4Fe-4S]-assembly machinery, in particular with the bacterial proteins IscA and SufA. Isa1 and Isa2 are the yeast orthologues to the aforementioned bacterial proteins [55] and the interference of copper with the iron-sulphur assembly machinery was also observed in *Saccharomyces cerevisiae* [56].

The response to copper-overload is mediated by Cu-chelator proteins, named metallothioneins, a class of small cysteine-rich proteins that have the ability to bind metals, thereby diminishing the toxic intracellular pool of free Cu(I) ions [57]. In addition, superoxide dismutase (SOD1) plays a dual role in detoxifying copper: on the one hand it helps the cell dealing with copper-generated oxidative stress, and on the other hand, being a copper containing protein, it contributes to the decrease of cellular free copper [31, 58] (Figure 1.3).

Excess of copper also triggers the post-translational regulation of the metal high-affinity uptake system, by a mechanism involving Cu-binding to the C-terminal of Ctr1 [59]. This interaction triggers degradation of Ctr1 at the plasma membrane [60]. Indeed a C-terminus truncated version of Ctr1 is functional in transporting copper but displays increased sensitivity to growth in Cu-enriched medium [59]

To fight the toxic effects of copper accumulation, fungal pathogens, such as *Candida albicans*, besides the mechanisms above mentioned also use Cu-export pumps (Crp1, in *C. albicans*) to transport copper out of the cell [61, 62].

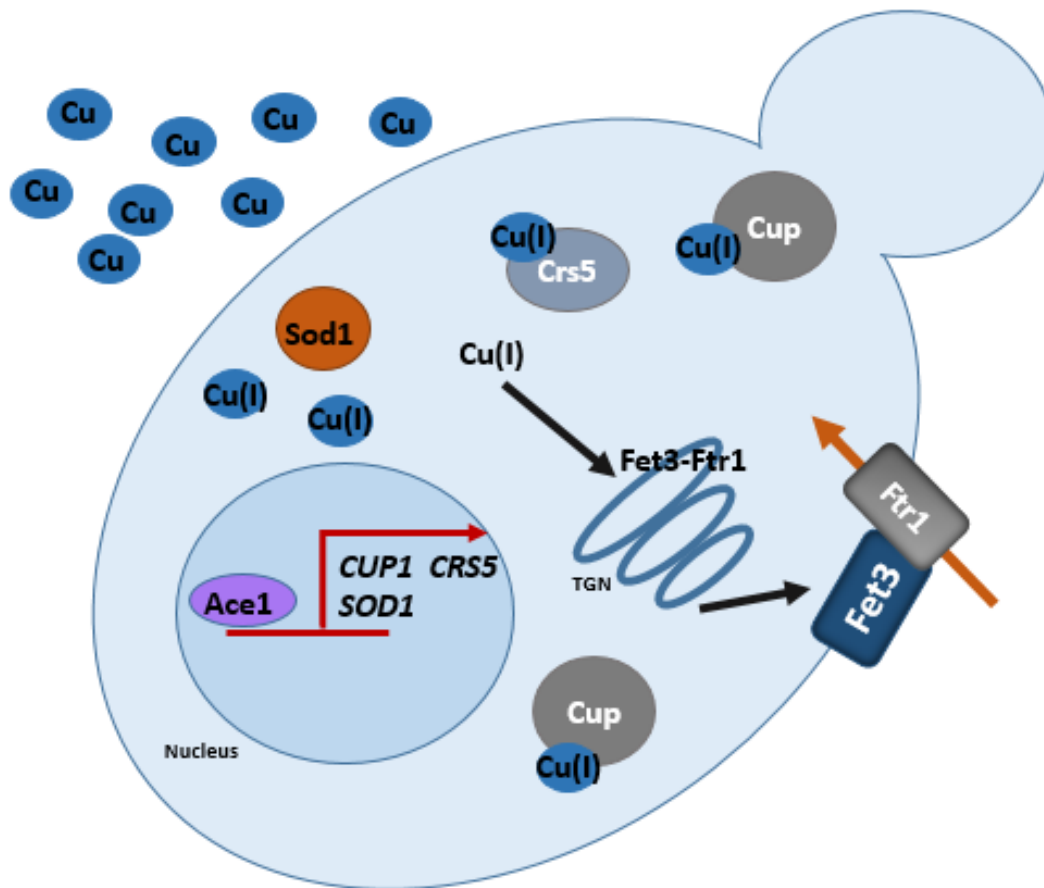


Figure 1.3 - The yeast response to toxic levels of copper. When cells are exposed to copper surplus Ace1 activates the expression of copper-detoxification genes that allow cells to cope with high copper levels. Metallothioneins, encoded by *CUP1* and *CRS5* genes, act as intracellular copper chelators and SOD1 neutralises copper-generated ROS.

1.4 Transcriptional Regulation of Copper homeostasis

The transcriptional regulation of copper homeostasis is orchestrated by two copper-responsive transcription factors: Ace1 and Mac1 [39, 63].

When cells are subjected to copper-depletion, Mac1 activates the expression of genes involved in copper uptake, such as the high-affinity copper transporters, *CTR1/CTR3* and the metalloreductases, encoded by *FRE1*, *FRE2* and *FRE7* genes [64] [39, 65]. Mac1 binds to two tandem TTTGCTCA sequences present in the promoter region of these genes [64]. When copper levels are adequate, Mac1 transcriptional activity is inhibited, by the binding of this metal to the copper regulatory domain of Mac1, causing its release from the DNA with consequent inhibition of gene expression [66].

Yeast cells exposed to high concentrations of copper (above 1 μ M) activate Ace1, a transcription factor involved in the regulation of copper detoxification genes [32, 58]. When copper binds to Ace1, the protein undergoes a conformational change that allows it to bind the cis-element TTNGCTG. Four Cu(I) ions bind to the cysteine-rich regulatory domain of this transcription factor [67]. Ace1 activates the expression of metallothioneins encoded by the *CUP1* and *CRS5* genes [58, 68]. The yeast genome contains 15 tandem copies of *CUP1* [69] and Jensen *et al.* have demonstrated that Cup1 plays the major role in copper detoxification, as cells harbouring a single copy of this gene are more effective in detoxifying copper than cells with one copy of *CRS5* [33]. Interestingly, the tandem amplification of the *CUP1* gene is related with an evolutionary gain of tolerance to copper [70]. In addition to copper-chelators, Ace1 also plays a role in activating the expression of the cytosolic Cu, Zn-SOD1, encoded by *SOD1* [58].

1.5 Crosstalk between copper and iron homeostasis

Several interconnections between iron and copper metabolism are known. In humans, copper is present in multicopper oxidases with ferroxidase activity, ceruloplasmin and hephaestin, two proteins essential for systemic iron homeostasis. Ceruloplasmin mobilises and oxidises iron prior to its association with transferrin, and hephaestin, mainly produced in enterocytes, transfers iron across the basolateral membrane into the circulation [71, 72].

Also in the yeast *Saccharomyces cerevisiae* copper is necessary for the oxidase activity of Fet3, a membrane ferroxidase and the yeast ortholog of ceruloplasmin, that together with Ftr1 mediates the high-affinity uptake of Fe(III) [73]. The assembly of the Fet3-Ftr1 complex occurs in the TGN. Ccc2 a membrane P-type ATPase is responsible for the transport of copper into TGN's lumen [26] Yeast mutant strains lacking Ccc2 have no functional Fet3, as without copper the apoFet3-Ftr1 maturation is impaired and the complex does not reach the plasma membrane [74]

Interestingly, *FET3* gene expression is induced by copper [63, 75]. Indeed, Fet3 appears to have a protective role in copper-detoxification, since deletion of *FET3* renders cells highly sensitive to copper excess [75, 76]. Li and Kaplan proposed that yeast cells lacking Fet3 try to re-establish iron homeostasis by upregulating low-affinity iron transporters, which transport iron but also copper, thereby leading to toxicity [77]. Later, Shi *et al.* demonstrated that Fet3 can use copper as a substrate, and proposed that the oxidation of Cu (I) to the less toxic species Cu(II), by Fet3 may constitute another line of protection mediated by this enzyme against copper toxicity [76].

In yeast, Aft1 is the major regulator of the transcriptional response to iron deficiency. Aft1 is constitutively expressed and in low-Fe conditions accumulates in the nucleus where it binds the DNA consensus sequence PyPyCACCC of its target genes [78]. When intracellular iron levels rise, Aft1 is inactivated by an ISC-intermediate, in a mechanism not yet fully understood, causing the nuclear export of this transcription factor [79]. In conditions of iron deficiency, Aft1 is the main regulator of *FET3* gene expression [78] and Gross *et al.* suggested that under copper surplus conditions Aft1 also upregulates *FET3* [63]. Moreover, the role Aft1 as a regulator of genes involved in copper homeostasis extends beyond *FET3* regulation, as it also controls the expression of *CCC2*, *CTR2* and *FRE1* genes [80, 78].

Objectives

In the yeast *Saccharomyces cerevisiae*, the multicopper oxidase Fet3 appears to have a pivotal role in the crosstalk between copper and iron homeostasis, as it is required to overcome both iron deficiency and copper overload. Accordingly, *FET3* expression is induced in those conditions in a way dependent on Aft1 [63, 78]. At an initial stage of this work, however, while screening for metal-sensitive mutant strains, we found that the mutant *fet3* is more sensitive to copper than the mutant *aft1*. This data indicates that a yet unidentified transcription factor is regulating *FET3* expression under copper surplus conditions. In this context, the main aims of this study were to:

1. Identify the transcription factor that regulates *FET3* expression under copper excess growth conditions.
2. Provide further insights into the interplay between iron and copper homeostasis.

II.

Materials and Methods

2.1 Yeast strains and Growth conditions

Table 1 - Strains used in this study

Strain	Genotype	Source
By4742	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0</i>	Euroscarf
By4742 <i>aft1</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YGL071W:: HIS</i>	Unpublished data
By4742 <i>fet3</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YMR058W::kanMX4</i>	Euroscarf
By4742 <i>ace1</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YGL166W::kanMX4</i>	This study
By4742 <i>arn4</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YOL158C::HIS</i>	This study
BY4742 <i>fet4</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YMR319C::HIS</i>	Unpublished data
By4742 <i>fre1</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YLR214W::HIS</i>	This study
By4742 <i>aft1fet3</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YGL071W:: HIS; YMR058W::kanMX4</i>	This study
By4742 <i>aft1ace1</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YGL071W::HIS; YGL166W::kanMX4;</i>	This study
By4742 <i>fet3fet4</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YMR058W::kanMX4; YMR319C::HIS</i>	This study
By4742 <i>fet3arn4</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YMR058W::kanMX4; YOL158C::HIS</i>	This study
By4742 <i>fet3fre1</i>	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 uraΔ0; YMR058W::kanMX4; YLR214W::HIS</i>	This study
Fet3-TAPTag	BY4741 MAT α <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; YMR058W-TAP::HIS</i>	Yeast Collection (Thermo Scientific-Open Biosystems)
Fet3-TAPTag	BY4741 MAT α <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; YMR058W-TAP::HIS; YGL166W::kanMX4</i>	This study

Saccharomyces cerevisiae strains used in this study are listed on table 1. The deletion mutants constructed in this study were generated by the micro-homology PCR

method [81], using primers listed in supplementary table S1. All the deletion mutants were confirmed by PCR analysis of genomic DNA using upstream and downstream specific primers, generally A1 and A4, which are listed in supplementary table S1. Strains were grown at 30°C in synthetic complete media (SC) (SC: 0.67% ammonium sulphate-yeast nitrogen base without amino acids (Difco), 0.60% Bacto™ Casamino Acids (DIFCO) 2% D-glucose, supplemented with the appropriate amino acids, according to the strains auxotrophic markers) or SC lacking specific components (SD), to ensure plasmid retention or after transformation to select positive mutants.

For long term storage, yeast strains were maintained at -80°C in 43.5% glycerol stock. For continued use strains were plated in YPD (0.3% (w/v) yeast extract, 1% peptone, 1% glucose/dextrose, 2% agar, dissolved in bi-distillated sterile water), grown for 2 days at 30°C and stored at 4°C.

Phenotypic growth assays were carried out by growing cells overnight and inoculating them at 0.1 OD₆₀₀/mL. Cells were grown until they reached early exponential phase (OD₆₀₀ = 0.4-0.6). Five 10-fold serial dilutions were performed to obtain samples ranging from, approximately, 5x10³ to 10 cells. 5 µL of each dilution were spotted in SC or YPD medium containing the concentrations of the mentioned drugs. Growth was recorded after up to 3 days at 30°C. YPDGE was used to assay mitochondrial function, this medium contains almost exclusively non-fermentable carbon sources [82].

To perform the growth curves, 4 biological replicates of each strain were pre-inoculated overnight in 3mL of SC media at 30°C. Cells were inoculated at OD₆₀₀=0.1 with or without 2 mM of CuSO₄. Optical densities at 600nm (OD₆₀₀) were recorded for 24hours.

Escherichia coli strain *XLI-Blue* recA1 edA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F9proAB lacIqZDM15 Tn10 (Tetr)] (Stratagene) was used as the host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation.

2.2 Plasmids

To construct the p*FET3*-*LacZ* plasmid, *FET3* promoter sequence was amplified by PCR with specific primers Fw_p*FET3* and Rv_p*FET3*, which contain XbaI and KpnI restriction sites, respectively. The PCR product was digested with XbaI and KpnI. The resulting fragment was inserted into XbaI and KpnI digested yEP356R vector, which contains the *lacZ* reporter gene, using T4 DNA ligase (New England BioLabs). For successful ligations 15ng of vector were used and ideally 1µg of insert fragment. The ligation product was used to transform *E. coli* competent cells and transformants were selected by plating cells in LB agar plates supplemented with Ampicillin 100µg/mL

2.3 Yeast competent cells preparation

The procedure used for the competent yeast cells preparation was the one described by Gietz *et al.* [83], with few modifications.

Cells were grown overnight in YPD, to reach stationary phase, and then inoculated at 0.2 OD₆₀₀/mL, in 25mL YPD media. Cells were then grown 5 hours at 30°C until OD₆₀₀=0.7-0.8. The cell culture was centrifuged at 3600 rpm for 2 minutes, supernatant was discarded and the pellet was washed with bi-distillated sterile water and a similar centrifugation was performed. Cells were resuspended in 1 mL LiAc/TE solution (100 mM Lithium acetate, TE 1X) and transferred to a 2 mL eppendorf. After centrifugation, cells were resuspended in 400 µL of LiAc/TE solution and stored at 4°C for up to 5 days.

2.4 Yeast transformation

Approximately, 1 µg of the disruption cassette DNA or 300 ng of plasmid DNA were mixed with 5 µg of carrier DNA (salmon sperm DNA [SIGMA]) and 300 µL of PEG3350 solution (40% PEG3350; 1X TE; 100 mM lithium acetate), and added to 50 µL of competent cells. The mixture was incubated for 30 minutes at 30°C, 40µL of DMSO was added, followed by heat shock at 42°C for 15 minutes. 800 µL of bi-distillated sterile water was added followed by centrifugation at 5000 rpm for 1 minute. Cells were resuspended in 1 mL of YPD, and incubated for 3 hours at 30°C. Cells were then collected by centrifugation using the same conditions as above stated, resuspended in 200 µL of bi-distillated sterile water and plated in appropriate selective media.

2.5 Western blot assays

Western blot analyses were performed using early exponential phase cells (Fet3-TAPTag and *ace1* Fet3-TAPTag, challenged with the indicated concentrations of CuSO₄ and harvested at the indicated time points. Total protein extracts were obtained from 30 mL of cell culture using Cell Lysis Buffer (50 mM HEPES , pH7.5; 100 mM KCl; 1mM EDTA;: 0.1% NP-40; 1 % glycerol) supplemented with 1mM PMSF and 1X protease inhibitors (ROCHE) and glass beads, as follows:

After harvesting, cells were washed with 800 µL of cold bi-distillated sterile water and centrifuged at 14000 rpm for 1 minute. The supernatant was discarded and cells were re-washed with 800 µL Cell Lysis Buffer and centrifuged in the same conditions. Glass beads and 200 µL Cell Lysis Buffer were added to the remaining pellet and the mixture was vortexed for 15 minutes a 4°C. After centrifugation at 14000 rpm for 30 minutes at 4°C, the supernatant (the protein extract) was collected to a new tube. The protein extracts were quantified using the Bradford method (BRADFORD BIORAD), and the absorbance was read at 595nm, in a SmartSpec™ 3000 spectrophotometer (BIORAD).

Approximately, 100 µg of each protein sample was resolved by SDS-PAGE. The resolving 12% poly-acrylamide gel was prepared with 1.2mL acrylamide/Bis-Acrylamide (37.5:1), 1mL Tris-HCl 1.5 M pH8.8, 1.75 mL H₂O, 20 µL SDS 20%, 40 µL APS 10% and 4µL TEMED. After polymerization of the resolving gel, the stacking 5% poly-acrylamide gel was added (0.625 mL Acrylamide/Bis-acrylamide mix 40% (39:1); 0.63 mL Tris-HCl 1M pH608, H₂O 3.645 mL, 25 µL SDS 20%, 50 µL PSA 10% and 5 µL TEMED). Each sample was loaded into a lane along with a molecular-weight marker (Precision Plus Protein™ Standards: Dual Colour [BIORAD]). The electrophoresis was performed at 100 V in a BIORAD Mini-Protean® II with running buffer 1 X (STOCK 10X: Tris 250 mM, glycine 2M and 1% (w/v) SDS), for 2hours or until the samples reached the bottom of the gel.

After the run, the gel and the nitrocellulose membrane were readily placed in Transfer Buffer 1X for 10 minutes (20 mL Transfer Buffer Stock: 20mL methanol; 185µL SDS 20%; make up with bi-distillated water to a total of 100mL). The transfer system was assembled in the following way: 6 sheets of Hybond™ blotting paper (Amersham™) paper wet in buffer, nitrocellulose membrane (Amersham), SDS-page gel and 6 sheets of

Hybond™ blotting paper. The transfer to the membrane was performed during 50 minutes at 15 V in a semi-dry system (ATTA Trans-Blot SD Transfer Cell [BIORAD]). The success of the protein transfer to the nitrocellulose membrane was monitored by membrane incubation with Ponceau S solution (30% (w/v) Ponceau S and 30% (w/v) sulphosalicylic acid) diluted 1:10 in bi-distilled water. The membrane was washed and then incubated with 30mL of a solution of PBS 1X, Tween-20 0.1% with 5% low-fat milk, for 45 minutes at room temperature, to block unspecific interactions with the antibody. Protein levels were detected by incubation with the primary antibody for 1hour at room temperature and with the Horseradish peroxidase-conjugated secondary antibody in the same conditions. The antibodies used in this study are listed in Table 2. Between antibodies' incubation and after the incubation with the secondary antibody, the membrane was washed 3 times during 10 minutes with PBS 1X Tween-20 0.1% solution. The detection system used was the chemo-luminescent SuperSignal® West Fento Maximum Sensitivity Substrate (Thermo). The membrane was then exposed to an autoradiograph film (Hyperfilm MP, Amersham). 3-PhosphoGlycerate Kinase (Pgk1) was used as loading control. Western-blots were repeated at least twice with different protein extracts.

Table 2 - Antibodies used in this study

Protein Detected	Primary Antibody	Secondary Antibody
Fet3-TAPTag	Antibody Anti-TAP (Sigma) (Anti-TAPTag 1:5000 in PBS 1X Tween-20 0.1%)	Horseradish peroxidase- conjugated anti-Rabbit IgG (Santa Cruz Biotechnology) (Anti-rabbit IgG (1:5000) in PBS 1X Tween-20 0.1%)
Pgk1	Antibody anti-3-phosphoglyceric Phosphokinase (Pgk1) (novex) (Anti-Pgk1 1:5000 in PBS 1X Tween-20 0.1%) (Life Technologies)	Horseradish peroxidase- conjugated anti-Mouse IgG (Santa Cruz Biotechnology (Anti- mouse IgG (1:5000) in PBS 1X Tween-20 0.1%))

2.6 Measurements of β -galactosidase activity

The procedure used for the measurement of β -galactosidase activity was the one described by Möckli *et al.*[84], with few modifications.

WT(BY4742) and *ace1* strains were transformed with pFET3-LacZ plasmid. The transformed cells were grown in SD medium (without uracil) to early exponential phase ($OD_{600} = 0.5$), and incubated with 0.5 mM of $CuSO_4$ for 30 minutes. Cells were harvested by centrifugation resuspended in LacZ Buffer (10.6 mg/mL $Na_2HPO_4 \cdot 2H_2O$; 5.5 mg/mL $NaH_2PO_4 \cdot H_2O$; 0.75 mg/mL KCl and 0.25 mg/mL $MgSO_4 \cdot 7H_2O$; pH 7, dissolved in H_2O) supplemented with 270 μ L β -mercaptoethanol per 100 mL LacZ Buffer. Cells were permeabilized by adding 60 μ L of chloroform and vortexing for 45 seconds. 100 μ l of ortho-Nitrophenyl- β -galactoside (ONPG) solution (4 mg/mL dissolved in KPO_4 Buffer 0.1M (0.1M: 61 mL K_2HPO_4 0.2M; 39 mL KH_2PO_4 0.2M) sterile water was added to cells and enzymatic activity was assayed by following the degradation of the colorimetric substrate ONPG at A_{420m} , using the following equation:

$$\beta - Galactosidase \ activity = \frac{(Abs_{420} - (1.75 \times Abs_{550})) \times 1000}{t \times V \times OD_{600}}$$

Where t stands for time in minutes and V for the total reaction volume. Enzyme activity was normalized against optical density (OD_{600}). Absorbance measurements were performed in an Epoch BioTek® equipment

2.7 Measurement of total iron and copper intracellular content

Triplicates of the indicated strains were grown in 3 mL of SC medium and cultured until reach stationary phase. Cells were then diluted to a final $OD_{600} = 0.1$ in 40 mL of SC and left untreated or treated with 2mM of $CuSO_4$ for 15 hours. Cells were then collected by centrifugation at 4000 rpm for 5 minutes. The pellet was washed with 5 mL EDTA 10mM pH8, and twice with miliQ water (Q-POD® MiliQwater – Milipore). The pellet was resuspended with 5mL miliQ water and OD_{600} was measured. 4mL of the cell resuspensions were transferred to HS23204N tubes (Heathrow Scientific® LLC) and centrifuged at 4000g for 5 minutes. Supernatant was discarded and 1mL of Nitric Acid 10% (v/v) was added to the cells. The samples were digested for 18h at 80°C. Samples were centrifuged at 1200rpm for 5 minutes and 2mL of the supernatant were sent to analysis. Samples were analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) at the service of Mass Spectrometry of REQUIMTE. Data obtained were normalized against OD_{600} .

2.8 qRT-PCR

Biological triplicates of the indicated strains were grown until early exponential phase ($OD_{600} = 0.4 - 0.5$), induced with 2mM of $CuSO_4$ and harvested (30mL) at the indicated time points, by centrifugation at 3000 rpm for 2 minutes. Total cellular RNA content was extracted using the phenol-chloroform method. Cells were collected by centrifugation and the pellet was resuspended in 1 mL of cold sterile bi-distilled water, transferred to a microcentrifuge tube and again centrifuged in the same conditions, to remove the supernatant. 350 μ L of Acidic phenol: chloroform (1:1) and 350 μ L of LETS Buffer (LETS Buffer: 0.1 M LiCl; 0.01M Na_2EDTA ; 0.01M Tris-HCl pH 7.4; 0.2% SDS, dissolved in bi-distilled sterile water) was added to the samples. Samples were then vortexed 5 minutes at 4°C, and centrifugation at 14000rpm to separate the organic from the aqueous fraction. The aqueous fraction was transferred to a microcentrifuge tube, 1 mL of ethanol 100% was added and RNA was precipitated overnight at -20°C. RNA was collected by centrifugation at 14000 rpm for 15 minutes and the pellet was resuspended in 200 μ L of ethanol 70% and centrifuged at 14000 rpm for 30 minutes. The pellet was air-dried and then resuspended in 100 μ L of RNase free water and incubated 15 minutes at 65°C to help the solubilisation of the pellet. RNA samples were treated with DNase (TURBO DNase [Ambion®]) according to manufacturer's instructions, at 37°C during 40minutes. RNA samples were then purified using RNeasy® Mini Kit (QIAGEN). cDNA synthesis was performed using "Transcriptor Reverse Transcriptase" (ROCHE). qRT-PCR reactions were prepared using the oligonucleotides described in table S1. Detection of the qRT-PCR products was done using the DNA intercalating compound SYBR Green (LightCycler LC-Faststart DNA MASTER SYBR Green I, (ROCHE)) and the qRT-PCR reaction was performed in a LightCycler® 480 equipment (ROCHE). Data was analysed with the software LightCycler® Software 4.1. Gene expression was normalized relatively to *ACTIN* mRNA levels. Two technical replicates were performed for each sample.

III. Results

3.1 Aft1 is not the major regulator of *FET3* gene under copper excess conditions

Gross *et al.* while identifying the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays noticed that elevated copper levels also induced the expression of the high affinity iron uptake system composed of Fet3 and Ftr1 [63]. The authors further suggested that copper-induced expression of *FET3* and *FTR1* arose from an indirect copper effect on cellular iron pools and was mediated by Aft1 [63].

In this work we have examined the growth phenotype of wild-type, (WT) *aft1* and *fet3* strains in the presence of copper excess (Figure 3.1). Unexpectedly, we found that a strain lacking Fet3 is more sensitive to copper surplus conditions than a strain carrying the *AFT1* gene disrupted (Figure 3.1). If Aft1 is the main regulator of *FET3* expression under such conditions, one would expect that the *aft1* mutant to be more sensitive than, or as sensitive as, the *fet3* mutant. This finding suggests that *FET3* has another yet unidentified regulator under copper loading conditions. Moreover, an epistatic analysis revealed that Fet3 cannot be downstream of Aft1 as the double mutant *aft1fet3* has a more pronounced growth defect in copper surplus compared to *fet3* and *aft1* single mutants (Figure 3.1).

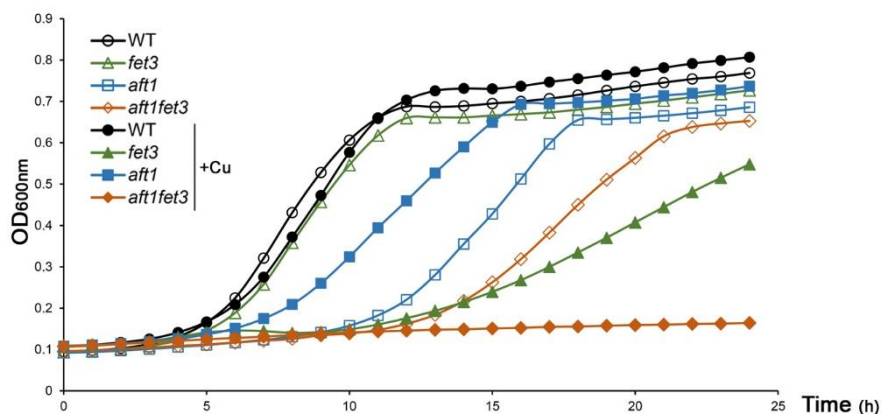


Figure 3.1 - Under copper overload, Aft1 is not the major regulator of *FET3* gene. A: Growth exhibited by the WT, *aft1*, *fet3* and *aft1fet3* strains in SC media was recorded over a period of 24 h. Closed symbols and open symbols represent strains grown in media containing or not 2mM of CuSO₄, respectively. Each colour represents one of the used strains: WT is represented in black, *aft1* in blue, *fet3* in green and *aft1fet3* in orange.

3.2 Copper accumulation observed in the *fet3* mutant strain is dependent on Aft1

Aiming at understanding why the *fet3* mutant is more sensitive to copper excess than the *aft1* strain, we measured, by inductively coupled plasma atomic emission spectroscopy (ICP-AES), the intracellular copper and iron concentrations of both strains in copper overload conditions (Figure 3.2 A and B, respectively). We observed that cells lacking *FET3* are iron depleted (Figure 3.2 B) and accumulate over 80% more copper than *aft1* or wild-type cells (Figure 3.2 A), which is consistent with *fet3* sensitivity to high copper. This finding has been reported by Li *et al.* [77], but here we show that *fet3* excessive accumulation of copper is mediated by Aft1. Indeed, deletion of this transcription factor from the *fet3* background, avoids copper accumulation (Figure 3.2A). The activation of Aft1 in the *fet3* mutant is probably due to the iron depletion observed in this strain, after copper treatment (Figure 3.2B).

In an attempt to identify the players involved in the above mechanism, we search in the literature for Aft1 targets known to encode membrane proteins and previously associated with copper transport. We found *FET4*, *FRE1* and *ARN4* as promising candidates. Fet4 is a cell surface low affinity iron transporter able to transport other divalent metals, including copper [40, 85]. Fre1 is a membrane metalloreductases involved in the uptake of iron and copper [64, 78]. Arn4 is a siderophore-iron transporter, whose substrate is enterobactin [86]. *ARN4* expression increases in high copper and its deletion renders cells resistant to copper [87]. To assess the involvement of these proteins in copper accumulation of *fet3* mutant, *FET4*, *FRE1* and *ARN4* genes were deleted from the *fet3* background, and phenotypic growth assays were performed under copper loading conditions (Figure 3.2C). Deletion of each of the gene increase the resistance to copper compared with *fet3*, with the effect of *FRE1* being the most pronounced (Figure 3.2C). None of the deletions, however, was able to fully restore cells growth in high copper. Interestingly we observed that supplementation of the growth medium with zinc or iron attenuates *fet3* sensitivity to copper (Figure 3.2 D and E, respectively), which is consistent with the involvement of low-affinity metal transporters in this phenomenon.

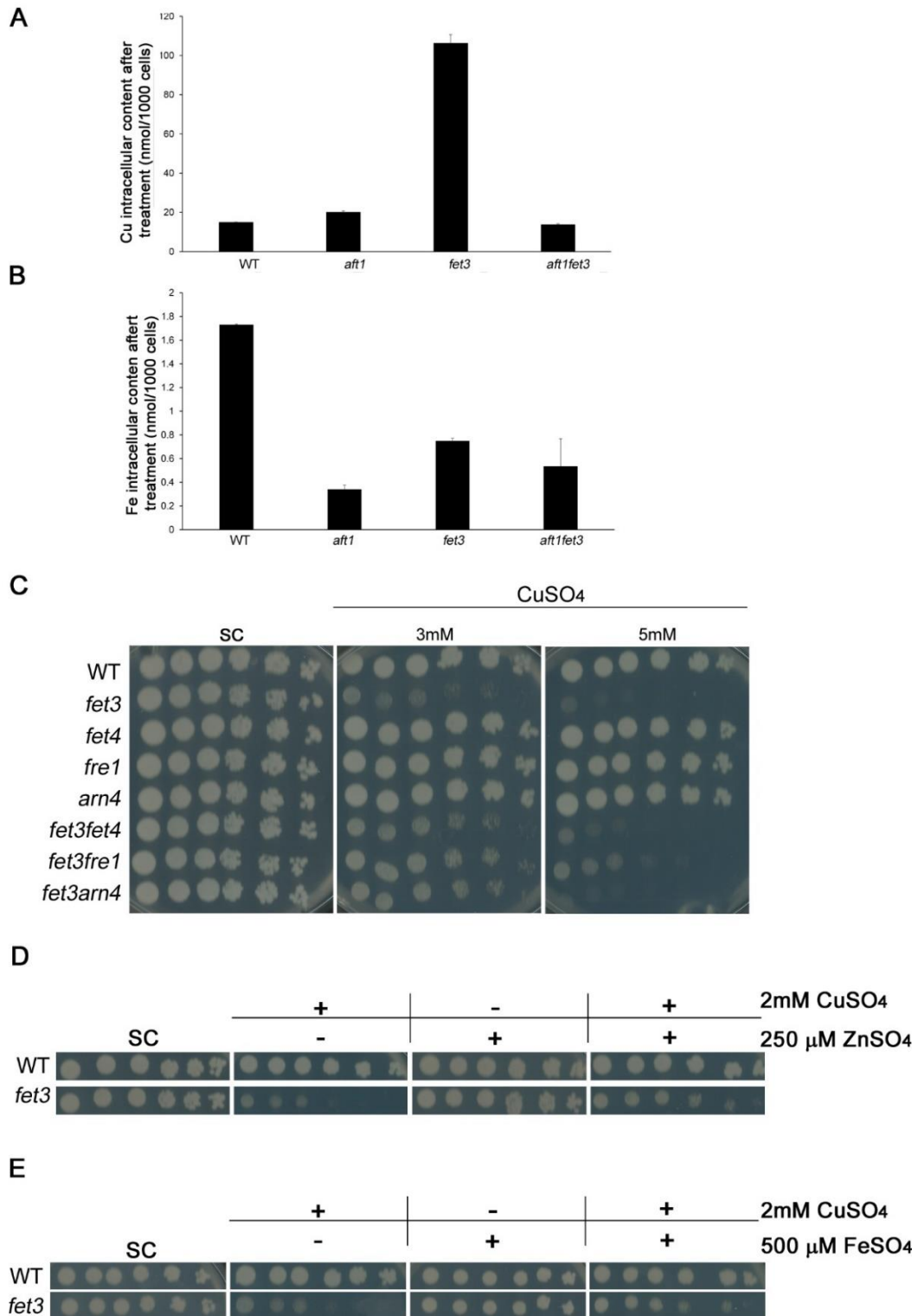


Figure 3.2 - *fet3* copper accumulation is dependent on Aft1. A: Copper (Cu) content of WT, *aft1*, *fet3* and *aft1fet3* strains were determined by ICP-AES, in SC medium after treatment with 2mM CuSO₄ for 15 h. B: Iron (Fe) content of WT, *aft1*, *fet3* and *aft1fet3* strains were determined by ICP-AES, in SC medium after treatment with 2mM CuSO₄ for 15 h. C: Growth sensitivity of WT, *fet3*, *fe4*, *arn4*, *fre1*, *fet3fet4*, *fet3arn4* and *fet3fre1* strains in SC plates and SC plates containing 3 or 5mM CuSO₄. D: Growth sensitivity of WT and *fet3*, in SC plates and SC plates containing 2mM of CuSO₄ supplemented or not with 250µM ZnSO₄. E: Growth sensitivity of WT and *fet3*, in SC plates and SC plates containing 2mM of CuSO₄ supplemented or not with 500µM FeSO₄.

3.3 Ace1 regulates *FET3* expression under high-copper conditions

Because the transcriptional response to copper overload is dependent on Ace1 [32, 58], we put forward the hypothesis that Ace1 could play as well a role in regulating the expression of *FET3*. To test this, we first analysed *FET3* promoter sequence using the online tool YEASTRACT [88] and found a putative Ace1 binding-site located at -578 bp from the ATG (+1) (Figure 3.3A). Next, a plasmid including the promoter region of *FET3* fused to the *lacZ* reporter gene (*pFET3-lacZ*) was generated and used to transform the WT and *ace1* mutant strains. We observed that β -Galactosidase activity was higher in the WT compared to the mutant strain (Figure 3.3B). The dependence of *FET3* on Ace1 was confirmed by the analysis of the levels of *FET3* transcripts in both strains, either in the absence or presence of copper, by qRT-PCR (Figure 3.3C). Under non-stressed conditions (time point 0) both strains exhibit similar levels of *FET3* mRNA, which is in agreement with the hypothesis that Ace1 only regulates *FET3* under copper overload. To further demonstrate an Aft1-independent role of Ace1 in *FET3* regulation, *AFT1* was deleted from the *ace1* background and *FET3* mRNA levels were followed by qRT-PCR. As depicted in Figure 3.3D, in the absence of Aft1, it was possible to observe a marked induction of *FET3* expression that was, however, completely abrogated upon deletion of *ACE1*.

Finally, we have also assessed the protein expression of a TAP-tagged version of Fet3 driven by its native promoter. To this end, the *ACE1* gene was deleted from a strain with a TAP-tagged genomic version of *FET3* (WT) and Fet3 expression was monitored by Western blot using an anti-TAPtag antibody. In agreement with the gene expression data, we observed that protein levels were consistently higher in the WT compared to the *ace1* mutant strain (Figure 3.3 E).

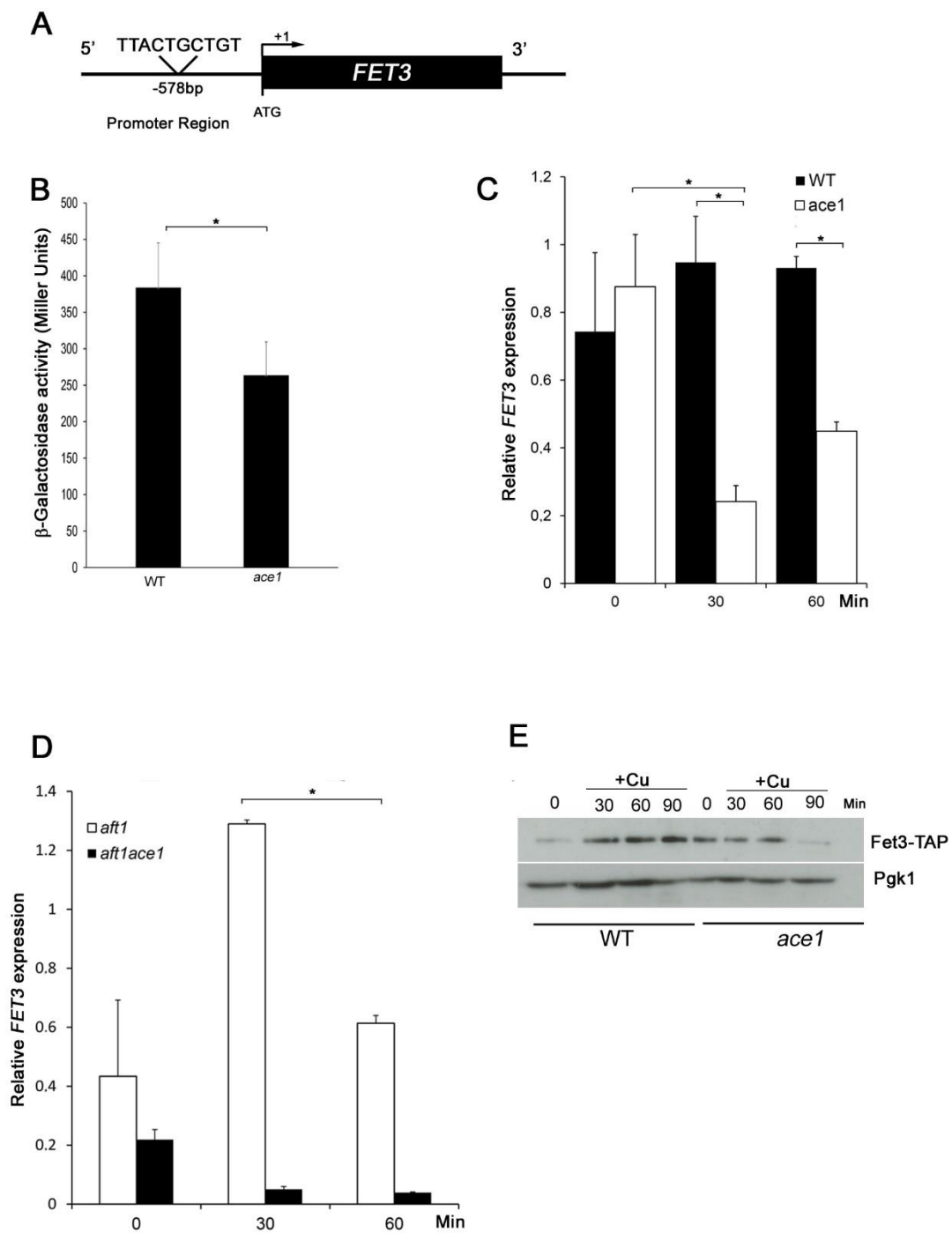


Figure 3.3 - *Ace1* regulates *FET3* expression under copper excess conditions. A: Schematic representation of the *FET3* promoter region containing the one putative *Ace1*-binding site located at -578 upstream of the ATG. B: WT and *ace1* mutant strains were transformed with the *pFET3-lacZ* plasmid. Cells were grown exponentially in SD medium and challenged with 0.5mM CuSO_4 for 30 min. β -Galactosidase activity was assayed as detailed in Materials and Methods. C: WT (wild-type) and *ace1* strains were challenged with 2mM CuSO_4 and harvested at the indicated time points. The expression of *FET3* was monitored by qRT-PCR. D: *aft1* and *aft1ace1* strains were challenged with 2mM CuSO_4 and harvested at the indicated time points. The expression of *FET3* was monitored by qRT-PCR. E: The WT *Fet3*-TAP and the *ace1* *Fet3*-TAP strains were incubated with 2 mM CuSO_4 or the indicated times. *Fet3* protein levels were analysed by Western-blot with an anti-TAP antibody. *Fet3* protein levels were normalized to *Pgk1*. Significance of differences was calculated with the t-test (* $p < 0.05$).

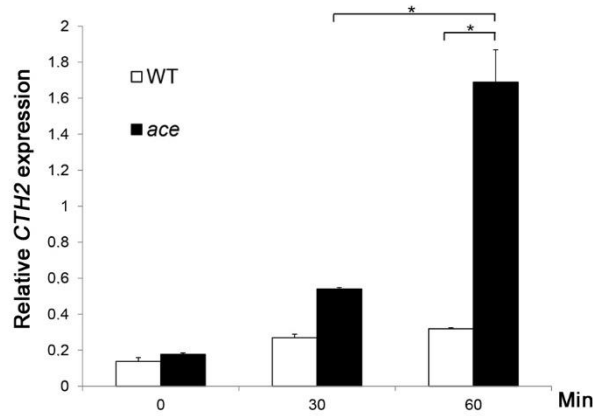
3.4 Ace1 restricts Aft1 activation under copper excess conditions

In this work we show that in the absence of *FET3* cells accumulate high amounts of copper in an Aft1 dependent manner (Figure 3.2A), which is deleterious to the cell. We suggest that iron depletion caused by deletion of *FET3* leads to the activation of Aft1 that in turn activates genes involved in low-affinity metal uptake. In this context it is reasonable to hypothesize that Ace1 activation of *FET3* under copper loading serves to prevent cells from sensing iron depletion, which would activate Aft1 and, consequently, low-affinity iron transporter that would transport copper to the cell.

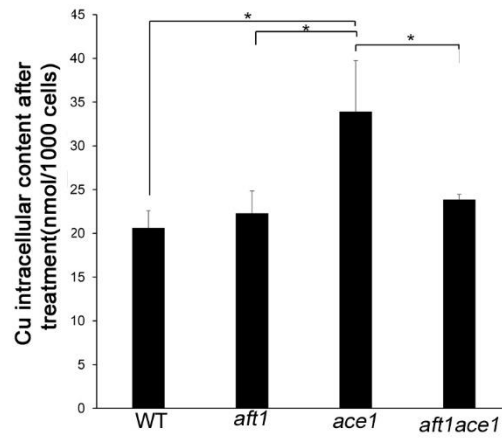
As a first approach to test this hypothesis, Aft1 activity in WT and *ace1* strains was monitored by evaluating the expression of *CTH2*, a target gene of Aft1 [89], by qRT-PCR (Figure 3.4 A). After copper treatment, the expression of this gene is upregulated in both strains, but a highly pronounced increased in *CTH2* mRNA levels (about 9-fold) was observed in *ace1* cells 60 minutes after copper exposure, suggesting that Aft1 is more active in this strain (Figure 3.4A). In this sense, the unexpected increase of *FET3* mRNA levels in *ace1* cells observed after 60 minutes of induction with copper (Figure 3.2 C) may also be mediated by Aft1. Corroborating our hypothesis we found that *ace1* cells exposed to a prolonged copper treatment (15 hours) exhibit higher intracellular copper and iron levels compared to WT, as measured by ICP-AES. (Figure 3.4 B and C). As expected, copper and iron contents in the *ace1* strain were dependent on Aft1, suggesting a role of Ace1 in preventing Aft1 activation.

Overall these results suggest that Ace1 regulates *FET3* gene to prevent Aft1 hyper-activation.

A



B



C

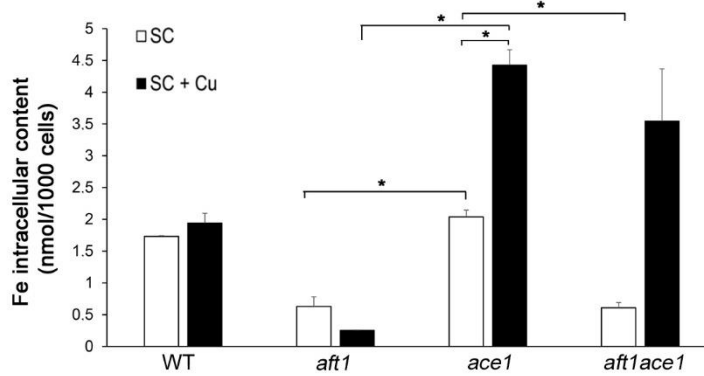


Figure 3.4 - Ace1 prevents Aft1 hyper-activation under copper excess conditions. A: WT (wild-type) and *ace1* strains were challenged with 2mM CuSO₄ and harvested at the indicated time points. The expression of *CTH2* was monitored by qRT-PCR. B: Copper content of WT, *aft1*, *ace1* and *ace1aft1* strains, after treatment with 2mM CuSO₄ (+Cu) for 15 was determined by ICP-AES. C: Fe content of WT, *aft1*, *ace1* and *ace1aft1* strains were determined by ICP-AES, in SC medium or after treatment with 2mM CuSO₄ (+Cu) for 15 h. Significance of differences was calculated with the t-test (* p<0.05)

3.5 Copper rescues the growth defect exhibited by the *aft1* strain

Remarkably, we found that copper alleviates the growth deficiency exhibited by the *aft1* mutant under normal growth conditions (Figure 3.1A), being the effect more evident the higher was the concentration of copper tested (Figure 3.5A). As Ace1 regulates *FET3* in copper surplus (figure 3.3) and as iron supplementation also attenuates *aft1* growth defect (Figure 3.5B), we first reasoned that copper could relieve the iron depletion sensed by the *aft1* strain. To test this hypothesis, we measured by ICP-AES the intracellular levels of iron in WT and *aft1* strains, after copper treatment. Contrary to our expectation, we observed that the iron content of the *aft1* mutant was decreased by approximately 50% in the presence of copper (Figure 3.5C). As Aft1 regulates several genes of copper metabolism we also tested, whether *aft1* cells could be copper deficient, before copper treatment. ICP-AES analyses of copper contents led us to rule out this possibility, as intracellular copper levels are similar in both strains (Figure 3.5D).

Because differences in iron and copper levels between *aft1* and WT strains could not explain the growth fitness of Aft1 after of copper supplementation, we decided to explore whether cellular processes dependent on copper were affected in the mutant *aft1*. Copper is an essential player in respiratory metabolism and oxidative stress regulation, as it is a cofactor for cytochrome C oxidase (CcO) and Cu,Zn-Superoxide dismutase (SOD1), respectively [28, 90]. Iron-depleted cells and *aft1* mutants are hypersensitive to oxidative stress and defective in respiratory growth [91]. Accordingly, SOD1 is induced in high copper conditions by Ace1 [58] and copper supplementation increases growth fitness when cells are cultured in medium containing a non-fermentable carbon source [36, 92]. As such, we next tested if copper was rescuing *aft1* growth defect by promoting the respiratory metabolism and/or strengthening the oxidative stress response.

To examine the respiratory metabolism, the growth of WT and *aft1* strains was inspected in medium containing almost exclusively non-fermentable carbon sources (YPDGE), which is commonly used to assess mitochondrial function [82]. We observed that *aft1* is sensitive to growth in YPDGE, a finding which is consistent with the knowledge that *aft1* strains are defective in mitochondrial function [91], and we found that copper supplementation to the medium rescues growth (Figure 3.5E).

Finally, as a first approach to understand whether the oxidative stress response could be enhanced in the *aft1* strain upon copper addition, we monitored the growth of WT and *aft1* strains in the presence of paraquat and copper, (Figure 3.5F). Paraquat is a redox-cycling drug that produces superoxide anion [93]. We noticed that *aft1* is sensitive to the drug, but the growth deficiency is significantly attenuated by copper (Figure 3.5F).

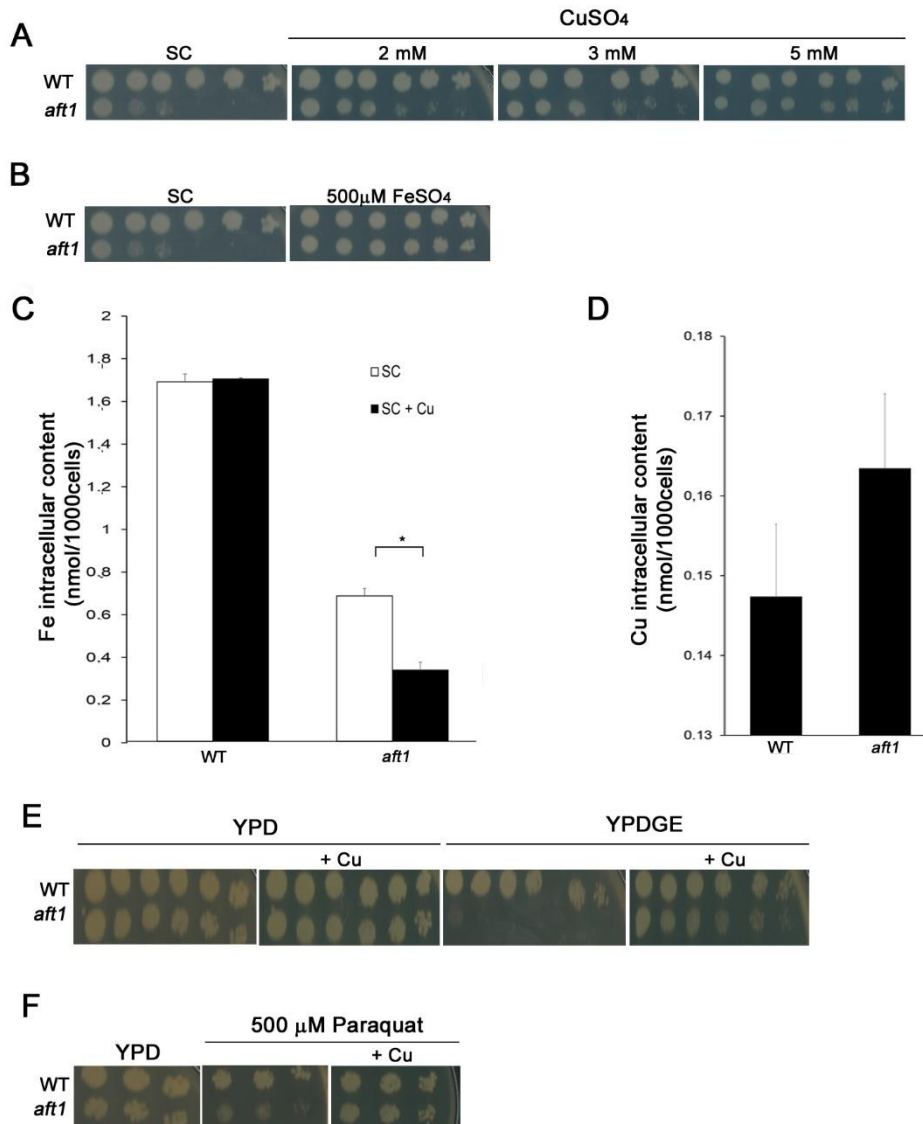


Figure 3.5 - Copper rescues the growth defect exhibited by the *aft1* strain. A: Exponentially growing WT and *aft1* were serially diluted and spotted onto SC plates and SC plates supplemented with 2, 3 or 5 mM CuSO₄. Growth was recorded at 48 hours incubation at 30°C. B: Exponentially growing WT and *aft1* were serially diluted and spotted onto SC plates and SC plates supplemented with 500 μM FeSO₄. Growth was recorded at 48 hours incubation at 30°C. C: Fe content of WT and *aft1* strains were determined by ICP-AES, in SC medium or after treatment with 2 mM CuSO₄ (+Cu) for 15 h. D: Copper content of WT and *aft1* strains were determined by ICP-AES, in SC medium. E: Growth sensitivity of WT and *aft1* in YPD and YPDGE plates containing 2 mM CuSO₄ (+Cu). F: Growth sensitivity of WT and *aft1* in YPD containing 500 μM of Paraquat, supplemented with 2 mM CuSO₄ (+Cu). Significance of differences was calculated with the t-test (* p<0.05).

IV. Discussion

In *Saccharomyces cerevisiae*, the response to copper overload is regulated by Ace1, a copper-responsive transcription factor that activates Cu-detoxification genes, such as *CUP1* and *CRS5*, which encode proteins involved in the sequestration of free copper ions but also *SOD1* that mediates the detoxification of reactive oxygen species generated by Cu-driven Fenton reactions and acts as a cytosolic buffer of free copper ions [31, 32, 58]. In this study another role of Ace1 in copper detoxification was unveiled.

The data here presented clearly shows that in conditions of copper overload, Ace1 is the major regulator of *FET3* gene. Indeed both protein and transcript levels of Fet3 are decreased in the mutant *ace1* (Figure 3.3). Other authors had already reported that copper induced the expression of *FET3*, yet the mechanism underlying this regulation remained unclear [63, 75]. Interestingly, the idea that Ace1 might play a role in *FET3* regulation was addressed by Gross *et al.* [63]. The authors demonstrated that *ace1* cells failed to display copper-induced expression of *FET3*. They also showed that a reporter construct containing two putative Ace1-binding sites found in *FET3* promoter was not able to drive the expression of the reporter gene. These findings, together with the dependence of *FET3* on Aft1, led them to conclude that a possible transient copper-induced decrease in cellular iron pools would activate Aft1 and, consequently, *FET3* expression. However, since the putative Ace1 sites were not shown, we cannot exclude the possibility that the chosen sequences were not relevant for Ace1 mediated gene activation, which would explain the discrepancy between our results and those of Gross *et al.* Moreover, in this work, two lines of evidence suggest that the dependence of *FET3* on Ace1 is not mediated by Aft1. First, the *fet3* mutant is more sensitive than the *aft1* mutant (Figure 3.1). Second, *FET3* expression is induced in the *aft1* mutant in an Ace1-dependent manner (Figure 3.3 D).

Several authors had reported that Fet3 is important for Cu-detoxification and that its deletion leads to copper accumulation, which results in toxicity and cell death [76, 77]. Here we demonstrate that the hyper accumulation of copper in the *fet3* mutant is fully dependent on Aft1 (Figure 3.2 A). We propose that Aft1 activation in such conditions is triggered by the iron-deficiency status of *fet3* cells (Figure 3.2B). In such conditions, Aft1 would induce the expression of low-affinity iron transporters that would massively transport copper, given its abundance in the extracellular milieu. We show that at least three Aft1 targets (*FET4*, *ARN4* and *FRE1*) may be involved in this process, suggesting that the excessive uptake of copper observed in *fet3* mutant is probably the consequence of the upregulation of several low-affinity membrane transporters (e.g. *FET4*) and/or

genes involved in iron uptake (e.g. *ARN4* and *FRE1*), rather than the action of a single protein.

The finding that Aft1 activity could be deleterious to cells under copper excess, led us to put forward the hypothesis that Ace1 regulation of *FET3* gene is serving the purpose of avoiding Aft1 activation. If copper induces a transient decrease of intracellular iron pools, the activation of *FET3* via Ace1 would re-establish iron homeostasis without the need of Aft1 activation. According to this hypothesis, after copper addition, we observed a drastic increase in the expression of Aft1 target gene *CTH2* in the *ace1* mutant (Figure 3.4 A), suggesting that *ace1* mutants cells experience iron depletion and consequently hyper-activate Aft1 (Figure 3.4A). Accordingly, copper and iron accumulation observed in the *ace1* mutant, after prolonged treatment with copper (15 hours), is dependent of Aft1 (Figure 3.4 B and C, *ace1* strain vs. *aft1ace1* strain).

Interestingly, we found that the double mutant *aft1fet3* is more sensitive to copper (Figure 3.1) than *fet3* and *aft1* single mutants, although it has a lower copper content as compared to *fet3* (Figure 3.2 A). These data suggest that Fet3 role in copper toxicity goes beyond the avoidance of copper uptake by low-affinity iron transporters. In line with these results, the Thiele group proposed that Fet3, being a copper-protein, also detoxifies copper by scavenging copper ions [75].

Finally in this work we also report that copper increases *aft1* mutant fitness (Figure 3.5 A). Iron-deprived cells are defective in growing in non-fermentable carbon sources, such as glycerol, since the metabolic response to iron-deprivation leads to the downregulation of iron-consuming pathways, one of which is oxidative phosphorylation [91, 94, 95]. Accordingly, our data indicate that in the *aft1* mutant the respiratory metabolism is affected and that copper, in an as yet unidentified way, compensates this deficiency (Figure 3.5 E). Similar results were reported by Blaiseau *et al.*, who found that the sensitivity of the *aft1* mutant to raffinose was rescued by copper addition [91]. In addition we also found that the *aft1* mutant is sensitive to paraquat, a superoxide anion producer drug, and that copper slightly alleviates this growth phenotype (Figure 3.5 F) [93]. One plausible explanation for this finding is that activation of *SOD1* gene by Ace1 [58] contributes to superoxide anion detoxification. Both, deficient respiratory metabolism and/or deficient detoxification of superoxide anion, may account for *aft1* mutant growth failure under normal growth conditions.

V.

**Conclusions
and Future
Perspectives**

Ace1 is the transcription factor that in the yeast *Saccharomyces cerevisiae* orchestrates copper detoxification. In this work we have identified a novel target of Ace1 unveiling a novel line of protection mediated by Ace1 against copper toxicity.

Overall, our findings suggest that under copper overload conditions:

1. Aft1 is not the major regulator of *FET3* gene expression.
2. Aft1 mediates copper accumulation observed in the *fet3* mutant.
3. Ace1 regulates *FET3* gene.
4. Aft1 is hyper activated in the absence of Ace1.

In light of the data described herein, we proposed that under copper-overload, Ace1 is activated leading to the upregulation of *FET3* gene. Induction of *FET3* gene serves a dual purpose: on the one hand, as Fet3 is a copper-containing protein, it contributes to diminish the pool of free copper ions; on the other hand it prevents cells from sensing iron depletion, thereby avoiding the hyper-activation of Aft1 and preventing further accumulation of copper ions (Figure 5.1).

In a near future it would be interesting to:

- Perform Chromatin Immunoprecipitation (ChIP), to elucidate if the Ace1 site found in *FET3* promoter is functional.
- Measure by qRT-PCR the dependence of *FET3* expression on Aft1 under copper surplus.
- Generate the triple mutant *fet3fet4arn4*, the quadruple mutant *fet3fet4arn4fre1* and measure intracellular copper levels in these mutants and in the double mutants described in this work, in order to give further insights into the contribution of each protein to copper-accumulation.

- Construct the double mutant *aft1sod1* and assayed its growth in medium supplemented with copper, to evaluate if activation of SOD1 is on the basis of *aft1* fitness induced by copper

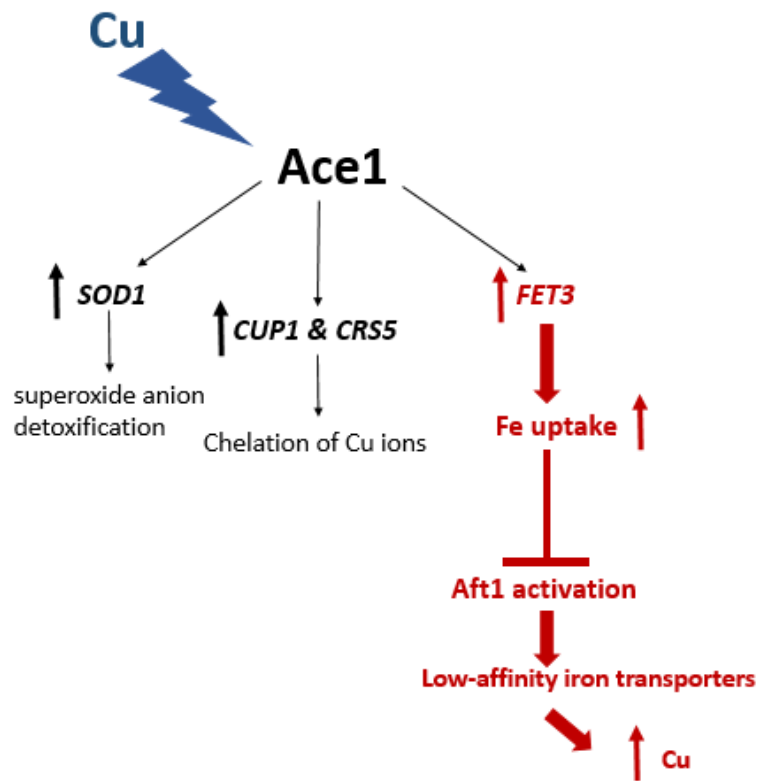


Figure 5.1 - Model for the role of Ace1 in copper detoxification. Under copper (Cu) overload conditions, Ace1, the transcription factor involved in the detoxification of copper, activates the expression of copper detoxification genes such as *SOD1*, *CUP1* and *CRS5*. In this study, we have showed that Ace1 regulates *FET3*, a multicopper oxidase essential for Fe uptake. This regulation serves as to restrict Aft1 hyper-activation, whose target expression, can lead to cell death. The novel pathway for Ace1-mediated copper detoxification proposed in this study is depicted in red.

VI. Appendix

Table S3 - List of oligonucleotides (primers) used in this study. Highlighted in bold are the sequences complementary to the histidine marker (HIS) or kanamycin (KAN).

Primer	Sequence	Use
<i>A1_AFT1</i>	5'CTCATGAAAAC TGCGTCC 3'	Mutant confirmation
<i>A4_AFT1</i>	5'GCGCCACTGAAAACACTA 3'	Mutant confirmation
<i>AFT1_KO_HIS_Fw</i>	5'ATGGAAGGCTTCAATCCGGCTGACATAGAACATGCGTCAA TCGTACGCTGCAGGTC 3'	Deletion mutant
<i>AFT1_KO_HIS_Rv</i>	5'CTAATCTTCTGGCTTCACTACTTCAACTCGCCGTTCACTT AGGGAGACCGGCAGAT 3'	Deletion mutant
<i>A1_ACE1</i>	5'ATCTAGGGATGAGCTGAACG 3'	Mutant confirmation
<i>A4_ACE1</i>	5'GAACAAACAACCGCCAATAT 3'	Mutant confirmation
<i>ACE1_KO_Kan_Fw</i>	5'GATGTAATTTATTAGACGGCGGCTTGATAAAAGAGGACTG ATAATCAGTGTATTCACAG CGTACGCTGCAGGTCGAC 3'	Deletion mutant
<i>ACE1_KO_Kan_Rv</i>	5'CGGGAGAACAACAACCGCCAATATATGTATATGTATATT TATATAGATGTCCAGATGCAG CATAGGCCACTAGTGGATC TG 3'	Deletion mutant
<i>A1_FRE1</i>	5'AAAGTGCCTGATCTTGCGATG 3'	Mutant confirmation
<i>A4_FRE1</i>	5'CTCTCTCAACAGGCAAAGGAAG 3'	Mutant confirmation
<i>FRE1_K7_HIS_Fw</i>	5'GTAATATAATCATCTAATTTCTCGCATATTCACGCCGACG GAAGAACGAGCCGGATCAATAT CGTACGCTGCAGG 3'	Deletion mutant
<i>FRE1_K7_HIS_Rv</i>	5'TTATCCGATAAAGCAACAAAATAACAAAAGTATGCGTGC AAGAATTATTAACAAGGGGCCT TAGGGAGACCGGCAGAT 3'	Deletion mutant
<i>A1_ARN4</i>	5'ACGGCTTACAATTGCACCC 3'	Mutant confirmation
<i>A4_ARN4</i>	5'CTACGGGAAATGACAGGTGCG 3'	Mutant confirmation

<i>ARN4_K7_HIS_Fw</i>	5'GGTTTGGGTCCTAGCAGAACTCTAATATAGCAAAAAAGTG CTCGTGAATGTCTCTCTGAAATCGTACGCTGCAGG 3'	Deletion mutant
<i>ARN4_K7_HIS_Rv</i>	5'AGAGAATTCTAGTTCAGGAAAGAGCTTAACACTATATTTG TATCGCTAGTACTCTCCAGCTTAGGGAGACCGGCAGAT 3'	Deletion mutant
<i>A1_FET4</i>	5'TGGCTCGAACAAACGACAAAA 3'	Mutant confirmation
<i>A4_FET4</i>	5'TCGGAATTCCACGTCAAGGT 3'	Mutant confirmation
<i>FET4_K7_HIS_Fw</i>	5'ATATAGAAACCTAATAAGACCCTTATTATTAAACATTCGT TAATCAAATTATGGGTAAAAATCGTACGCTGCAGG 3'	Deletion mutant
<i>FET4_K7_HIS_Rv</i>	5'TTAACTCCCTCATAAGTAACATGAGGAATTCTTCCCCTA ATGCTAAAATGTTCAATGAAGCTATTTTTCTTAGGGAGACC GGCAGAT 3'	Deletion mutant
<i>qFET3_Fw</i>	5'ACGGTGTGAATTACGCCTTC 3'	qRT-PCR
<i>qFET3_Rv</i>	5'TTGGAAGCGTGACCATGTA 3'	qRT-PCR
<i>qCTH2_Fw</i>	5' AGGTATGCTGCTGGAGCTGT 3'	qRT-PCR
<i>qCTH2_Rv</i>	5' GAGGGCCATGAAGGTATCAA 3'	qRT-PCR
<i>ACT1_Fw</i>	5'CTATTGGTAACGAAAGATTCAG 3'	qRT-PCR
<i>ACT1_Fw</i>	5'CCTTACGGACATCGACATCA 3'	qRT-PCR
<i>Fw_pFET3</i>	5'GCTCTAGACAAAATTCAGCGCCTGCGT3'	<i>pFET3-lacZ</i>
<i>Rv_pFET3</i>	5'GGGGTACCCGAGAAAAGCAAAACGGCT3'	<i>pFET3-lacZ</i>

VII.

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