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The cases of cobalt, cadmium and iron

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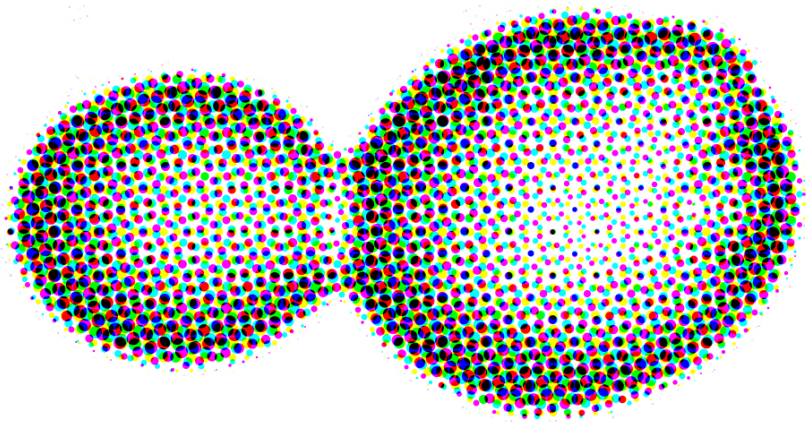

Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
July, 2017

UNIVERSIDADE NOVA DE LISBOA

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"You never fail until you stop trying"

Albert Einstein

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Foreword

The Genomics and Stress laboratory, at ITQB, headed by Prof. Claudina Rodrigues-Pousada, has been focused on the identification and characterization of stress-responsive genetic programs regulating metal toxicity in the yeast *Saccharomyces cerevisiae*, with special emphasis on those controlled by a set of eight transcription factors – the Yap family. Over the last years, in our laboratory, several metal detoxification pathways coordinated by different Yap members have been shown to be crucial for yeast survival under metal stress conditions. The work presented in this thesis further reinforces the relevance of the Yap family in this process with special emphasis on Yap1. The work began with the investigation of Yap1 role in cobalt surplus detoxification, the results of which are described in Chapter II. During this study it became clear that Yap1 may as well function as a repressor of transcription, which set the ground for the work described in Chapter III, where the repressive activity of Yap1, was shown to be the first line of cellular defense against cadmium toxicity. At the same time, taking advantage of the expertise acquired by working with metal detoxification pathways in *Saccharomyces cerevisiae* and due to the relevance of metals in infection, the work was also performed using *Candida glabrata*, a yeast of medical importance. Therefore, in Chapter IV a yet unexplored mechanism used by this organism to cope with iron loading is discussed and an association between iron loading and fluconazole antifungal activity was brought to light and started to be address. This last part of the work was performed in close collaboration with Professor Patrick Van Dijck (VIB, University of Leuven).

Finally, in Chapter V the findings of this thesis are summarized and the directions to which future research may proceed are discussed.

List of Publications

Publications included in this thesis:

Caetano SM, Menezes R, Amaral C, Rodrigues-Pousada C & Pimentel C (2015) Repression of the Low Affinity Iron Transporter Gene *FET4*: a novel mechanism against cadmium toxicity orchestrated by Yap1 via *ROX1*. *J Biol Chem* **290**, 18584-18595.

Pimentel C, **Caetano S**, Menezes RA, Figueiredo I, Santos C, Ferreira RB & Rodrigues-Pousada C (2014) Yap1 mediates tolerance to cobalt toxicity in the yeast *Saccharomyces cerevisiae*. *BBA-General subjects* **1840**(6): 1977-86.

Other publications:

Mazzola D[#], Pimentel C[#], **Caetano S**, Amaral C, Menezes R, Santos CN, Eleutherio E & Rodrigues-Pousada C (2015) Inhibition of Yap2 activity by MAPKAP kinase Rck1 affects yeast tolerance to cadmium. *FEBS Lett.* **589**, 2841-2849.

Pimentel C, Vicente C, Menezes RA, **Caetano S**, Carreto L & Rodrigues-Pousada C (2012) The role of the Yap5 transcription factor in remodeling gene expression in response to Fe bioavailability. *PLoS One* **7**(5): e37434.

Abstract

While toxic metals are detrimental to health, essential metals may be looked at as double-edge swords, as their essential nature is counterbalanced by the toxic effects they can exert on cells, when accumulating beyond homeostatic levels. The molecular bases of the toxicity of essential and non-essential metals, as well as the mechanisms that cells have evolved to cope with them, are not yet well understood.

In this work, we have explored the molecular mechanisms put at play by the yeast *Saccharomyces cerevisiae* to detoxify excess of cobalt and cadmium. We show that the major transcriptional regulator of the oxidative stress response in yeast, Yap1, is relevant for the detoxification of cobalt excess. Analysis of the transcriptional profile of cells stressed with high concentrations of cobalt revealed induction of the oxidative stress response genes triggered by Yap1. Activation of Yap1, however, is not exclusively mediated by cobalt-generated reactive oxygen species, since under anoxia cobalt also triggers Yap1 nuclear localization. Yap1 induces the *PHO84* gene, which encodes a phosphate transporter known to be involved in cobalt uptake, and accordingly *yap1* knockout cells accumulate lower levels of cobalt. This apparent paradoxal role of Yap1 in cobalt detoxification puts into question whether phosphate uptake may fulfill a role in the oxidative stress response. Moreover, we found Yap1 as repressor of *FET4* gene, a cell surface low affinity iron transporter which can transport other divalent metals, including cobalt and toxic cadmium ions. Although this repression does not significantly impact on cobalt uptake, we show

that this is the first line of defense against cadmium toxicity, as it impairs its cellular uptake. The repression of *FET4* expression by Yap1 is not a direct event, but it is mediated by *ROX1*, a known transcriptional repressor of *FET4*. Interestingly, we noted that, when challenged with cadmium, yeast knockouts of *YAP1* and *ROX1* genes have post-transcriptional mechanisms, dependent on the 5′-3′exoribonuclease Xrn1, which compensate the derepression of *FET4*. This constitutes a new aspect of cellular defence against cadmium toxicity.

We have also investigated whether the players orchestrating iron overload adaptation in *Saccharomyces cerevisiae* are conserved and operating in the opportunistic human fungal pathogen *Candida glabrata*, a close relative of *S. cerevisiae*. In the latter, Ccc1, a vacuolar transporter which effects the accumulation of iron in the vacuoles, mediates iron detoxification. In *Candida glabrata*, however, we suggest that Ccc1 cannot be the major effector of iron-excess detoxification. A phenotypic screening of the *C. glabrata* mutant collection unveiled several other proteins, which may as well be involved in this process.

Finally, our results indicate that iron-loading conditions endow *S. cerevisiae* and *Candida glabrata* more tolerant to the antifungal fluconazole. Interestingly, in *S. cerevisiae*, impaired adaptation to iron loading (*ccc1* knockout) suppresses that effect. These findings clearly indicate a strong association between fluconazole and iron worth being further investigated. We have started to dissect the mechanisms underlying iron-mediated alleviation of fluconazole activity by performing a phenotypic screening of the *C. glabrata* mutant collection.

Resumo

Os metais tóxicos são prejudiciais à saúde, no entanto também os metais essenciais podem ser nefastos, quando se acumulam nas células acima dos seus níveis homeostáticos. As bases moleculares da toxicidade dos metais essenciais e não essenciais, bem como os mecanismos que as células possuem para limitar a sua toxicidade, são ainda pouco conhecidos.

No presente trabalho, explorámos os mecanismos moleculares utilizados pela levedura *Saccharomyces cerevisiae* para desintoxicar o excesso de cobalto e cádmio. Assim, demonstrámos que o principal regulador transcricional da resposta ao stress oxidativo na levedura, Yap1, é relevante para a desintoxicação do excesso de cobalto. A análise do perfil transcricional de células induzidas com altas concentrações de cobalto revelou a ativação do Yap1 e consequentemente, dos genes de resposta ao stress oxidativo. A ativação do Yap1, no entanto, não é exclusivamente mediada por espécies reativas de oxigénio geradas por cobalto, uma vez que em condições de anóxia o cobalto induz a localização nuclear do Yap1. O factor de transcrição Yap1 também induz a expressão do gene *PHO84* que codifica um transportador de fosfato conhecido por estar envolvido na captação de cobalto, e, consequentemente, as células mutantes *yap1* acumulam níveis mais baixos de cobalto. Este aparente papel paradoxal de Yap1 na desintoxicação de cobalto levou-nos a questionar se a absorção de fosfato poderia desempenhar um papel na resposta ao stress oxidativo. Além disso, demonstrámos que o Yap1 atua como repressor do gene *FET4* que codifica um transportador de ferro de baixa afinidade. Este transportador permite a entrada

na célula de outros metais bivalentes, incluindo o cobalto e iões tóxicos de cádmio. Embora esta repressão não tenha um impacto significativo na absorção de cobalto, mostrámos que é a primeira linha de defesa contra a toxicidade provocada por cádmio, pois previne a sua entrada na célula. A repressão da expressão de *FET4* por Yap1 não é direta, mas sim mediada por *ROX1*, um conhecido repressor da transcrição deste gene. Curiosamente, observámos que, na presença de cádmio, as estirpes mutantes nos genes *YAP1* e *ROX1* (*yap1, rox1)* possuem mecanismos de pós-transcrição, dependentes da 5'-3' exoribonuclease Xrn1, que compensam a ausência da repressão do gene *FET4*. Este mecanismo constitui uma nova defesa celular contra a toxicidade provocada por cádmio.

Foi também investigado neste trabalho se os participantes na adaptação ao excesso de ferro em *Saccharomyces cerevisiae* são conservados e operacionais em *Candida glabrata*, um fungo patogénico filogeneticamente próximo de *S. cerevisiae*. Em *S. cerevisiae*, Ccc1, um transportador vacuolar que efetua a acumulação de ferro no vacúolo, medeia a desintoxicação de ferro. Em *Candida glabrata* concluímos que, além do Ccc1, deveriam existir outras proteínas relevantes na adaptação ao excesso de ferro. Assim, usando uma coleção de mutantes de *C. glabrata*, efectuou-se um rastreio fenotípico revelando várias outros candidatos, que poderão estar envolvidos na resposta ao excesso de ferro.

Finalmente, os nossos resultados indicam que em condições de excesso de ferro as leveduras S. *cerevisiae* e *Candida glabrata* são mais tolerantes ao antifúngico fluconazol. Curiosamente, em *S. cerevisiae*, a incapacidade de

adaptação ao excesso de ferro (estirpe com o gene *CCC1* deletado) suprime esse efeito. Estes resultados indicam uma forte associação entre o fluconazol e o ferro. Neste sentido, começámos, neste trabalho, a explorar os mecanismos subjacentes à diminuição da atividade de fluconazol mediados pelo excesso ferro, tendo identificado vários genes que poderão participar neste processo.

Abbreviations

As	arsenic
Ag	silver
BER	base excision repair mechanism
Bi	bismuth
Cd	cadmium
CFEM	common in fungal extracellular membrane protein superfamily
CNS	central nervous system
Co	cobalt
Cr	chromium
CRD	C-terminal cysteine rich domain
Cu	copper
CWI	cell wall integrity
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
Fe	iron
GSH	glutathione
GPI	glycosylphosphatidyliinositol
Hg	mercury
HO^-	hydroxide anion
HO•	hydroxyl radical
H_2O_2	hydrogen peroxide
MAPKAP	MAPK-activated protein kinase
min	minutes
MMR	mismatch repair mechanism
Mn	manganese
Mo	molybdenum
MRPs	multidrug resistance-associated proteins
MTs	metallothioneins
NEM	N-ethyl maleimide
NER	nucleotide excision repair mechanism
NES	nuclear export sequence
NETs	neutrophil extracellular traps
NHEJ	non-homologous end-joining mechanism
Ni	nickel
O•	singlet oxygen

$O_2\bullet^-$	superoxide anion
O_2	oxygen
Pb	lead
PCs	phytochelatins
PDRE	pleiotropic drug response elements
Sb	antimony
Se	selenium
Rh	rhodium
RNA	ribonucleic acid
NER	nucleotide excision repair mechanism
ROS	reactive oxygen species
RTG	retrograde pathway
Te	tellurium
TFs	transcription factors
TOR	target of rapamycin pathway
Trx	thioredoxin
UV	ultraviolet radiation
Zn	zinc
Yap	AP-1 like factor
YRE	Yap responsive element

Latin abbreviations

et al	*et alia*, and other people
vs	*versus*, as opposite to

Amino acids

Ala	A	Alanine	Leu	L	Leucine
Arg	R	Arginine	Lys	K	Lysine
Asn	N	Asparagine	Met	M	Methionine
Asp	D	Aspartate	Phe	F	Phenylalanine
Cys	C	cysteine	Pro	P	Proline
Gln	Q	Glutamine	Ser	S	Serine
Glu	E	Glutamate	Thr	T	Threonine
Gly	G	Glycine	Trp	W	Tryptophan
His	H	Histidine	Tyr	Y	Tyrosine
Ile	I	Isoleucine	Val	V	Valine

Chapter I

GENERAL INTRODUCTION

1. Metal toxicity

Metals are essential for life, as several biological functions rely on them. Metals like sodium, potassium and calcium are crucial for nerve conduction and muscle contraction, whereas iron, copper, manganese, zinc, cobalt and nickel are incorporated into proteins and have a determinant role in protein structure and catalytic activity [1, 2]. On the other extreme are nonessential metals such as cadmium, lead, mercury, among others, which can be highly toxic [1]. However, even essential metals can be toxic whenever present in high, non-homeostatic, concentrations. Cells have therefore evolved sophisticated molecular mechanisms aiming at, on the one hand, avoiding toxicity of nonessential metals, and on the other, maintaining the adequate concentration of essential metals [3, 4].

Human exposure to metals occurs in a day-by-day basis since metals are used in many industries, such as in the production of batteries, cosmetics, paper and pulp preservatives, pharmaceuticals, paint pigments, plastic coatings, electroplating, among others [3]. The main metal contamination result from activities in the mines, industrial effluents, wastewaters and insect and/or disease control drugs applied to crops [5]. Besides anthropogenic pollution, metals can also contaminate the surroundings by natural means such as soil erosion and natural climate alterations of the earth's crust. Metal pollution represents a significant problem given that metal toxicity affects the population in a nutritional and environmental manner [5]. Furthermore, human exposure to metals is associated with several diseases. Metal exposure can be classified as chronic or acute depending on the exposure period length. Chronic exposure can

lead to mental retardation, birth defects, psychosis, autism, allergies, cardiovascular disease, dyslexia, weight loss, hyperactivity, brain, kidney, lungs and liver damage, cancer, osteoporosis, among others [6-9], whereas acute exposure to metals can cause nausea, vertigo, loss of appetite, headache, abdominal pain, fatigue, sleeplessness, arthritis, and hallucinations [5].

Metal toxicity depends on each metal's physicochemical properties and ligand preferences. Redox-active metals such as iron (Fe), chromium (Cr), copper (Cu) and cobalt (Co) can take oxygen and sulphur as their ligands, whereas redox-inactive metals such as cadmium (Cd) and mercury (Hg) prefer sulphur as ligand [4, 10, 11]. Redox-active metals can induce oxidative stress by participating in Fenton-type reactions; redox-inactive metals imbalance the antioxidant pool of the cell [4, 10, 11]. In general, metals induce cellular toxicity by generating oxidative stress, impairing the DNA repair system and inhibiting protein folding and function [4, 12, 13].

1.1 Metal-induced oxidative stress

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the amount/efficacy of the antioxidant defences in the cell [14].

Metals can lead to the formation of ROS by participating in Fenton-type reactions (1). This reaction takes place between a redox-active metal, for instance iron (Fe^{2+}), and hydrogen peroxide (H_2O_2), which naturally occurs in aerobic organisms as a result of the oxidative metabolism (1) [13, 15].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO\bullet \quad (1)$$

This reaction leads to metal oxidation (Fe^{3+}) with the formation of hydroxyl radical ($HO\bullet$) and hydroxide anion (HO^-). The superoxide radical ($O_2^-\bullet$) formed during oxidative metabolism or by autoxidation of some metals, can then reduce Fe^{3+} to Fe^{2+}, with the formation of O_2 (2) [13, 15, 16].

$$Fe^{3+} + O_2^-\bullet \rightarrow Fe^{2+} + O_2 \quad (2)$$

The combination of these two reactions, (1) and (2), is designated Haber-Weiss reaction (3) [13, 15, 16].

$$O_2^-\bullet + H_2O_2 \rightarrow O_2 + HO\bullet + HO^- \quad (3)$$

One of the primary targets of ROS is polyunsaturated fatty acids, which are constituents of the cell membrane. Consequently, metal induced-ROS can lead to the initiation of non-enzymatic lipid peroxidation and formation of lipid peroxides [17]. Lipid peroxidation is a chain reaction that involves the formation and propagation of lipid radicals leading, most probably, to membrane damage [18]. Also, proteins are targets of metal-generated ROS and their effects at this level include protein denaturation, loss of function, cross-linking and aggregation [19]. Moreover, the production of $HO\bullet$ by metals can cause DNA damage. This oxygen-activated species possesses the capacity to react with all components of nuclear chromatin, to modify DNA bases and deoxyribose, to produce DNA-protein crosslinks and to cause DNA depurination and DNA strand scission [13].

As mentioned before, redox-inactive metals such as Cd, Hg and Pb can not participate in Fenton-type reactions, but induce oxidative stress by depleting the antioxidant pools of cells and inhibiting specific enzymes [3, 4, 16, 20]. Depletion

of the glutathione (GSH) levels, a tripeptide considered to be the main cellular antioxidant molecule, is one of those mechanisms [21]. GSH chelates metals and metalloids leading to a decrease of the free GSH cytosolic pool, thereby unbalancing the redox environment of the cell [21]. Metals such as Cd also have an inhibitory effect on antioxidant enzymes such as catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase [3]. Finally, redox-inactive metals may perturb the cell iron metabolism, leading to increased free Fe levels, which in turn can enhance Fenton-type reactions [4].

1.2 Metal inhibition of the DNA repair machinery

Several metals are able to indirectly induce DNA damage as, even at low non-cytotoxic concentrations, they have the ability to diminish and/or inhibit the activity of several enzymes involved in the DNA repair pathway [12, 13]. The impairment of DNA damage repair mechanisms increases the accumulation of DNA mutations and may ultimately lead to cancer [12]. It has been reported that Cd has the ability to inhibit DNA nucleotide excision repair (NER) [22], non-homologous end-joining (NHEJ) [23], base excision repair (BER) [24], and mismatch repair (MMR) [23]. Cd inhibition of DNA mismatch repair causes recombination, base-substitution, and frame-shift mutations [25]. Cd and Ni are thought to interfere with the DNA damage recognition process. The presence of these metals diminishes DNA binding of proteins required to recognize the damage and block the beginning of the repair process [26, 27]. Ni was also shown to compete with the Fe present in catalytic sites of relevant DNA repair enzymes,

replacing it and leading to enzyme inactivation [28]. The presence of arsenite (AsIII) in the cell inhibits the Poly(ADP-ribose) polymerase-1 (PARP-1) enzyme, which leads to the malfunction of the DNA break repair process [29]. Cobalt blocking of the incision step of UV-generated pyrimidine dimer lesions was found to repress DNA repair [30]. Cr and As were also identified as presumed inhibitors of the repair of UV-induced DNA damages [31].

1.3 Metal-induced protein damages

Metals can interfere with protein function and structure by (i) binding to thiol, imidazole, carboxyl or other protein functional groups, catalyzing oxidation of amino acid side chains, or (ii) displacing metal ions from metalloproteins [32]. Furthermore, metals may target non-folded nascent proteins and interfere with folding, perturbing general protein homeostasis and eventually cell viability [32, 33]. Misfolded proteins may aggregate and/or interact inappropriately with other cellular components, becoming cytotoxic. It is well known that many neurodegenerative and age-related disorders are directly related to protein misfolding and aggregation [34]. In fact, growing evidences suggest that metals can enhance aggregation of some disease-associated proteins inducing the progression of neurodegenerative disorders [35-37]. Particularly, As can directly bind to cysteine residues of some chaperones leading to inhibition of their activity. It has been suggested that this effect determines the cell overall folding capacity, resulting in widespread protein misfolding and aggregation [38]. Another example is Cd, which besides interfering with protein folding also

disturbs calcium metabolism, inducing endoplasmic reticulum (ER) stress [39, 40]. ER stress is defined as the accumulation of unfolded proteins in the ER [40-42]. Interestingly, it was reported that Cr promotes the formation of protein aggregates by generating mRNA mistranslation [43]. The exact mechanism is not yet well understood, but misincorporation of amino acids may surely be the cause of protein misfolding and aggregation.

2. Metal detoxification

To avoid metal toxicity, organisms have developed several cellular mechanisms to limit metal reactivity.

Metal arrest in the vacuole is particularly important for organisms such as plants and yeasts. After entering the cell, metals are directed to the organelles where they are necessary or they are alternatively, stored in the vacuole [44]. Essential metals can be posteriorly effluxed from vacuoles to maintain the proper function of the cell [1].

Metal binding to proteins such as metallothioneins is another mechanism of metal detoxification, common to several organisms, from humans to yeast. Metallothioneins are low molecular mass cysteine-rich proteins able to bind several metal ions; these proteins can also scavenge free radicals protecting cells against oxidative stress [45]. The tripeptide glutathione (GSH) also serves this purpose as it can chelate metal ions resulting in a complex that can be taken up by vacuolar transporters [4]. In humans, metal-glutathione complexes can be

possibly exported from the cell by members of the MRP (multidrug resistance-associated proteins) family [46], or, in the case of As and Sb, GSH can be used to co-transport these metals out of the cell [47]. In plants, GSH is a precursor of phytochelatins (PCs), which are enzymatically synthesized Cys-rich peptides that limit the free metal ions cellular concentration [48]. In human cells, and in the particular case of Fe, ferritin can "buffer" and capture the intracellular labile Fe pool, being the major player in maintaining cellular Fe homeostasis [49].

2.1 The yeast *Saccharomyces cerevisiae* as an eukaryotic model organism for metal metabolism and detoxification studies

Saccharomyces cerevisiae has been used in research for more than one hundred years and is generally regarded as the most well understood eukaryotic organism [50, 51]. Several significant advantages justify its extensive and continued use. First, it is a non-pathogenic unicellular organism that divides rapidly and can be grown on defined media, which gives the complete control over the chemical and physical experimental conditions [52, 53]. Second, yeast cells, as any sexual eukaryote, have a cell cycle alternating between haploid and diploid states [50, 53]. Third, its genome is sequenced and it is easily manipulated, which has allowed the development of several molecular biological tools for genetic, biochemical and cell biology studies [1, 52]. Finally, there is a high conservation between several genes of *S. cerevisiae* with those of distant eukaryotes.

Altogether the above arguments support the use of *S. cerevisiae* as an up-to-date model organism to unravel the molecular details of several eukaryotic pathways, where metal metabolism and detoxification are included. In fact, *S. cerevisiae* is nowadays considered a model organism to study mitochondrial dysfunction and consequent associated diseases [54]. Due to the importance of Fe in mitochondrial function, the insights provided by the human mitoferrin and frataxin homologs, Mrs3/4 and Yfh1, respectively were determinant to understand Fe uptake and trafficking in yeast as in humans [1, 55, 56]. Another example is the yeast Golgi copper transporter Ccc2, a homolog to the human Atp7a and Atp7b proteins. Mutations in *ATP7A* and *ATP7B* genes are related with Menkes and Wilson diseases respectively, and the inability of these mutated proteins to deliver copper to the secretory pathway was demonstrated by complementation assays of a yeast mutant strain lacking *CCC2* gene[1, 57].

2.2 Mechanisms of metal detoxification in yeast

In *S. cerevisiae*, metal compartmentalization in the vacuole is a common mechanism of metal detoxification. The membrane of this organelle has several transporters involved in the uptake of different metals. Ycf1, a member of the ATP-binding cassette (ABC) family of transporters, is the major player in the vacuolar sequestration of glutathione-conjugated metals and xenobiotics [4]. Yeast mutant cells lacking the *YCF1* gene are sensitive to Cd, Hg, Pb and As(III) [58-60]. Ycf1 has two paralogues, Bpt1 and Vmr1, which seem to play a significant role in metal detoxification in the absence of Ycf1 [4, 61]. Another

vacuolar transporter, Zrc1, is determinant in zinc (Zn) homeostasis. This transporter is a member of the cation diffusion facilitator (CDF) family of transporters, which transports Zn and Co, and probably Cd and Ni, to the vacuole [62-65]. Cot1 is also a vacuolar member of the CDF family that transports Zn and Co, and whose overexpression confers resistance to rhodium (Rh) [63]. Finally, the transporter Ccc1 is involved in internalization of Fe and Mn in the vacuole and deletion of its gene renders cells sensitive to Fe excess [66].

A second mechanism of metal detoxification is metal export through the plasma membrane. Contrarily to prokaryotes, that have several export routes conferring tolerance to metal and metalloids, in yeast there are only a few. Acr3 is a member of the bile/arsenite/riboflavin transporter (BART) superfamily [67], and it extrudes As(III) from the cytosol, being probably the most important As detoxification system in yeast [58, 68]. Pca1, a P-type cation-transporting ATPase, is a Cd transporter responsible for the cellular export of this metal. Pca1 was identified in a Cd resistant strain [69], but, in most laboratory strains, it is not functional due to a Gly970Arg mutation [70]. Also the ABC transporter family member Yor1 is thought to be involved in Cd-GSH complex efflux from the cell, given that *yor1* mutant cells exhibit sensitivity to this metal [71].

Finally, metal chelation by low-molecular-weight cysteine-rich peptides and proteins such as GSH and metallothioneins is another important metal detoxification process in yeast. Through the generation of glutathione-conjugated metals species, metal ions are reduced, preventing the induction of oxidative stress [72]. These species can be used afterwards as substrates for vacuolar or

membrane transporters, as mentioned above. In *S. cerevisiae*, there are two types of metallothioneins, Cup1 and Crs5. Cup1 is a polypeptide able to bind Cu, Cd and Zn [73]. Cup1 seems to have a preponderant role in Cu detoxification as *cup1* mutant yeast cells are sensitive to Cu, but not to other metals and metalloids [74]. Crs5 is a protein also responsive to Cu excess and the absence of *CRS5* gene renders cells slightly sensitive to Cu [75]. Moreover, besides Cu, Crs5 was shown to bind Cd and Zn, being essential for yeast tolerance to mixed Cu/Zn overload [76].

2.3 Transcriptional regulation of metal detoxification

To correctly produce enzymes, metal-transporters and compounds required for cellular metal detoxification, the expression of the corresponding genes must be precisely synchronized. In this context, transcription factors (TFs) have a major role, making the bridge between cytotoxic-sensors and transcriptional activation of metal detoxification genes.

2.3.1 The Yap family

The yeast AP-1 (Yap) family of *S. cerevisiae*, is a set of eight transcription factors (Yap1 to Yap8) belonging to the b-ZIP superfamily (Figure 1.1) [77]. Of those, four members, Yap1, Yap2, Yap5 and Yap8 were reported to play a role in metal detoxification.

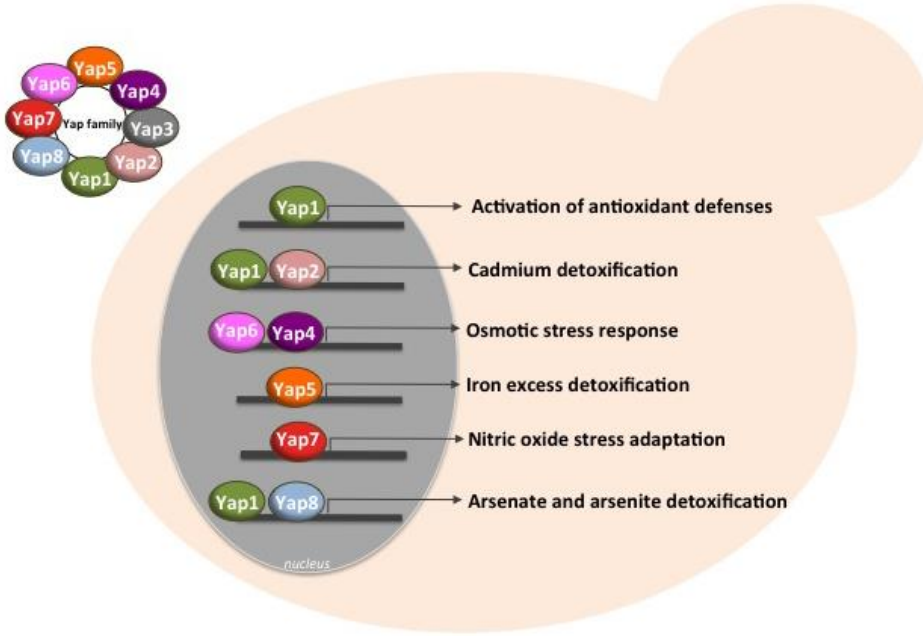

Figure 1. 1 Diagram representing the involvement of several members of the Yap family in the stress response. The Yap family members involved in metal detoxification are Yap1, Yap2, Yap5, and Yap8. A special attention will be given to these transcription factors in this thesis.

Yap1

Yap1 is the main regulator of the oxidative stress response in yeast cells, and, as such, has a pivotal role in mitigating metal-generated ROS. Under non-stressed conditions, Yap1 shuttles between the nucleus and the cytoplasm, but upon stress conditions, it accumulates in the nucleus, resulting in the increased expression of its target genes [78, 79]. Azevedo *et al* showed that Yap1 possesses two distinct molecular sensors, one senses H_2O_2 and the other one thiol-reactive compounds, such as N-ethyl maleimide (NEM) (Figure 1.2) [80]. The first leads to the formation of an intramolecular disulphide bond between Yap1 N- and C-terminal

cysteine residues (Cys303 and Cys598) and requires Ybp1 (Yap1-binding protein) [81, 82], as well as the glutathione peroxidase Gpx3/Orp1, which is the primary sensor in transducing H_2O_2 signal to Yap1 (Figure 1.2) [83, 84]. The second, is regulated by the direct binding of thiol-reactive compounds to the residues Cys598, Cys620 and Cys629 located in the C-terminal cysteine rich domain (CRD) of Yap1 (Figure 1.2) [85]. Both types of modifications induce a conformational change of the protein, which prevents the recognition of the nuclear export sequence (NES) by the exportin Crm1, promoting Yap1 nuclear accumulation (Figure 1.2) [86]. Gulshan *et al* suggested the co-existence of two different Yap1 pools in the cell; one associated with Ybp1, which responds to H_2O_2, and the other one non-Ybp1-associated, which is activated by thiol-reactive compounds [87]. In the nucleus, Yap1 binds to the DNA sequence T(T/G)ACTAA termed Yap1 response element (YRE) present in the promoter region of its target genes. Some examples of oxidative stress response genes regulated by Yap1 are *GSH1* (gamma glutamylcysteine synthetase) [88], *GLR1* (glutathione reductase) [89], *GPX2* (2-cys peroxiredoxin [90]) [91], *TRX2* (thioredoxin) [92], *TRR1* (thioredoxin reductase) [93], *SOD1* (superoxide dismutase) [94], *CTT1* (catalase T, present in the cytosol) [95], among others. Yap1 contribution to metal detoxification, however, is not restricted to the induction of the cell antioxidant defenses. As mentioned before, Yap1 also regulates the expression of *YCF1* gene, the vacuolar transporter of metal-glutathione conjugates, thereby contributing to vacuolar compartmentalization of metal species [96].

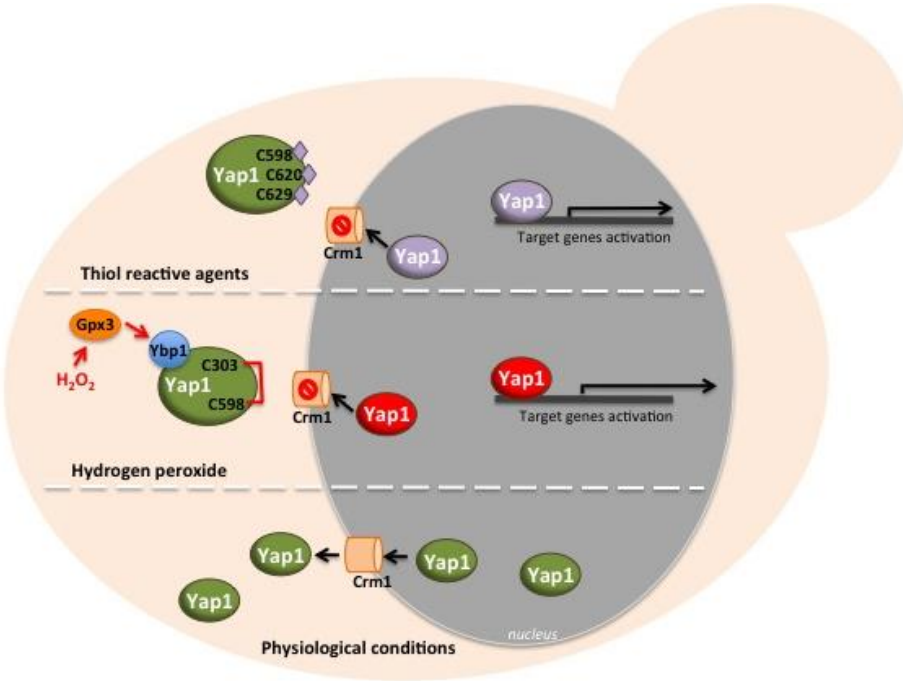

Figure 1. 2 Schematic representation of Yap1 activation. Yap1 has two distinct molecular sensors: one for Hydrogen peroxide (H_2O_2) and the other for thiol-reactive compounds. In the first panel is depicted the activation of Yap1 by thiol-reactive agents. These compounds bind to Cys598, Cys620 and Cys629, thereby preventing the recognition of the nuclear export signal (NES) by Crm1. In the second panel is depicted the activation of Yap1 by H_2O_2, which is dependent on Gxp3/Orp1 and Ybp1 proteins. H_2O_2 induces the formation of a disulfide bond between Cys303 and Cys598 of Yap1, preventing the recognition of the NES by Crm1. In both cases, the conformational change leads to Yap1 accumulation in the nucleus and posterior gene activation. In the last panel is represented the shuttling of Yap1 between the nucleus and cytoplasm, occurring under physiological conditions.

Yap2

Yap2, also known as Cad1, is a paralog of Yap1. In the absence of Yap1, overexpression of Yap2 confers resistance to cadmium toxicity [97]. Under unstressed growth conditions, Yap2 shuttles between the nucleus and the cytoplasm. In the presence of Cd, three cysteine residues of Yap2 C-terminus

(Cys391, Cys356 and Cys387) bind the metal, resulting in the retention of this regulator in the nucleus [85]. Although Yap2 is clearly a Cd-responsive transcription factor, deletion of the corresponding gene does not increase yeast sensitivity to this metal and for this reason the exact function of Yap2 in Cd detoxification remains to be clarified [77]. In our laboratory it was shown that Cd triggers Yap2-dependent regulation of *FRM2* gene (a type II nitroreductase), whose product is thought to function in lipid metabolism [98], suggesting that Yap2 may play a role in the regulation of this pathway [77, 85]. We have also shown that deletion of *RCK1* gene, a MAPK-activated protein kinase (MAPKAP) previously shown to interact with Yap2 [99-101], renders cells more resistant to Cd toxicity, whereas removal of *YAP2* from *rck1* mutant background increases its sensitivity [102]. Our data also revealed Rck1 as an inhibitor of Yap2 activity. Indeed, in the absence of *RCK1* gene, the Yap2 target, *FRM2*, is over-induced. Accordingly, Rck1 reduces Yap2 protein half-life and shortens the time period that Yap2 remains in the nucleus upon Cd treatment [102]. Our results also indicated Cd induction of the cell wall integrity (CWI) genes *SLT2*, *RLM1* and *CHS1*, suggesting that most probably this metal damages the glucan structure of yeast cell wall. Moreover, the down-regulation of these genes after Cd treatment in a *yap2* mutant strain indicates a potential regulatory role of Yap2 in the expression of CWI genes [102].

Yap5

Yap5 was shown to be involved in Fe-excess detoxification, by regulating the expression of the vacuolar Fe transporter *CCC1* [103]. We have shown the partial regulation of *CCC1* gene by Yap5 [104]. A novel target of this regulator was identified in our laboratory – the glutaredoxin Grx4, a Fe-S containing protein. Because Grx4 plays a key role in the nuclear export of Aft1 (the main regulator of Fe uptake genes) [105, 106], we suggested that under Fe-loading conditions Yap5 induction of *GRX4* gene expression is required to avoid the activation of Aft1 targets, by impairing its nuclear activation [104]. Later, Li *et al* described a novel target of Yap5, *TYW1* which encodes a cytosolic Fe-S cluster-containing enzyme involved in the synthesis of wybutosine [107]. The authors suggested that the up-regulation of *TYW1* gene by Yap5 provides protection against Fe excess due to sequestration of Fe into Tyw1 Fe-S clusters and diminishing free cytosolic Fe levels [107].

Yap8

Yap8 is an activator of the genes encoding *ACR2*/*ARR2* and *ACR3*/*ARR3*, which are essential in arsenic compounds detoxification. *ACR2* codes for an arsenate (AsV) reductase, whereas *ACR3* encodes an arsenite (AsIII) and antimonite (SbIII) exporter [108-110]. Similarly to Yap1 and Yap2, Yap8 activity is regulated at the cellular localization level. Our laboratory demonstrated that, under non-stressed conditions this transcription factor is predominantly localized in the cytoplasm and after As exposure it accumulates in the nucleus [110]. The

cysteine residues Cys132, Cys137 and Cys274 of Yap8 bind As which alters the protein conformation, leading to the inhibition of the nuclear export by Crm1 [110]. Two residues of the basic region of Yap8, Leu26 and Asn31, are essential for Yap8 DNA-binding specificity to its response element (TGATTAATAATCA [109, 111]) present in the promoter region of *ACR2* and *ACR3* genes [112]. In the presence of arsenic compounds, Yap8 suffers another post-transcriptional modification, which allows this regulator to escape the ubiquitin-proteasome pathway through a mechanism involving its stabilization by the E4-ubiquitin ligase Ufd2 [113].

2.3.1 Other transcription factors involved in metal detoxification

Ace1

When yeast cells are exposed to copper excess, the major player in the activation of detoxification genes is the transcription factor Ace1. In these conditions, Ace1 activates (i) *CUP1* and *CRS5* metallothioneins expression [114, 115], which bind free Cu, limiting its toxicity [73], (ii) the expression of superoxide dismutase gene (*SOD1*) in order to scavenge Cu-driven ROS as well as to buffer free Cu ions [73, 75, 116] and (iii) *FET3* expression to hinder Aft1 activity in an attempt to prevent accumulation of toxic Cu levels in the cell mediated by this regulator (our unpublished results).

Met4

The transcription factor Met4 is the most important regulator of the sulfur assimilation and glutathione (GSH) biosynthesis pathways [72]. As mentioned before, GSH is a determinant molecule, which confers protection against both metal toxicity and oxidative stress [4, 72]. Met4 is particularly important upon Cd exposure since it brings into motion the global sulfur-sparing response. This regulator orchestrates the decrease of sulfur-rich proteins and favors sulfur-depleted isoenzymes in order to enable the recycling of sulfur-containing proteins into the GSH biosynthetic pathway [117].

Zap1

Yeast cells grown in zinc-limiting conditions and supplied with a small dose of this metal, quickly accumulate large amounts of Zn, a phenomenon designated as "zinc shock" [118]. Surprisingly, in these conditions, the Zap1 transcription factor, responsible for the activation of Zn-limiting responsive genes [119], regulates the expression of *ZRC1*, a vacuolar transporter involved Zn overload detoxification [62, 64, 65]. This proactive mechanism of Zn homeostasis assures zinc shock protection of Zn-deficient cells returning to normal conditions [118].

3. Metals in fungal infections

Metals are strategic players of the immune system. To prevent the process of infection, the host is able to thwart pathogens growth by (i) restricting their access

to essential metals, a process often designated as nutritional immunity; or (ii) exposing them to metal overload [120, 121]. Therefore, pathogens have effective metal assimilation and detoxification mechanisms to survive and grow within the infected host.

Iron

Iron is an essential metal for humans being involved in several cellular processes. Despite its biological importance, when in excess Fe can potentiate cellular toxicity by catalyzing Fenton reactions, due to its ability to cycle between two redox stages, ferrous (Fe^{2+}) and ferric (Fe^{3+}) [122], and by this reason the host needs to thoroughly control the levels of free Fe. Inside the cell, Fe is stored in ferritin, a spherical 24-subunit protein with a Fe binding capacity of up to 4,500 atoms [120, 122, 123]. In healthy individuals, transferrin, a Fe-binding glycoprotein, binds all the Fe released into the blood plasma, limiting its toxic free radical production and facilitating its transport to target cells. Consequently, this also limits Fe availability to pathogens [122, 123]. During infection, there is a host reinforcement of Fe restriction orchestrated by hepcidin, a peptide hormone produced in the liver, which posttranscriptionally controls the Fe levels in the plasma [124-126]. In addition, neutrophils and macrophages can also synthetize hepcidin in response to infection, putting forward a restriction of Fe at the infection locus [127]. Moreover, phagocytes also restrict Fe to phagocytized pathogens by decreasing the Fe content in the phagolysosomes [128, 129].

To overcome the host Fe-withholding defense mechanism, fungi have

developed mechanisms to enhance Fe acquisition. Since in humans the majority of Fe is complexed in hemoglobin inside erythrocytes, some organisms have evolved mechanisms allowing erythrocytes lysis [122]. *Candida albicans* can use heme/hemoglobin as a Fe source. The exact molecular mechanism by which erythrocytes are bound and lysed is not yet understood. Nevertheless, the assimilation of heme and/or hemoglobin after release from erythrocytes is already well characterized. The uptake of heme/hemoglobin by *C. albicans* is dependent on the Rbt5 family members *RBT5*, *PGA10/RBT51*, *CSA1*, *CSA2* and *PGA7*, which belong to the CFEM (Common in Fungal Extracellular Membranes) protein superfamily (Figure 1.3) [130]. Rbt5, an O-mannosylated glycosylphosphatidyliinositol (GPI) anchored protein, has the ability to bind heme and hemoglobin, being localized in the cell wall and plasma membrane [131-133]. The use of hemoglobin as a Fe source requires the binding of this molecule to the cell wall receptors Csa1, Pga10/Rbt51 and Rbt5 (Figure 1.3) [132]. Hemoglobin is then transported to the vacuole *via* an endocytic process dependent on Myo5, a type I myosin, and on the ESCRT complex components, Vma11 and Vps41 [133]. Heme binds Rbt5 and is directed to the vacuole through a Rbt5-Pga7 dependent process (Figure 1.3) [134], *via* a similar endocytic process [135]. *C. albicans* also secretes the protein Csa2 which functions as a "hemophore", capturing heme in the surrounding environment [120, 136]. In the vacuole, Fe is released from heme and/or hemoglobin by the action of Hmx1 oxigenase (Figure 1.3) with the production of α-biliverdin [137]. Fungi, such as *Paracoccidioides*, also express Rbt5 and are able to use heme/hemoglobin as a Fe

source. In the case of *Cryptococcus neoformans*, the Cig1 receptor plays a similar role to Rbt5, although it does not seem to be related to this receptor due to the lack of the typical CFEM motif [120]. *Aspergillus fumigatus* and *Candida glabrata* are examples of non-using heme/hemoglobin organisms [138, 139].

In addition, several pathogenic fungi can produce siderophores, which are small organic molecules with affinity for ferric Fe. The *SIDA* and *SID1* genes of *A. fumigatus* and *H. capsulatum*, respectively, are involved in the first step of the biosynthetic pathway of siderophores, which have been shown to be essential for the virulence of both strains [140-142]. In the particular case of *A. fumigatus*, the production of intracellular siderophores is also involved in hyphal and conidial Fe storage [143]. Some fungi, however, are not able to synthesize siderophores, but express transporters enabling the use of siderophores produced by other microorganisms [144, 145]. Like *S. cerevisiae, C. albicans* and *C. neoformans* are non-producing siderophores fungi. The *C. neoformans* genome encodes at least six siderophores transporters, but only the transporter Sit1 was so far characterized [146]. In the case of *C. albicans*, the assimilation of siderophores occurs *via* the cell surface transporter, Sit1/Arn1 (Figure 1.3) [147]. In *C. glabrata*, Sit1 is also involved in the assimilation of siderophores [138].

Ferritin can also be used as an Fe source by fungal pathogens. *C. albicans* is able to capture ferritin by the expression of the hypha-associated cell wall gene, *ALS3* (Figure 1.3) [148]. Although *C. albicans* Als3 binds ferritin, the release of Fe from this protein is only possible after acidification of the surroundings, suggesting that this mechanism is pH-mediated [148]. Once released from

ferritin, Fe enters the reductive Fe assimilation pathway. *C. albicans* possesses a family of ferric reductases that reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron [144], which can be reoxidized by a multicopper oxidase to ferric Fe. The latter can then enter the cell *via* Ftr1, a high-affinity Fe permease (Figure 1.3) [130].

C. albicans can use transferrin as a Fe source (Figure 1.3) [149]. Although the transferrin receptor was not yet identified, it was suggested that this fungus requires direct contact with transferrin in order to use it [149]. Ferric Fe is subsequently taken up *via* the reductive iron pathway [150].

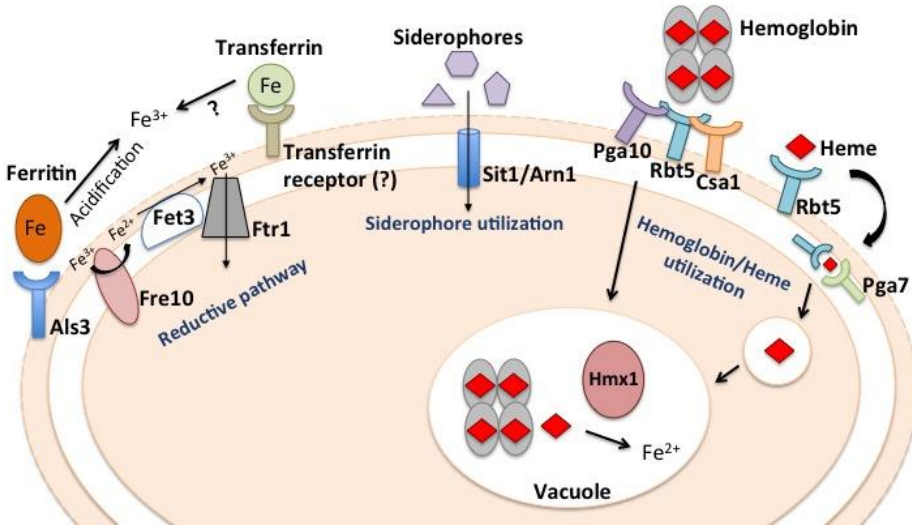

Figure 1. 3 Schematic representation of *C. albicans* mechanisms of Fe acquisition. Hemoglobin, heme, transferrin, and ferritin, as well as siderophores may be used as Fe sources. The question mark " ?" indicates still uncovered steps. In the reductive pathway, Fre10 is used as an example of the ferric reductases family. Although *C. albicans* is not able to produce siderophores, it can assimilate siderophores produced by other species.

Despite the thight regulation of Fe availability in humans, increased Fe levels are commonly associated with several diseases and conditions, such as hemochromatosis, hepatic disease, acute leukemia among others [151, 152]. Not

surprisingly, growing evidences point towards clinical connections between those diseases and fungal infections [151].

Copper

Enzymes involved in several biological processes, such as respiration and free radical detoxification, rely on Cu to properly function, making it an essential metal for life [153, 154].

In humans, the uptake and distribution of Cu is a target of tight homeostatic regulation. Inside the cell, copper is complexed with metallothioneins (MTs) and cuproenzymes, and in the plasma it is transported by albumin [155, 156], or it is bound to ceruloplasmin, a multicopper oxidase which oxidizes Fe^{2+} to Fe^{3+}, to be loaded into transferrin [153].

Due to the toxic potential of Cu, this metal is used by the host's macrophages to kill pathogens. The biocide activity of Cu metal relies on its ability to produce ROS *via* Fenton-type reactions and to mediate the disruption of Fe-S clusters, leading to the inactivation of essential enzymes [153]. Wagner *et al* demonstrated for the first time that the increase of copper concentration in phagolysosomes is used by macrophages as a means to kill *Mycobacterium* [157]. A similar phenomenon was suggested to occur during *C. albicans* infections since these fungi exhibit response signals of high Cu exposure after macrophage engulfment [158]. To counteract the negative effects of Cu overload, pathogenic fungi have developed several strategies. *C. albicans* possesses a Cu extrusion P-type ATPase in the plasma membrane, Crp1, which is not found in *S. cerevisiae*, *C. neoformans*

or *Schizosaccharomyces pombe* [159]. This extrusion pump has been shown to be the foremost important Cu detoxification mechanism, being responsible for the high Cu tolerance observed in *C. albicans* [159]. During infection of human lungs, *C. neoformans* senses high levels of Cu and activates the expression of two metallothioneins genes, *CMT1* and *CMT2*, shown to be essential for virulence and survival of this fungus inside the host [160].

Restriction of Cu as part of the host's nutritional immunity response is still a controversial subject. Mammals have four metallothioneins isoforms, being one of them, MT3, almost exclusive to the central nervous system (CNS). The fact that these proteins can be released by astrocytes to the extracellular environment, suggests a potential involvement of MTs in host restriction of Cu in the brain [161]. Accordingly, there are some examples of Cu deprivation responses in pathogens during infection. One example occurs in the brain after *C. neoformans* dissemination leading to meningoencephalitis. In this environment, the fungus activates Cu uptake transporters, contrarily to what is observed in the lung, as mentioned before [162, 163]. Another example is found during kidney infection by *C. albicans*. During the progression of infection, *C. albicans* activates the expression of *CTR1* gene, which encodes a Cu uptake transporter and uses a non-copper superoxide dismutase, Mn-Sod3, to spare copper but still resist host generated-ROS [164, 165].

Zinc

Zinc is the second most abundant transition metal in the human body. It plays

a relevant biological role since it is structurally and/or catalytically essential for several enzymes involved in diverse cellular processes, such as transcription, cell division, programmed cell-death, T-cell differentiation and maturation [166-168], among others [155, 169].

In humans, albumin and α-2-macroglobulin bind Zn in the plasma [170, 171]. Cellular Zn transport is mainly mediated by two families of transporters: ZnT (soluble-linked carrier 30 – SLC30) and Zip (Zrt- and Irt- like proteins – SLC39A). The first decreases cellular Zn levels, by mediating its extrusion from the cell or its influx to intracellular vesicles. The second promotes the increase of Zn in the cytoplasm, conveying Zn transport from the extracellular fluid or Zn export from intracellular vesicles [172]. Metallothioneins have been also implicated in Zn homeostasis, acting as a reservoir during Zn deficiency and as a buffer when Zn levels are high [173].

Zinc restriction and sequestration was first described as a mechanism of nutritional immunity in bacterial infections [121]. Calprotectin, also designated calgranulin, is a heterodimer composed of two subunits, S100A8 and S100A9, with Zn and Mn binding capacities [174]. The metal binding capacity confers calprotectin antibacterial [174] and antifungal roles [120]. Calprotectin constitutes approximately 45% of the total cytosolic protein content of neutrophils [175]. It was shown that calprotectin is secreted, and due to the high infiltration ability of neutrophils, this protein reaches high concentrations during the course of fungal infections [120]. The neutrophil-induced calprotectin exposure is observed during a process designated NETosis (Figure 1.4). In this

process, neutrophils undergo programmed cell death with the release of NETs (neutrophil extracellular traps) to the surroundings [120]. NETs are spider web-like structures, comprising DNA with proteins associated to it among which is calprotectin (Figure 1.4) [176]. The metal-sequestration capability of calprotectin seems to be the ruling mechanism of the antifungal activity of NETs, as NETs derived from calprotectin-deficient mice demonstrated remarkably reduced antifungal activity comparing to wild-type mice [176].

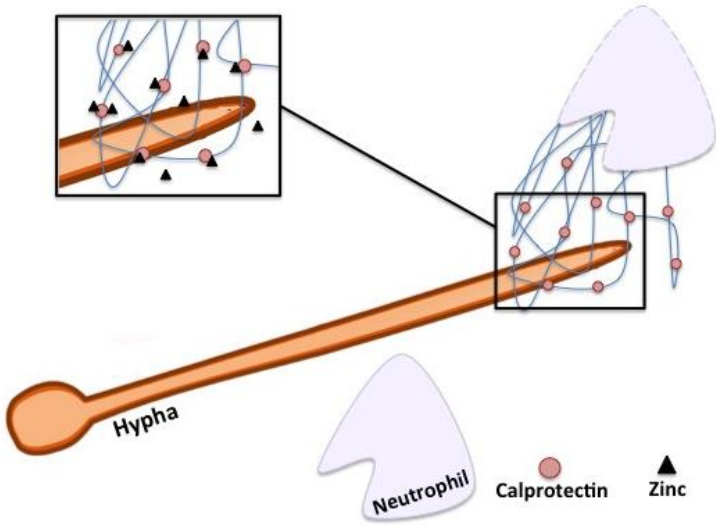

Figure 1. 4 Representation of the NETosis process. Neutrophils release NETs (neutrophil extracellular traps), which have a high calprotectin-content. The high Zn binding capacity of calprotectin leads to Zn sequestration and, consequently, Zn restriction in fungi's surrounding. (Adapted from [120]).

Zn deprivation occurs inside macrophages, as a weapon against fungal pathogens and at least two findings support this idea. First, there is evidence that phagocytized *C. albicans* up-regulates the expression of the Zn-transporter gene *ZRT2*, suggesting exposure to low Zn levels [177]. Second, it was demonstrated that Zn is mobilized to the Golgi apparatus of macrophages when *Histoplasma*

capsulatum is phagocytized, whereas in macrophages without phagocytized yeast, Zn is diffused throughout the cell [178].

C. albicans secretes the Pra1 protein, which functions as a "zincophore" system, to sequester Zn from host cells [179]. Surprisingly, in this yeast, Zrt1, a cell surface Zn transporter, also functions as a receptor for Pra1 [179, 180]. Interestingly, the absence of cell surface Zn transporters can lead to fungal virulence attenuation. This phenomenon has been reported in *A. fumigatus* after deletion of the *ZRFA* Zn-transporter gene [181], as well as, in *Cryptococcus gattii* lacking both Zn-transporter genes, *ZIP1* and *ZIP2*, [182].

4. Thesis scope

Over the last years, one of the research focus area of the Genomics and Stress laboratory has been the investigation of the mechanisms underlying the detoxification of essential and non-essential metals in *Saccharomyces cerevisiae*, with particular emphasis on those orchestrated by the Yap family. The main goals of this work were:

- To study the role of Yap1 in cobalt and cadmium detoxification;
- To investigate whether the players that orchestrate iron-loading detoxification in *Saccharomyces cerevisiae* are conserved and operational in the opportunistic human fungal pathogen *Candida glabrata*, a close relative of *S. cerevisiae*. If not, novel players should be identified.
- To further explore our preliminary data suggesting a link between fluconazole antifungal activity and yeast response to iron loading.

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Chapter II

YAP1 MEDIATES TOLERANCE TO COBALT TOXICITY IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Abstract

Cobalt has a rare occurrence in nature, but may accumulate in cells to toxic levels. In the present study, we have investigated whether the transcription factor Yap1 fulfils a role in cobalt detoxification. As expected, we found that cobalt generates oxidative damage in yeast cells, especially in the absence of Yap1. Strikingly enough however, when challenged with high concentrations of cobalt, *yap1* mutant cells accumulate lower levels of this metal. Accordingly, microarray analysis revealed that the expression of the high affinity phosphate transporter, *PHO84*, a well-known cobalt transporter, is compromised in the *yap1* mutant. Moreover, we uncovered Yap1 as a repressor of the low affinity iron transporter, *FET4*, which may as well mediate the cellular uptake of cobalt.

Altogether, our work demonstrates that cobalt activation of Yap1 alleviates the oxidative damage caused by this metal. Yap1 partially controls cobalt cellular uptake *via* the regulation of *PHO84*. Although *FET4* repression by Yap1 has no effect on cobalt uptake, under cobalt surplus conditions, we anticipate it may be its first line of defense against other toxic metals. Our results emphasize the important role of Yap1 in mediating protection against cobalt-induced oxidative damages and reveal new routes for cell protection provided by this regulator.

1. Introduction

Cobalt is a biologically important metal as it is an essential enzyme cofactor in most living organisms. However, a tight homeostatic control of cobalt is a preponderant requirement, since when present in excess, this metal may be life threatening. Overexposure to cobalt often occurs in several industrial occupations and, in humans, it can cause several diseases such as contact dermatitis, asthma, pneumonia and lung cancer [1].

Cobalt toxicity arises not only from the ability of cobalt ions to generate reactive oxygen species (ROS) *via* Fenton-type reactions, but also from its competition with other essential metals such as iron, calcium and zinc, for the binding to macromolecules possibly resulting in the inhibition of their proper function [2]. In addition, the chemical affinity of cobalt for sulfur atoms has also been invoked as a possible mechanism of cobalt toxicity, due to the likely dysfunction of proteins whose activity depends on thiol groups [3]. The exact mechanisms used by organisms to protect from cobalt toxicity remain to be elucidated. In the yeast *Saccharomyces cerevisiae*, cobalt may enter the cell through the low affinity iron transporter, Fet4, *via* the manganese transporter Smf2, or *via* the phosphate transporter Pho84 [4-6]. In line with the mechanisms of cobalt-induced toxicity, the cellular response to cobalt excess includes the activation of the major iron responsive transcription factor, Aft1 [7, 8]. One target of Aft1 is the *COT1* gene that codes for a vacuolar transporter [8, 9]. Cot1 presumably renders protection against the damaging effects of cobalt through cobalt sequestration in the vacuole [9, 10]. Nevertheless, overexpression of *COT1*

gene does not confer increased cobalt tolerance to cells deficient in high affinity iron transporter system, which met their iron requirements by increasing the expression of low affinity iron transporters [10].

In a second line of defence against cobalt surplus, yeast also increases the expression of genes involved in oxidative stress response [8]. The yeast transcription factor Yap1, is an essential regulator of the cellular response to oxidative stress, being activated upon exposure to hydrogen peroxide or to thiol modifying agents [11]. Moreover, Yap1 also plays a prime role in cadmium and arsenic detoxification by regulating the *YCF1* gene that encodes a vacuolar ATP binding cassette (ABC) transporter which functions as a vacuolar glutathione S-conjugated pump [12, 13]. Regulation of Yap1 occurs mainly through the control of its nuclear localization. Yap1 contains a C-terminal non-canonical nuclear export signal (NES), containing three cysteine residues, which are important for the interaction with the exportin protein Crm1 [14-16]. Crm1 rapidly exports Yap1 to the cytoplasm under normal growth conditions [15, 16]. When exposed to oxidants Yap1 undergoes oxidation induced conformational changes; as a consequence NES becomes masked, the Yap1-Crm1 interaction is lost and Yap1 becomes localized into the nucleus, activating its targets [17].

In this work, we have investigated the role of Yap1 in the response of yeast cells exposed to cobalt excess. We observed that in the absence of Yap1, cells exhibit a higher sensitivity towards cobalt and therefore we sought to understand how cobalt activates Yap1 and how Yap1 contributes to cobalt tolerance. Using genetic and biochemical approaches, we show that cobalt induces oxidative stress

in yeast cells and that Yap1 is required to alleviate the oxidative damages. The transcriptomic analysis of a strain lacking *YAP1* gene revealed novel targets of this regulator involved in the low affinity metal uptake (*PHO84* and *FET4*).

2. Material and methods

2.1 Strains, plasmids and growth conditions

The yeast strains and plasmids used in this study are listed in Table 2.1 and Table 2.2, respectively. Yeast strains were grown in synthetic media (SC: 0.67% ammonium sulfate-yeast nitrogen base without amino acids (Difco), 2% glucose, supplemented with the appropriate selective amino acids) or SC lacking specific requirements (SD). Phenotypic growth assays were carried out by spotting 5 µl of early exponential phase cultures (OD_{600}=0.4) sequentially diluted (approximately 5×10^3 to 50 cells) in medium containing the indicated $CoSO_4$ concentrations. Growth was recorded after 2 days at 30°C. The bacterial *Escherichia coli* strain XL1-Blue (Stratagene) was used as a host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation. The anaerobiosis assay was performed with cultures transferred to a glove box, for at least 24 h, where the O_2 concentration was kept below 1 ppm. Low and high-phosphate media were prepared as described in [18].

Table 2. 1 Strains used in this study.

Strain	Genotype	Source
BY 4742	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0*	EUROSCARF
FY 1679-01B	MATa *ura3-52 leu2 trp1 hisS3 gal2*	EUROSCARF
BY 4742 *yap1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 yap1Δ::kan*	EUROSCARF
YPH98 *orp1*	MATa *ura3-52 lys2-801^{amber} ade2-202^{ochre} trp1Δ1 leu2Δ1 orp1Δ*	[19]
BY 4741	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY 4741 *yap1*	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 yap1Δ:: kan*	EUROSCARF
BY 4741 *pho4*	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 pho4Δ:: leu2*	Prof. Valeria Culotta
BY 4741 *yap1pho4*	MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 yap1Δ:: his3 pho4Δ:: leu2*	This study

Table 2. 2 Plasmids used in this study.

Designation	Use	Source
cp-GFP-HA-YAP1	see Figure 2.2 A	[17]
GFP-cCRDYap1	see Figure 2.2 B	[17]
cCRD-Yap1-TAP-tag	see Figure 2.2 C	[20]
c-Myc– YAP1	see Figure 2.3A	[17]

2.2 Fluorescence microscopy

Cells transformed with *GFP-YAP1* [14] or *GFP-cCRDYap1* [20] were grown to early exponential phase and challenged with 2 mM $CoSO_4$ for the indicated time points. DAPI (4′,6-diamidino-2-phenylindole) was added as a DNA marker at the final concentration of 5 μg/ml before microscopy. After washing with PBS, cells were resuspended in a solution of DABCO [(1,4-diazadicyclo[2.2.2]octane) in

75% (v/v) glycerol and 0,25× PBS (Sigma-Aldrich)]. GFP signals were analysed in living cells with a Leica DMRXA fluorescent microscope equipped with a Roper Scientific Micro-Max cooled CCD (charge-coupled device) camera and MetaMorph software (Universal Imaging Inc.).

2.3 Immunoblot assays

Wild-type (WT) and *yap1* strains containing *cCRD-Yap1-TAP-tag* plasmid [20] were grown until early exponential phase (OD_{600} =0.4–0.5) and untreated or exposed to 10 mM $CoSO_4$ for 30 min. Cells were harvested and protein extracts were generated from cell cultures using cell lysis buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM KCl, 10% glycerol, 0.1% NP40) supplemented with protease inhibitors (Roche). Protein extracts were incubated 30 min at room temperature and 5 min at 37°C with 20 mM of methoxypolyethylene glycol maleimide (MAL-PEG, Sigma-Aldrich). Samples were resolved by reducing 10% SDS-PAGE and immunoblotted with a horseradish peroxidase-bound antiperoxidase rabbit IgG (Life Technologies). Regarding the protein carbonylation assay, WT and *yap1* strains were grown until early exponential phase cells, either untreated or exposed to 2 mM $CoSO_4$ and collected after 60 and 90 min of treatment. Cell cultures were resuspended in cell lysis buffer [50 mM Tris buffer pH7.5, 100 mM NaCl, 5 mM MgCl2, 5% β-mercaptoethanol and protease inhibitors (Roche)]. To evaluate the presence of carbonyl groups, an OxyBlot™ Protein Oxidation Detection Kit (Intergen) was used. The samples were analysed by immunoblotting and

processed as described in [12], using rabbit anti-dinitrophenol antibody. Pgk1 was used as a loading control [21].

2.4 Microarray and quantitative real-time PCR analyses

Total RNA from early log-phase cultures untreated or exposed to 2 mM of $CoSO_4$ for 1 h, was purified and used for DNA microarray analysis, as previously described [21]. SAM analyses were performed using the algorithm implemented in MeV from TM4 software [22]. The array design, spotting protocol, raw data and pre-processed data from all hybridizations were submitted to the Array Express Database (E-MEXP- 3874). Searches for putative Yap binding sites were carried out using the YEASTRACT database [23]. Gene clustering was performed according to the Munich Information Center for Protein Sequences database (MIPS) functional catalog (http://mips.helmholtz-muenchen.de/proj/funcatDB/).

For qRT-PCR experiments, RNA was isolated from early log-phase cultures that were either untreated or exposed to 2 mM $CoSO_4$ and harvested at the indicated time points. RNA samples were treated with DNase (TURBO™ DNase-free; Ambion) according to the manufacturer's instructions and purified by on-column DNAse I digestion (RNase-Free DNase Set; Qiagen). Total RNA (1 μg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). qRT-PCR reactions were performed in the Light Cycler 1.5 Real-Time PCR System (Roche), using Light Cycler Fast Start DNA Master SYBR Green I (Roche). Relative standard curves were constructed for each gene, using

triplicate serial dilutions of cDNA. The relative expression of the genes was calculated by the relative quantification method with efficiency correction, using the LightCycler Software 4.1. Actin (*ACT1*) gene was used as a reference gene. All assays were made at least in duplicate. The primers used in this assay are listed in Table 2.3.

Table 2. 3 Primers used in the qRT-PCR analysis.

Gene	Primers
ACT1	5'cta ttg gta acg aaa gat tca g 3'
	5' cct tac gga cat cga cat ca 3'
GPX2	5'cgg aag taa tgc tga ctc tgt c 3'
	5' ggt cca agg acg atg gtt tt 3'
GSH1	5' agg cgt ggt gaa aaa gtt tg 3'
	5' tcg cct ctt tgt ctt ctg gt 3'
TRX2	5' ggt cac tca att aaa atc cgc ttc 3'
	5' cga cga ctc tgg taa cct cct tac 3'
SOD1	5' agc caa cca ctg tct ctt acg a 3'
	5' aca cca ttt tcg tcc gtc ttt a 3'
PHO84	5' cgt tct agg gtt ggc atg tt 3'
	5' ttc gcc atc ttg ttc ttg tg 3'
TRR1	5' cca cac tcc agc aac aaa ga 3'
	5' tcc tgg aac gga ggt taa tg 3'
SPL2	5' ggt cac cag cat aag gga ag 3'
	5' tgc gat gtt cta cgt gga gt 3'

2.5 Measurement of cobalt and phosphorous

Strains were grown in SC media with 2 mM of $CoSO_4$ for 20 h, collected by centrifugation and washed with 10 mM EDTA and metal-free water. The total Co and P were measured by inductively coupled plasma (ICP) atomic emission spectroscopy. Data were normalized against OD_{600}.

2.6 Glutathione (GSH) and glutathione disulphide (GSSG) quantification

The WT and mutant strains were grown to early log-phase, exposed to 2 mM of $CoSO_4$ and harvested after 1 h. GSH and GSSG were quantified by HPLC after sample derivatization, as described in [24]. GSH and GSSG concentrations in mM/cell were calculated on the basis of an average yeast cell volume of 4.5×10^{-14} [25].

3. Results

3.1 Yap1 is required for resistance to cobalt

We have started to investigate the role of Yap1 in response to cobalt excess by monitoring the growth of a *S. cerevisiae* strain lacking *YAP1* (*yap1*) and of its isogenic wild-type (WT) strain, in the presence of cobalt (Figure 2.1 A). The *yap1* mutant strain was more sensitive than the WT to a wide range of cobalt concentrations (Figure 2.1 A and B). Reintroduction of *YAP1* into the *yap1* mutant strain rescued its cobalt sensitivity, confirming that cobalt high-toxicity was in fact due to the loss of Yap1 function (Figure 2.1 C).

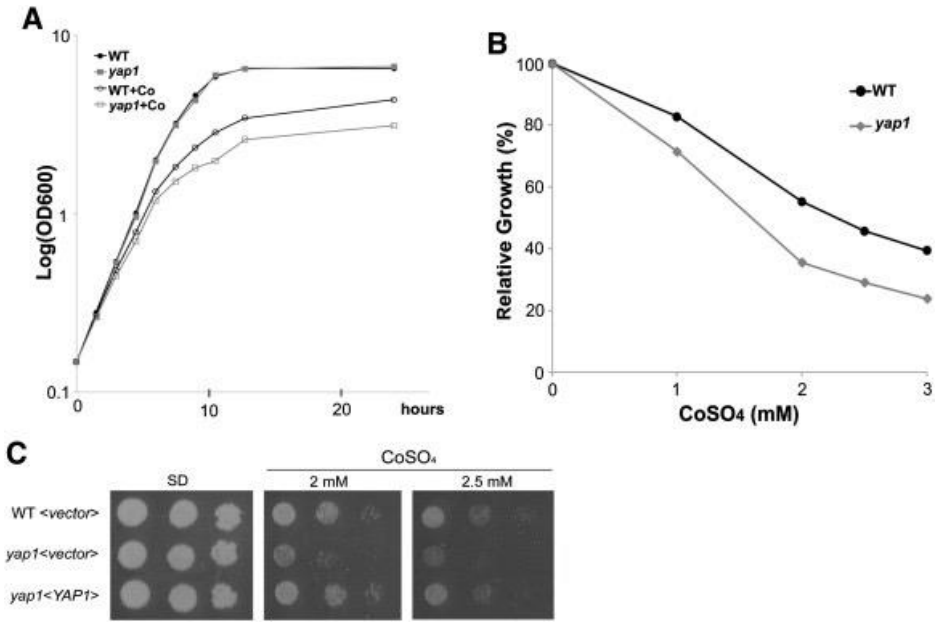

Figure 2. 1 Yap1 confers tolerance to cobalt excess. (A) The growth of wild-type (WT) and yap1 mutant strains in SC medium containing 2 mM of $CoSO_4$ was monitored by means of turbidity measurements. **(B)** WT and *yap1* cells were incubated in SC medium containing the indicated amount of $CoSO_4$ for 12 h and spread onto SC plates. Colony forming units were recorded (% survival relative to untreated cells). **(C)** *yap1* strain was transformed with a centromeric plasmid containing the *YAP1* gene (*yap1*<*YAP1*>), or the plasmid alone (*yap1*<*vector*>).WT strain was transformed with the empty plasmid (WT <*vector*>). Exponentially growing cells were harvested, serially diluted and spotted onto control SD plates or SD plates containing the indicated $CoSO_4$ concentrations.

3.2 Cobalt activates Yap1

Because Yap1 activation requires its accumulation in the nucleus, we followed the effect of cobalt in the cellular localization of a GFP-Yap1 fusion protein. Yap1 appeared predominately localized in the cytoplasm under non-stressed conditions. 10 min after cobalt treatment (2 mM of $CoSO_4$), a strong GFP nuclear staining was observed that persisted for up to 1 h (Figure 2.2 A). After 90 min,

nuclear staining was still detected, although with a lower intensity. After this time period, Yap1 is still able to sense cobalt as a second challenge with this stressor led to a novel induction of its targets, which suggests that the activation of Yap1 by cobalt is a reversible process (Figure 2.3). Overall, the kinetics of Yap1 localization in the cell is in according to the rapid and transient expression profile of Yap1 target genes, upon exposure to cobalt (Figure 2.7 A–E).

Azevedo *et al.* showed that Yap1 carries two distinct molecular redox sensors, one operated by H_2O_2 and the other by thiol-reactive compounds [20]. The former relies on the formation of an intramolecular disulphide bond between N- and C-terminal cysteine residues that requires Orp1/Gpx3, the primary sensor transducing the H_2O_2 signal to Yap1 [17, 19]. The latter, involves three Yap1 C-terminal cysteine residues that directly interact with the drug [20]. In order to understand which molecular device was operating in Yap1 activation by cobalt, we first examined whether a truncated GFP-Yap1 version, only bearing the C-terminal cysteine rich domain of Yap1 (*GFP-cCRDYap1*), was enough to drive its accumulation into the nucleus under cobalt treatment. As illustrated in Figure 2.2 B, the GFP-cCRDYap1 construct was found in the nucleus in the presence of cobalt. Consistent with this observation, cobalt induced the accumulation of Yap1 in the mutant *orp1* (Figure 2.2 B). Furthermore, we still observed Yap1 nuclear localization under anoxia after cobalt treatment (Figure 2.2 C), suggesting that Co-Yap1 activation may also occur *via* a ROS-independent mechanism.

As cobalt has high affinity for sulfhydryl groups [2], we next assessed Yap1 cCRD free sulfhydryl status by means of the high molecular mass cysteine-specific

alkylating agent, MAL-PEG. MAL-PEG increases the mass of a protein by 5 kDa for each alkylated cysteine. Proteins with exposed thiols are able to react with the MAL-PEG to form covalent complexes of greater molecular mass relative to non-alkylated proteins and thus their migration is retarded in subsequent electrophoretic gel. We used a TAP tagged version of the C-terminal cysteine rich domain of Yap1 (*cCRDYap1-TAP-Tag*) to transform a WT strain. Protein extracts were then incubated with MAL-PEG in the absence or presence of cobalt, analysed by SDS-PAGE and immunoassayed using an antiTAP-tag antibody (Figure 2.2 D). Yap1 cCRD from samples untreated with cobalt and incubated with MAL-PEG (lane 2) had a slower mobility than the protein from MAL-PEG unreacted samples (lane 1), indicating modifications of the polypeptide by the high molecular mass alkylating agent. The presence, however, of three bands in the former (lane 2) indicates that under the tested conditions Yap1 cCRD was present as a mixture of modified and non-modified molecules. Yap1 cCRD from protein extracts treated with cobalt and MAL-PEG (lane 3) exhibited a third band ("b" in Figure 2.2 D) of intermediate size between the total alkylated ("a" in Figure 2.2 D) and unalkylated ("c" in Figure 2.2 D) polypeptides.

These data suggest that cobalt activates Yap1, driving its nuclear accumulation presumably by binding to the Yap1 C-terminal cysteines.

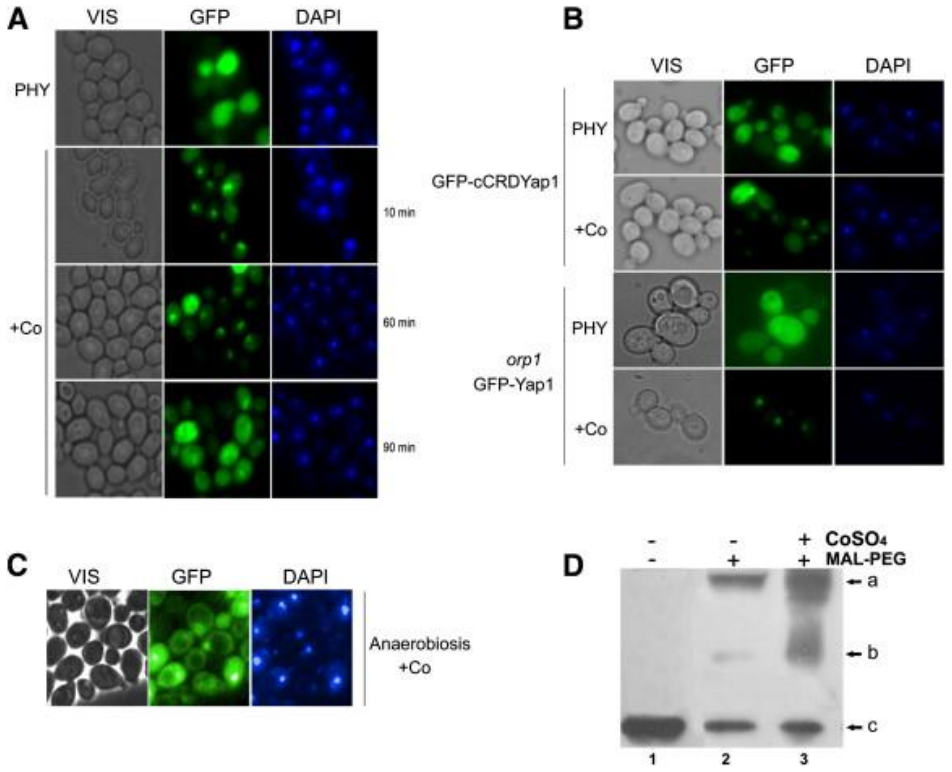

Figure 2. 2 Yap1 is activated by cobalt. (A) The *yap1* mutant expressing the fusion *GFP-YAP1* was induced with 2 mM of $CoSO_4$ and analysed for GFP staining at the indicated time points, by fluorescence microscopy. **(B)** The construct *GFP-cCRDYAP1*, containing the C-terminal cysteine rich domain of Yap1, was used to transform *yap1* strain (upper part of the panel). The *orp1* mutant was transformed with the fusion *GFP-YAP1* (lower part of the panel). Exponentially growing cells were treated with 2 mM of $CoSO_4$, for 10 min and fluorescence was recorded. **(C)** The localization of the GFP-Yap1 fusion was assayed under anaerobiosis after exposure to 2 mM of $CoSO_4$ for 30 min. PHY-physiological conditions **(D)** Protein extracts from cultures of a WT strain carrying a *cCRDYap1-TAP-tag* were left untreated (lanes 1 and 2) or were treated with $CoSO_4$ (lane 3). The proteins were solubilized in the absence (lane 1) or presence (lanes 2 ad 3) of 20 mM of MAL-PEG. The *cCRDYap1-TAP-tag* was revealed with labeled IgG.

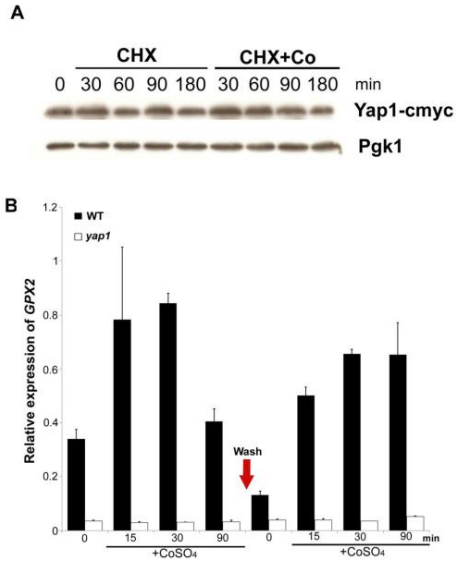

Figure 2. 3 Yap1 can be reactivated by a second cobalt challenge. (A) Cobalt does not dramatically interfere with Yap1 protein levels. Exponentially *yap1* mutant cells transformed with a c-Myc tagged version of Yap1 (Yap1-c-Myc) were first incubated with 2 mM of $CoSO_4$ or left untreated. Cells were next treated with the inhibitor of protein biosynthesis cicloheximide (100 µg/µl) and harvested at the indicated time points. The protein levels were subsequently monitored by Western blot using an anti-c-Myc antibody. **(B)** Yap1 is able to induce its targets upon a second cobalt challenge. Exponentially growing cells of WT and *yap1* were treated with 2 mM of $CoSO_4$. After 90 minutes, cells were washed with warm medium (30ºC) and subjected to a second treatment with cobalt (2 mM of $CoSO_4$). Cells were harvested at the indicated time points and RNA was extracted. The Yap1 target *GPX2* expression was analyzed trough qRT-PCR (see Material and Methods).

3.3 Yap1 targets under cobalt induced stress

Aiming at identifying Co-dependent targets of Yap1, we characterized the global changes in the transcriptome of *yap1* mutant cells grown under cobalt surplus conditions. Because all organisms encounter reactive oxygen species during the course of normal aerobic metabolism, which could lead to Yap1 activation, we also monitored transcriptomic changes of *yap1* mutant cells in the

absence of cobalt. DNA microarrays analyses were conducted by comparing RNAs isolated from the *yap1* mutant *vs* WT strains grown in the absence or presence of 2 mM of $CoSO_4$ for 60 min. Genes differentially expressed were sorted into functional categories according to MIPS (Tables S1 and S2). Figure 2.4 shows a schematic representation of the results of these experiments from triplicate Agilent DNA microarray studies. A total of 40 genes displayed an altered expression, by at least 1.5 fold, in the *yap1* strain, following growth shift to high-Co medium, with 30 transcripts being down-regulated. Remarkably, roughly 70% of these genes (27 out of 40) are already dependent on Yap1 under normal growth conditions (Figure 2.4). Among all the MIPS functional categories, the stress response and oxidative stress response categories were well represented in our microarrays, accounting for more than 40% (17 out of 40) of the total number of genes. We also found that Yap1 regulates the expression of 5 genes involved in iron homeostasis (Figure 2.4 and Tables S1 and S2), namely in plasma membrane iron uptake (*FIT2*, *FIT3* and *FET4*); mitochondrial iron import (*MRS4*) and iron-sulfur protein synthesis (*ISU2*) [7]. All of them are up-regulated by Yap1, with the exception of *FET4*, a low affinity iron transporter that has a broad metal specificity [10]. Indeed, *FET4* expression in the *yap1* mutant strain was up-regulated by 2 and 4.5 fold, in the absence and presence of cobalt excess, respectively (Tables S1 and S2), suggesting that Yap1 is a negative regulator of *FET4*. Nevertheless, we could not find any canonical YRE (Yap Response Element) [11] in the promoter region of *FET4* indicating that Yap1 is not most probably directly regulating *FET4* gene. Interestingly, we observed that Yap1 is a

positive regulator of two other genes involved in phosphate metabolism: *PHO84* and *SPL2* (Figure 2.4, Table S1 and S2, and Figure 2.5). Pho84 is a high affinity phosphate transporter [5, 26, 27] and Spl2 is a negative regulator of the low affinity phosphate transporters [28]. Neither *PHO84* nor *SPL2* have YREs in the respective promoter regions, suggesting that Yap1 may not directly orchestrate their regulation.

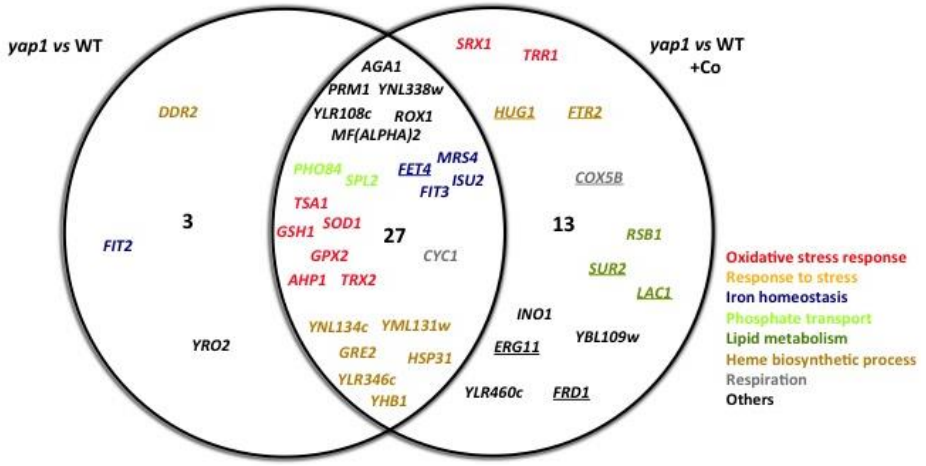

Figure 2. 4 Summary of genes whose expression is altered in the *yap1* mutant strain under normal or cobalt-induced stress conditions. In the *yap1* mutant, 40 genes showed altered expression levels by at least 1.5 fold, relative to the wild-type (WT), after treatment with 2 mM of $CoSO_4$, for 1 h (*yap1 vs* wt +Co). A total of 30 genes were already dependent on Yap1 under normal growth conditions (*yap1 vs* wt). The intersect represents the number of genes regulated by Yap1 in both conditions (27 genes). Down-regulated genes are underlined. Genes were sorted into functional categories accordingly to the indicated colour code.

These results indicate that the majority of genes regulated by Yap1 upon stress are already being regulated by this transcription factor under normal growth conditions. The main differences observed concern the up-regulation of a larger number of genes implicated in the stress response. Furthermore, our data also

unveil new targets of Yap1 involved in metal metabolism, pointing out towards new lines of cell protection given by this regulator.

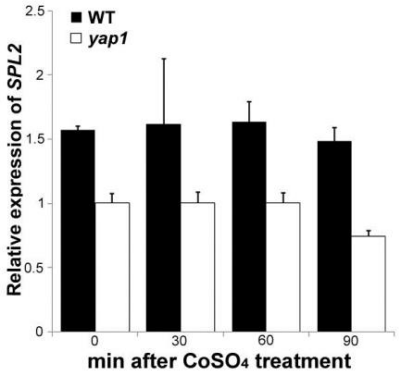

Figure 2. 5 Yap1 is a positive regulator of *SPL2* gene. Wild-type (WT) and *yap1* strains were upshifted to high-cobalt medium, by supplementation of SC medium with 2 mM of $CoSO_4$, and harvested at the indicated time-points. The expression of *SPL2* gene was assessed by qRT-PCR. Values are the mean of biological triplicates ± s.d.

3.4 Integration of Yap1-mediated gene regulation in the overall transcriptomic response of yeast cells to cobalt

In order to provide a clear picture of the relevance of Yap1 transcription factor in the overall yeast response to cobalt stress, we performed a genome-wide transcriptional analysis of *S. cerevisiae* exposed to this stressor. As such, we first compared the mRNA expression profile of wild-type (WT) cells upshifted to Co-supplemented medium (2 mM $CoSO_4$, 60 min) to cells grown under unstressed conditions. Cobalt challenge led to an increase in the steady-state levels of 242 genes (Table S3). Approximately 25% of these genes (61 out of 242) are related to stress response, with 26 genes assigned to the oxidative stress response category. As described by others [8], the metal category is also well represented (17 out of

242 genes) in the yeast response to cobalt, being 14 genes involved in iron homeostasis (Table S3).

We next searched for potential Yap1 regulated genes in the above dataset. To accomplish this task we used the YEASTRACT database [23] and compared our dataset with previous studies on the Yap1 regulon or search for the presence of YREs in the promoter region of the genes (Yap Responsive Elements). We found that 152 out of 242 genes possesses YREs in the corresponding promoter regions or have been already documented to be regulated by Yap1. However comparison of those Yap1-regulated candidates with the list of genes dependent on Yap1 under cobalt surplus conditions (Table S2) just retrieved 13 genes (Figure 2.6 and Table 2.4). Interestingly, only two of these genes (*INO1* and *SRX1*) are not dependent on Yap1 under normal growth conditions (Table 2.4) suggesting that Yap1 specifically regulates them under cobalt surplus. *INO1* however does not have YREs and as such Yap1 cannot be a direct regulator of this gene.

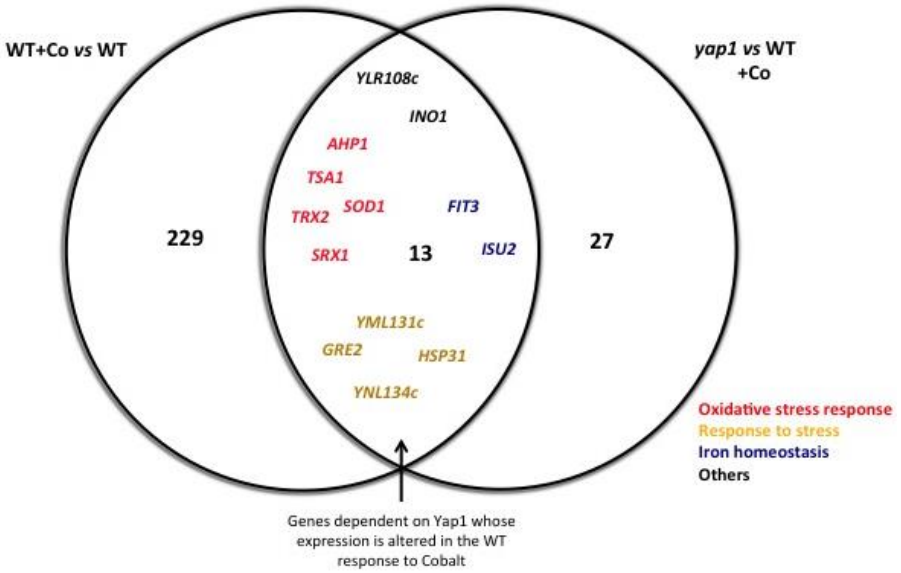

Figure 2. 6 Involvement of Yap1 in the overall transcriptomic changes observed in yeast cells exposed to cobalt. In the wild-type (WT) strain 242 genes were induced after treatment with 2 mM of $CoSO_4$, for 1 h (wt+Co *vs* wt). In the *yap1* mutant, 40 genes showed altered expression levels by at least 1.5 fold, relative to the WT, after treatment with 2 mM of $CoSO_4$, for 1 h (*yap1 vs* wt +Co). The intersection represents the number of genes dependent on Yap1 whose expression is altered in the WT response to cobalt (13 genes). Genes were sorted into functional categories accordingly to the indicated colour code.

Table 2. 4 Genes dependent on yap1 whose expression is altered in the wt response to cobalt.

Systematic name	Gene	YREs	Description	(1)	(2)
YLR109W	*AHP1*	−313	Thiol-specific peroxiredoxin	Y	Y
YOR383C	*FIT3*	−126	Mannoprotein	N	Y
YOL151W	*GRE2*	−777	3-methylbutanal reductase and NADPH-dependent	Y	Y
YDR533C	*HSP31*	−261	Possible chaperone and cysteine protease	Y	Y
YJL153C	*INO1*	–	Inositol-3-phosphate synthase	N	N
YOR226C	*ISU2*	−438	Required for synthesis of mitochondrial and cytosolic iron-	Y	Y
YJR104C	*SOD1*	−142	Cytosolic copper-zinc superoxide dismutase	Y	Y
YKL086W	*SRX1*	−144; −255; −294	Sulfiredoxin, contributes to oxidative stress resistance	Y	N
YGR209C	*TRX2*	−181; −210	Cytoplasmic thioredoxin isoenzyme	Y	Y
YML028W	*TSA1*	−180	Thioredoxin peroxidase	Y	Y
YLR108C		−343; −169	Protein of unknown function	Y	Y
YML131W		−500	Protein of unknown function	Y	Y
YNL134C		−289; −860; −748	Protein of unknown function	Y	Y

(1) Previously documented as dependent on Yap1?[a]

(2) Dependent on Yap1 under normal growth conditions?[b]

3.5 Cobalt generates severe oxidative damage in the absence of *YAP1*

To evaluate whether Yap1 was important to deal with the oxidative damage presumably generated by exposure to cobalt, we first monitored by qRT-PCR, the expression of genes coding for several antioxidant defences (*TRR1*, *TRX2*, *GSH1*, *SOD1* and *GPX2*) and confirmed their dependence on Yap1 (Figure 2.7 A–E). All tested genes were up-regulated after treatment with cobalt and were shown to be dependent on Yap1. Some level of Yap1-independent gene expression, however, was observed suggesting that other factors might be regulating their expression. The exception is the *GPX2* gene that encodes a 2-Cys peroxiredoxin [29] whose expression is completely abrogated in the *yap1* mutant (Figure 2.7 E).

In response to an oxidant challenge, yeast cells elevate GSH levels both by means of increased synthesis and by recycling the oxidized form (GSSG) to the reduced form (GSH) [30]. The compromised levels of *GSH1* gene expression in the *yap1* mutant, prompted us to further analyse, by HPLC, the cellular levels of reduced and oxidized glutathione in the wild-type (WT) and mutant strains subjected to cobalt treatment (Table 2.5). As expected, GSH levels were significantly compromised in the *yap1* mutant strain, being reduced from approximately 40% and 26% in unstressed and stressed samples, respectively, in comparison to the WT strain. Notably, GSSG/GSH ratios were decreased in both strains, after treatment with cobalt. This finding was rather unexpected as the utilization of GSH usually results in its conversion to the oxidized form (GSSG) and in the consequent increase of the GSSG/GSH ratio [12].

To examine the possible oxidative effects of cobalt treatment at the protein level, we next monitored the changes in the protein carbonyl status of yeast cells exposed to 2 mM of $CoSO_4$ for 60 and 90 min (Figure 2.7 F). Protein carbonylation is an early and largely used indicator of protein oxidation, and results from oxidative modifications on amino acid residues and by oxidative cleavage of the peptide chain [31]. Our results clearly indicate that the amount of carbonylated proteins increase after cobalt treatment, being more pronounced in the *yap1* mutant.

Overall our data indicate the existence a redox imbalance and oxidative damage triggered by cobalt excess which are more accentuated in cells lacking Yap1, highlighting the relevance of this regulator in cobalt induced stress response.

Table 2. 5 GSH measurements in WT and *yap1* strains upon cobalt induced stress.

Strain	GSH (mM/cell)	GSSG (mM/cell)	GSSG/GSH
WT	12.23 ± 0.03	3.35 ± 0.14	0.274 ± 0.011
WT+Co	8.11 ± 1.50	0.61 ± 0.09	0.077 ± 0.025
yap1	7.3 ± 1.10	1.87 ± 0.30	0.256 ± 0.003
yap1+ Co	6.0 ± 0.07	0.22 ± 0.07	0.037 ± 0.010

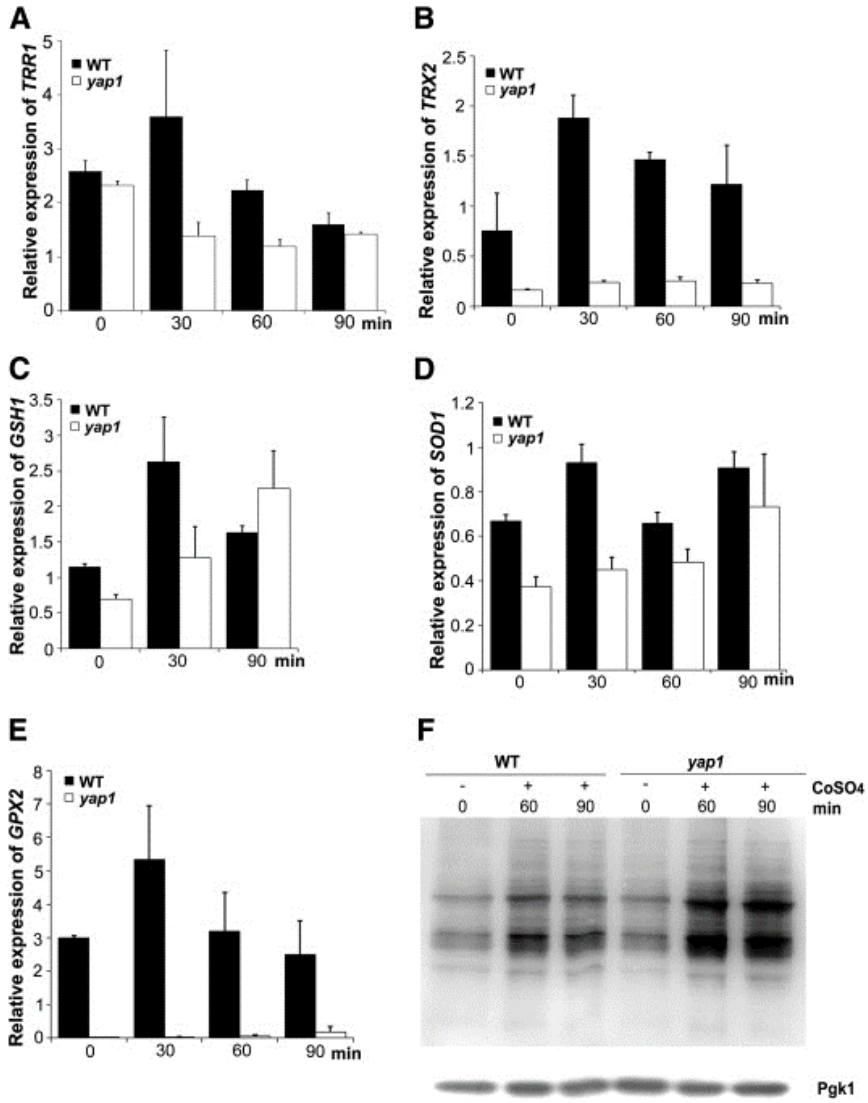

Figure 2. 7 Yap1 provides protection against cobalt-induced oxidative stress. (A–E) Wild-type (WT) and *yap1* strains were upshifted to high-cobalt medium, by supplementation of SC medium with 2 mM of $CoSO_4$, and harvested at the indicated time-points. The expression of the indicated genes was assessed by qRT-PCR. Values are the mean of biological triplicates ±s.d. *TRR1* - thioredoxin reductase gene; *TRX2* - thioredoxin isoenzyme gene; *GSH1* - gamma glutamylcysteine synthetase gene; *SOD1* - copper-zinc superoxide dismutase gene; *GPX2* - glutathione peroxidase gene. **(F)** The content of carbonyl groups in proteins after cobalt treatment were evaluated by derivatization with 2,4-dinitrophenylhydrazone (DNP), followed by

immunoblot with an anti-DNP antibody. Pgk1 protein levels were used as loading control. A representative experiment is shown.

3.6 Yap1 is a negative regulator of *FET4*

Li *et al.*, have demonstrated that yeast cells compensate for the absence of the high iron affinity transporter, Fet3, by up-regulating *FET4* gene. The overexpression of *FET4* observed in the *fet3* mutant strain, however, led to an increase of transient metal accumulation that renders this strain high sensitive towards metals [10].

Microarray analyses showed Yap1 as a negative regulator of *FET4* expression (Figure 2.4, Tables S1 and S2). Aiming to understand whether the uptake of cobalt *via FET4* could be contributing to the sensitivity exhibited by the *yap1* mutant, we first tested *FET4* dependence on Yap1 by qRT-PCR (Figure 2.8 A). Confirming our microarray data, *FET4* expression was induced in the *yap1* mutant, being however its induction no longer observed after 90 min of cobalt exposure.

We next monitored the total cellular concentrations of cobalt in cells exposed to 2 mM of $CoSO_4$. Surprisingly, we observed a 25% decrease of the cobalt levels in the *yap1* mutant strain relative to the WT, as measured by ICP-AES (Figure 2.8 B). In addition, the double mutant *yap1fet4* did not exhibit an increased resistance to cobalt, comparatively to the single mutant *yap1* (Figure 2.8 C).

Overall, these results indicate that overexpression of *FET4* observed in the *yap1* strain is not responsible for the toxicity displayed by this mutant, and suggest that another Yap1 target is involved in cobalt uptake.

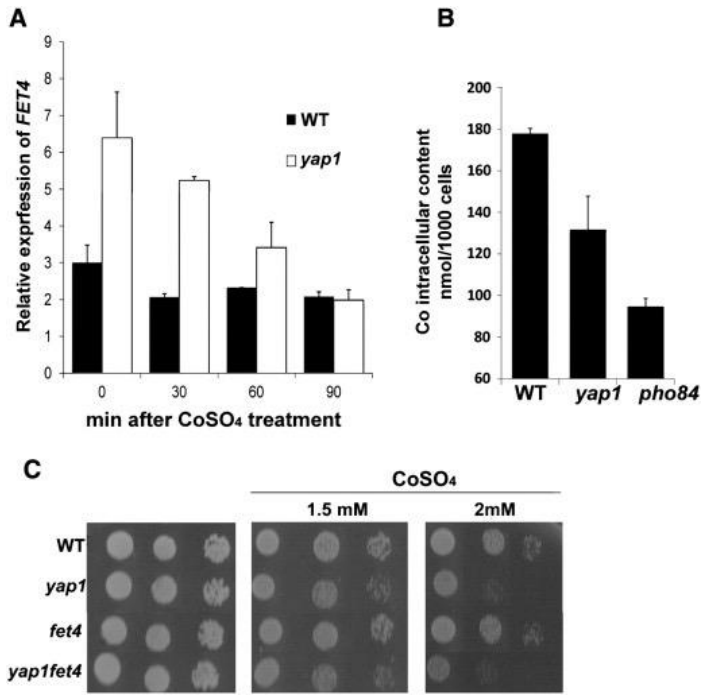

Figure 2. 8 The negative regulation of *FET4* by Yap1 does not contribute to *yap1* cobalt-sensitivity. (A) Wild-Type (WT) and *yap1* strains were treated with 2 mM of $CoSO_4$, and harvested at the indicated time-points. The expression of *FET4* gene was monitored by qRT-PCR. Values are the mean of biological triplicates ±s.d. **(B)** Cobalt accumulation in the WT (By4741), *yap1* and *pho84* mutants, after treatment with 2 mM of $CoSO_4$, was determined by inductively coupled plasma atomic emission spectroscopy. The data (±s.d.) are from at least two independent experiments **(C)** Exponentially growing cells from WT, single mutants *yap1* and *fet4*, and double mutant *yap1fet4* were harvested, serially diluted and spotted onto control SC plates or SC plates containing the designated $CoSO_4$ concentrations.

3.7 The lack of *YAP1* partially affects cobalt and phosphorus uptake

In an attempt to understand why the levels of cobalt were diminished in the *yap1* mutant, the microarray data were inspected as to find other Yap1-dependent genes known to be involved in metal homeostasis. In this search we

have found *PHO84* gene (Figure 2.4 and Tables S1 and S2) which codes for a high affinity inorganic phosphate transporter located at the yeast cell surface [32, 33]. In the absence of *PHO84* yeast cells become more resistant to several metals, including cobalt, as a consequence of a reduced metal accumulation [5, 27]. Accordingly, in this work, under the tested stress conditions, approximately 46% of cellular cobalt enters the cell *via* Pho84 (Figure 2.8 B). It is known that *PHO84* is regulated by the Pho4 transcription factor [34] and the microarray data (Figure 2.4) confirmed by qRT-PCR analysis (Figure 2.9 A), provide evidence that Yap1 is also regulating this gene even under physiological growth conditions. Indeed, in the double mutant *yap1pho4* the expression of *PHO84* is lower than in *pho4* and *yap1* single mutants (Figure 2.9 A). This finding may justify the lower cobalt uptake, observed in the *yap1* mutant (Figure 2.8 B). Further corroborating this assumption, we noted that the levels of total phosphorus were slightly but significantly compromised in the *yap1* strain, as measured by ICP-MS (Figure 2.9 B). Notably, whereas the deletion of *PHO84* from the *yap1* background completely rescues the growth sensitivity displayed by this strain, deletion of *PHO4* does not restore the normal growth (Figure 2.9 C). In our view, these findings point towards one of the following possibilities: or the basal levels of expression of *PHO84*, observed in the *yap1pho4* mutant, still upshift cobalt intracellular levels to toxic concentrations (Figure 2.9 A), or Pho4 is regulating other genes relevant for cobalt tolerance.

Together these data suggest that Yap1 partially controls cobalt and phosphorus uptake *via* the regulation of *PHO84*.

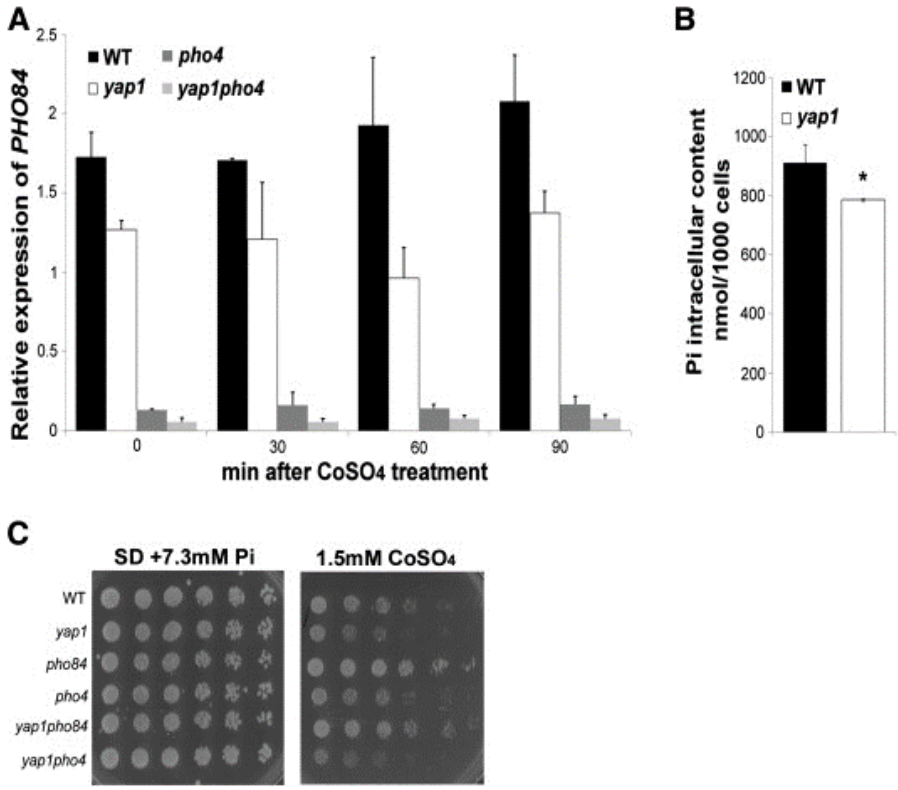

Figure 2. 9 Yap1 interferes with cobalt and phosphorus intracellular levels. (A) Wild-Type (WT), *yap1*, *pho4* and *yap1pho4* strains were challenged with 2 mM of $CoSO_4$ and harvested at the indicated time-points. The expression of *PHO84* gene was evaluated by qRT-PCR. Values are the mean of biological triplicates ± s.d. **(B)** WT and *yap1* strains were grown to mid-log phase and then upshifted to high-cobalt (2 mM $CoSO_4$), for 16 h. Phosphorus contents were determined by inductively coupled plasma atomic emission spectroscopy. The values are expressed in nmol/1000 cells and correspond to the mean of at least three independent measurements ± s.d.. Differences between WT and *yap1* strains are denoted as $*p<0.05$. **(C)** Exponentially growing cells from WT, single mutants *yap1*, *pho4* and *pho84* and double mutants *yap1pho4* and *yap1pho84* were harvested, serially diluted and spotted onto SC medium supplemented with cobalt.

3.8 Phosphate uptake alleviates superoxide stress

The role of Yap1 as a cobalt resistance determinant appears however to be contradictory to its role as *PHO84* activator. Why would Yap1, the major

oxidative surveyor of yeast cells, participate in the activation of a gene that contributes to the uptake of cobalt, a known oxidative damage inducer? One possibility is that phosphate uptake, somehow, counteracts the oxidative injury. Remarkably, growing evidence suggests that manganese-phosphate complexes act as potent scavengers of superoxide [35, 36]. On the other hand, Pho84 functions as a manganese phosphate transporter [5]. To test this hypothesis we growth the aforementioned strains in high (7.3 mM) or low (0.1 mM) phosphate medium supplemented with paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride), a superoxide generator. We observed that low phosphate concentrations render all the tested strains more sensitive to paraquat (Figure 2.10). In addition, deletion of *PHO4* or *PHO84* from the *yap1* background makes cells less resistant to this stressor (Figure 2.10). This data indicates that phosphate uptake should be relevant to cope with superoxide-induced stress.

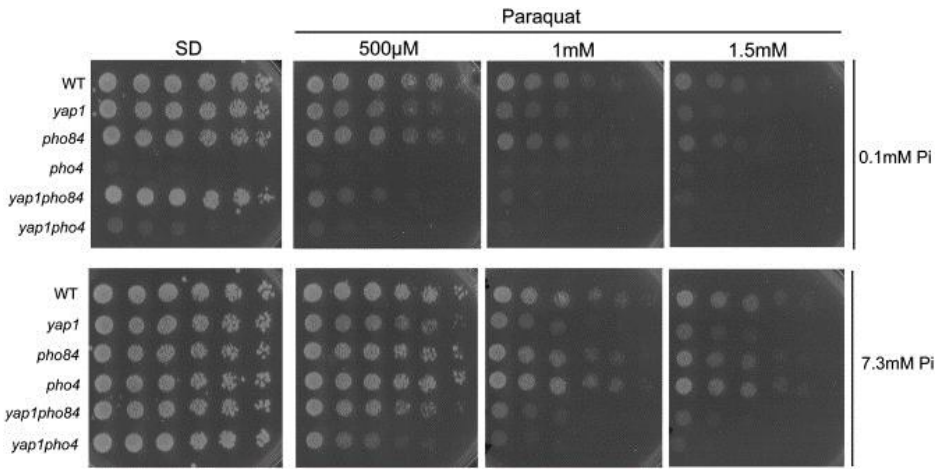

Figure 2. 10 Phosphate uptake alleviates superoxide stress. Exponentially growing cells from wild-type (WT), single mutants *yap1*, *pho4* and *pho84* and double mutants *yap1pho4* and *yap1pho84* were harvested, serially diluted and spotted onto high phosphate (7.3 mM) or low phosphate (0.1 mM) plates supplemented with the designated paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) concentrations.

4. Discussion

Our data revealed that the stress responsive bZIP regulator Yap1 is important for *Sacharomyces cerevisaie* tolerance to cobalt (Figure 2.1). We showed that Yap1 cCRD cysteine residues are crucial for sensing cobalt, probably serving as a binding site for this metal. As proposed for Cd mediated Yap1 activation [20], the putative binding of cobalt to Yap1 C-terminal cysteine residues may modify Yap1 NES, thereby inhibiting its interaction with the exportin Crm1 (Figure 2.2). As a result, Yap1 accumulates in the nucleus and drives the expression of its target genes.

As previously reported by other authors [37] we found that the regulatory role of this transcription factor is not restricted to stress, but Yap1 also controls gene expression under normal growth conditions (Figure 2.4). Consistently, we had shown that Yap1 possesses a high transactivation potential even in standard growth conditions [38]. ROS are by-products of aerobic metabolism, and therefore the activation of Yap1 under such conditions is not an unexpected finding. When cells are exposed to external stressors, however, the basal activation of Yap1 is likely insufficient to re-establish the redox homeostasis and yeast cells have therefore evolved a mechanism to keep Yap1 in the nucleus, assuring an increased and/or stress-specific expression of its targets. Our data further indicates that Yap1 is not the sole regulator of many of those genes, as basal expression was observed in the *yap1* mutant under normal and stress conditions (Figure 2.7 A–D). Indeed, Skn7 is a transcription factor which cooperates with Yap1 in the activation of H_2O_2-inducible target genes such as *TRR1* (thioredoxin reductase), *TRX2* (thioredoxin 2) and *SOD1* (cooper/zinc superoxide dismutase), among others [39, 40]. Together with Yap1, Met4, another transcription factor, was shown to regulate *GSH1*, the gene coding for the enzyme that catalyses the limiting step of glutathione biosynthesis, under glutathione depletion and cadmium injury [41, 42]. As such, it is possible that Yap1 functions in cooperation with these transcription factors in the activation of antioxidant genes under normal and cobalt stress conditions.

Yap1 only drives the expression of a very small subset of its known targets, under cobalt stress, (Figure 2.4). Remarkably only 13 of these genes were

detected in the wild-type (WT) response to cobalt (Table 2.4 and Figure 2.6) and just two of them (*INO1* and *SRX1*) are not dependent on Yap1 under normal growth conditions (Table 2.4). The exiguity of the data suggests that cobalt tolerance mediated by this transcription factor is cobalt-specific, but rather should relies on the role of Yap1 as a surveyor of cell injury under normal growth conditions. Alternatively, the relative low growth inhibition exerted by the used cobalt concentration (Figure 2.1) may as well explain the low number of genes dependent on Yap1 under such conditions.

Notwithstanding with the fact that the *yap1* mutant exhibits lower levels of intracellular cobalt (Figure 2.8 B), we noticed that disruption of *YAP1* increases protein carbonyl content of cells challenged with cobalt (Figure 2.7 F), highlighting its important role in the protection against cobalt mediated oxidative stress.

Our data point out a significant redox imbalance of cells treated with cobalt that was more pronounced in the absence of Yap1 (Table 2.5). Notably, the decrease of GSH levels observed after cobalt treatment is not reflected on GSSG levels increase. This finding suggests that when challenged with cobalt, GSH recycling might be impaired which may be itself a mechanism of cobalt toxicity.

Mitochondrial Fe–S proteins were identified as being major targets of cobalt ions within cells [43, 44]. Because Aft1 activation responds to the production of mitochondrial Fe-S clusters [45, 46], cobalt attack of the mitochondrial Fe–S enzymes has been proposed to be the mechanism underlying cobalt-induced Aft1 activation [44]. On the other hand, yeast cells depleted of GSH activate Aft1

and exhibit an intense iron starvation response [25, 47]. It is therefore possible that cobalt depleted GSH levels may as well contribute to Aft1 activation.

Accumulated evidences suggest that the role of Yap1 as a mediator of stress response is not confined to the activation of the cellular antioxidant defences. Indeed, Yap1 also controls the expression of *YCF1* which encodes a vacuolar pump important for arsenic and cadmium sequester into the vacuole [12, 48]. We here show that Yap1 also regulates several genes involved in iron homeostasis (Figure 2.4). Five of these genes (*FIT2*, *FIT3*, *MRS4*, *ISU2*) are targets of Aft2 [49, 50], a transcription factor that together with Aft1 coordinates the cellular response to iron deprivation in yeast [51]. Aft2 has been shown to be dependent on Yap1 [51], which may explain the down-regulation of the aforementioned genes observed in the *yap1* mutant.

Noteworthy, we showed that Yap1 is a negative regulator of the low affinity iron transporter gene, *FET4* (Figure 2.8). Although in the particular case of cobalt, this negative regulation had no effect on the intracellular cobalt content (Figure 2.8 B), we cannot rule out the possibility that Yap1-mediated negative regulation of *FET4* may be the first line of protection conferred by this regulator regarding other metals.

One of the most intriguing findings of this work was the identification of Yap1 as a regulator of the expression of *PHO84*, the gene that encodes the high affinity phosphate transporter (Figure 2.9). We have shown that Yap1-dependent regulation of *PHO84* may be an additional mean used by yeast cells to overcome oxidative stress induced by superoxide (Figure 2.10). This regulation is observed

under normal growth conditions possibly as a defence mechanism against superoxide generated during the aerobic metabolism (Figure 2.9 A). As such, the paradoxical increase of cobalt uptake mediated by Yap1 (Figure 2.8 B) may be a side effect of Yap1-dependent *PHO84* regulation under non-stressed conditions.

In conclusion these studies reiterate the relevant role played by Yap1 in providing protection against metal-induced oxidative stress and bring to light new possible Yap1-mediated mechanisms of stress tolerance.

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Chapter III

REPRESSION OF THE LOW AFFINITY IRON TRANSPORTER GENE *FET4*, A NOVEL MECHANISM AGAINST CADMIUM TOXICITY ORCHESTRATED BY YAP1 *VIA ROX1*

The author of this dissertation had a major contribution in this work, namely in the planning of the experimental work, in the execution and analysis of the experiments.

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In order to uniform the nomenclature in the present thesis, the original manuscript of this article was altered.

Abstract

Cadmium is a well-known mutagenic metal, which can enter cells *via* non-specific metal transporters, causing several cellular damages and eventually leading to death. In the yeast *Saccharomyces cerevisiae*, the transcription factor Yap1 plays a key role in the regulation of several genes involved in metal stress response. We have previously shown that Yap1 represses the expression of *FET4*, a gene encoding a low affinity iron transporter able to transport metals other than iron. Here, we have studied the relevance of this repression in cell tolerance to cadmium. Our results indicate that genomic deletion of Yap1 increases *FET4* transcript and protein levels. In addition, cadmium toxicity exhibited by this strain is completely reversed by co-deletion of *FET4* gene. These data correlate well with the increased intracellular levels of cadmium observed in the mutant *yap1*. Rox1, a well-known aerobic repressor of hypoxic genes, conveys the Yap1-mediated repression of *FET4*. We further show that, in a scenario where the activity of Yap1 or Rox1 is compromised, cells activate posttranscriptional mechanisms, involving the exoribonuclease Xrn1, to compensate the derepression of *FET4*. Our data thus reveals a novel protection mechanism against cadmium toxicity mediated by Yap1, which relies on the aerobic repression of *FET4* and results in the impairment of cadmium uptake.

1. Introduction

Cadmium contamination poses a threat to the environment and human health, because cadmium is extremely toxic and carcinogenic even at low concentrations [1]. Environmental contamination with this metal arises from both natural and anthropogenic sources. Human exposure to cadmium mainly occurs through contaminated dietary sources or by inhalation of tobacco smoke or polluted air, leading to its accumulation in the liver, kidney, and lungs [2].

The precise molecular mechanism of cadmium toxicity is not fully understood, but it is thought to cause injury primarily *via* oxidative-induced cellular damages. Cadmium is, however, unable to directly generate free radicals, but it is assumed to induce the formation of reactive oxygen and nitrogen species by two distinct mechanisms. One of those mechanisms involves the displacement of metals from proteins that, once unbound, may generate oxidative stress *via* Fenton-type reactions; the other relies on cadmium ability to inhibit the activity of antioxidant enzymes, such as superoxide dismutase and catalase [3, 4]. Other forms of cadmium cellular toxicity include the inhibition of the DNA mismatch repair system [5], the induction of iron deficiency [6-8], and the disturbance of the homeostasis of other metals [9-11].

Cadmium enters cells through transporters evolved for the uptake of essential metals, such as iron, zinc, manganese, and calcium [7, 12-16]. The budding yeast *Saccharomyces cerevisiae* has long been used as model organism to study the molecular mechanisms of cadmium toxicity and tolerance. The most relevant mechanism of cadmium detoxification in yeast relies on its accumulation in the

vacuole, which is strongly dependent on Ycf1, a vacuolar membrane transporter of the ABC family. Ycf1 transports cadmium conjugated with glutathione into the vacuole [17]. Two Ycf1 paralogues, Bpt1 and Vmr1, and the zinc transporter Zrc1 also play a minor role in vacuolar cadmium sequestration [18, 19]. Another cadmium detoxification system in yeast relies on Pca1, a plasma membrane P-type ATPase involved in cadmium efflux [20]. In addition, two other plasma membrane transporters, Alr1 and Yor1, have been implicated in cadmium detoxification; however, little is known regarding their mode of action [21, 22]. Also the yeast internal Ca^{2+} transporters, Pmr1 and Pmc1 can act as ancillary pathways to cope with cadmium toxicity, particularly when Ycf1 activity is compromised [23, 24].

In yeast, the transcriptional regulator Yap1 plays a central role in cadmium stress response, by activating antioxidant genes and by inducing the expression of *YCF1* [25]. Yap1 senses cadmium by means of the direct interaction of its C-terminal cysteine rich domain with the metal [26]. As a consequence, Yap1 nuclear export signal becomes masked, and the factor accumulates in the nucleus, activating its target genes [27].

In the previous Chapter we have shown that under normal growth conditions Yap1 is a negative regulator of *FET4.* Fet4 is a cell surface low affinity iron transporter able to transport other divalent metals, including toxic cadmium ions [13]. In this context, we put forward the hypothesis that Yap1-mediated negative regulation of *FET4* may be a novel line of protection conferred by this regulator against cadmium insult. We show herewith that Yap1 represses *FET4* gene

expression *via* Rox1, which results in a reduction of cadmium uptake. We also show that, when challenged with cadmium, *yap1* and *rox1* mutant strains trigger post-transcriptional mechanisms, dependent on the 5' – 3' exoribonuclease Xrn1 that compensates the derepression of *FET4* gene expression.

2. Material and Methods

2.1 Strains, plasmids and growth conditions

The yeast strains used in this study are listed in Table 3.1. All mutants constructed in this work were generated using the microhomology PCR method [28].

To construct the p*ROX1-lacZ* plasmid, a fragment comprising a 1-kb sequence upstream of *ROX1* ATG codon was amplified by PCR using the primers listed in Table 3.2. PCR product was first digested with BamHI, treated with Klenow, and next digested with SphI. The resulting fragment was cloned into the YEp356R vector previously digested with EcoRI, treated with Klenow, and cut with SphI. To generate the C-terminal HA-tagged version of *FET4* (*FET4-HA*), one fragment comprising 1 kb upstream from the ATG plus the *FET4* coding region and another including 0.5 kb downstream from the stop codon were amplified by PCR using the primers listed in Table 3.2. The *HA* sequence was inserted in frame with *FET4* coding region just before the TAG stop codon. Both fragments were inserted into the pRS416 vector, previously linearized with SmaI, by homologous recombination using the In-Fusion Advantage PCR cloning kit (Clontech). To construct the *p-ROX1* plasmid, *ROX1* gene was amplified by PCR with specific primers (Table 3. 2). The resulting fragment was inserted into the SmaI site of pRS416. The *MUTp-ROX1* plasmid was generated using as template *p-ROX1*, and the primers depicted in Table 3.2 were used in a PCR-directed mutagenesis reaction to mutate the YRE site located at -414 bp, as detailed in Ref. [29]. A similar and sequential strategy was used to construct *MUTp-FET4-HA*

using the primers listed in Table 3.2.

Yeast strains were grown in synthetic medium (SC) or medium lacking specific requirements (SD), as previously described [29]. Phenotypic growth assays were carried out by spotting 5 µl of cultures in early exponential phase ($OD_{600}\approx$ 0.4 – 0.5) sequentially diluted (5×10^3 to 50 cells) in medium containing the indicated concentrations of $CdCl_2$ and supplemented or not with $FeSO_4$. These assays were repeated at least twice. Cultures were grown for 2 days at 30 °C. The bacteria *Escherichia coli* strain XL1-Blue (Stratagene) was used as a host for routine cloning purposes. Standard methods were used for genetic analysis, cloning, and transformation.

Table 3. 1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
BY4742	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0*	Euroscarf
BY4742 *yap1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 yap1Δ::kanMX4*	Euroscarf
BY4742 *rox1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 rox1Δ:: kanMX4*	Euroscarf
BY4742 *fet4*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 fet4Δ:: kanMX4*	Euroscarf
BY4742 *xrn1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 xrn1Δ:: kanMX4*	This study
BY4742 *rnt1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 xrn1Δ:: kanMX4*	This study
BY4742 *yap1fet4*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 fet4Δ:: kanMX4 yap1Δ:: his3Δ1*	Chapter II
BY4742 *yap1xrn1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 xrn1Δ:: kanMX4 yap1Δ:: his3Δ1*	This study
BY4742 *yap1rnt1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 rnt1Δ:: kanMX4 yap1Δ:: his3Δ1*	This study
BY4742 *yap1rox1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 yap1Δ:: kanMX4 rox1Δ:: his3Δ1*	This study
BY4742 *yap1xrn1fet4*	MATα his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 yap1Δ:: kanMX4 rox1Δ:: his3Δ1	This study

Table 3. 2 Oligonucleotides used in this study.

Primer	Sequence	Use
ACT1	5' ctattggtaacgaaagattcag 3'	qRT-PCR
	5' ccttacggacatcgacatca 3'	
CTH2	5'aggtatgctgctggagctgt3'	qRT-PCR
	5'gagggccatgaaggtatcaa3'	
FET4	5'ggagaactgcctgtggaaaa3'	qRT-PCR
	5'ttctccggtgtaaggtggag3'	
FET3	5'acggtgtgaattacgccttc3'	qRT-PCR
	5'ttggaaagcgtgaccatgta3'	
ROX1	5'agggcttacaaccggaagat3'	qRT-PCR
	5'gctgttgctcgatttccttc3'	
CUP1	5'tgaaggtcatgagtgccaat3'	qRT-PCR
	5'gcatttgtcgtcgctgttac3'	
ARN2	5'aggtatgctgctggagctgt3'	qRT-PCR
	5'gagggccatgaaggtatcaa3'	
ACT1-ChIP	5'gatcctttccttcccaatctctcttg3'	ChIP
	5'gctcatgtagtagaagatcctatt3'	
ARN2	5'ggtatgctgctggagctgt3'	ChIP
	5'agggccatgaaggtatcaa3'	
ROX1-414bp	5'gcaaaacaattggaaatctgg3'	ChIP
	5'gaacaacaaaagaggcagca3'	
ROX1-897bp	5'tctacataatgcacgaaacttgg3'	ChIP
	5'cgcagtgtgtgttcctgtct3'	
ROX1p'1000	5'ctagcatgcagttgacctacattcaac3'	*pROX1*
A4-ROX1	5'ggatttcgcatcctagacca3'	
*ROX1*m Fw	5'tggcgattgaagacaaagaagaaa3'	*MUT-pROX1*
*ROX1*m Rv	5'tttcttctttgtcttcaatcgcca3'	
ROX1-ATG-codon	5'ctagcatgcagttgacctacattcaac3'	*pROX1-lacZ*
	5'cttggatccggattcattgttgattgtc3'	
FET4-ORF	5'gaattcctgcagcccctgtgcttgctgttc3'	*pFET4-HA*
	5'atgtacccatacgatgttccagattacgcttagcttcattgaaca3'	
0.5Kb-FET4-terminator	5'agcgtaatctggaacatcgtatgggtacatttttttccaacatcat3'	*pFET4-HA*
	5'actagtggatcccccgacatataagcggag3'	
*FET4*m1 Fw	5'gccttcttaattgagtttagcatc3'	*MUT-pFET4-HA*
*FET4*m1 Rv	5'gatgctaaactcaattaagaaggc3'	(Aft1 site1 deletion)
*FET4*m2 Fw	5'gttccgaaaacccactttttgttc3	*MUT-pFET4-HA*
*FET4*m2 Rv	5'gaacaaaaagtgggttttcggaac3'	(Aft1 site2 deletion)

2.2 Measurements of β-Galactosidase Activity

The BY4742 (wild-type) and the *yap1* mutant strains were transformed with the p*ROX1-lacZ* plasmid. Cells were grown in liquid SD medium until the early exponential phase in the presence or absence of 25 μM $CdCl_2$ and harvested after 15 min. Relative β-galactosidase activity was monitored as described in Ref. [29]. Enzymatic activity was assayed by following the degradation of the colorimetric substrate *O*-nitrophenyl-β-D-galactopyranoside at A_{420} and normalizing against OD_{600}. The results are the average of at least six biological replicates.

2.3 Immunoblot Assays

Wild-type, *yap1* and *rox1* mutant strains containing the *FET4-HA* or *MUTp-FET4-HA* plasmids were grown until the early exponential phase ($OD_{600}\approx$ 0.4–0.5) and exposed to 25 μM $CdCl_2$. Cells were harvested at different time points after treatment with $CdCl_2$. Total proteins were extracted from cell cultures as described in Ref. [29]. Proteins were resolved in a 10% SDS-PAGE and immunoblotted with horseradish peroxidase-bound anti-HA IgG (Roche). In what concerns PMSF treatment, cells were grown until the early exponential phase, exposed to 1.2 mM of PMSF for 90 min, and supplemented (or not) with $CdCl_2$ for 1 h. Pgk1 was used as loading control. Immunoblots were repeated at least twice with different protein extracts.

2.4 Quantitative Real Time RT-PCR Analyses

Cells were grown until the early exponential phase, cultures were left

untreated or treated with 25 μM $CdCl_2$. Cells were harvested at the indicated time points, and RNA was isolated. RNA samples were next treated with DNase (TURBO™ DNase-free; Ambion) according to the manufacturer's instructions and purified. Total RNA (1 μg) was reverse transcribed with transcriptor reverse transcriptase (Roche Diagnostics). Quantitative PCRs were performed in the Light Cycler 480 II real time PCR system (Roche), using Light Cycler 480 SYBR Green I Master (Roche). Relative standard curves were constructed for each gene, using triplicate serial dilutions of cDNA. The relative expression of the genes was calculated by the relative quantification method with efficiency correction, using the Light Cycler 480 software 1.5 [30]. Actin gene was used as a reference gene. All assays were made using biological and technical triplicates. Primers used in this assay are listed in Table 3.2.

2.5 ChIP Analysis

ChIP assays were carried out as previously described [29]. Cells transformed with a c-Myc-tagged version of Yap1 [27] and treated with cadmium were harvested at OD_{600}=0.6 and fixed with 1% formaldehyde. The cross-linking was stopped by addition of glycine, and cells were disrupted with a FastPrep®-24 instrument (MP Biomedical). Cell extracts were sonicated to yield DNA fragments with an average of 500 bp. c-Myc-tagged Yap1 was immunoprecipitated by incubating the cross-linked chromatin with a c-Myc antibody prebound to Dynabeads Pan mouse IgG (Invitrogen) for 16 h at 4°C. Immunoprecipitated proteins were eluted from the beads by heating the samples

in appropriate buffer, and fixation was reversed. Aliquots of total chromatin input (IN) and immunoprecipitated chromatin were simultaneously processed for subsequent normalization. After sample treatment with proteinase K and RNase A, DNA was purified. Quantification of specific DNA targets in the input and immunoprecipitated samples was per- formed by quantitative PCR. A standard curve, generated with a dilution series of the immunoprecipitated sample, was used to assess the PCR efficiency. The relative enrichment of a specific pRox-YRE in the immunoprecipitate was determined using the $\Delta\Delta C_T$ method through the calculation of $\log_2$ (immunoprecipitated/input). The primer sequences used are listed in Table 3.2. The primers *ROX1*-414 bp and *ROX1*-897 bp were used to amplify the regions of the *ROX1* promoter flanking both YRE sites located at -414 and -897 bp from the ATG, respectively. *ARN2* was used as a negative control.

2.6 Measurement of Cadmium and Iron

Strains were grown to early exponential phase ($OD_{600}\approx$ 0.4-0.5) in SC medium and left untreated or treated with 25 μM $CdCl_2$. Cells were harvested after 6 h of stress induction, collected by centrifugation, and washed with 10 mM EDTA and metal-free water. This time point was chosen because, in all strains, the maximum of growth inhibition was observed after 6 h of treatment with cadmium (data not shown). The total cadmium and iron content was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The data were normalized against OD_{600}. All assays were made using biological

triplicates.

3. Results

3.1 Yap1 regulation of *FET4* contributes to *S. cerevisiae* cadmium tolerance

Our previous work indicated that Yap1 is a negative regulator of *FET4* (see Chapter II), a gene encoding a low affinity iron transporter also involved in the transport of zinc, cobalt, manganese, and cadmium [13, 31]. To evaluate whether the uptake of cadmium *via FET4* could contribute to the well-known sensitivity exhibited by the *yap1* mutant to this metal, we first tested *FET4* dependence on Yap1 by qRT-PCR (Figure 3.1 A). As illustrated in Figure 3.1 A, *FET4* expression is induced in *yap1* unstressed cells. A sharp decrease of *FET4* levels was, however, observed after cadmium challenge. Indeed, after 20 min of cadmium exposure, the levels of *FET4* transcripts in the *yap1* mutant and WT strains were comparable. We have also assessed the protein expression of a HA-tagged version of Fet4 driven by its native promoter. In agreement with the gene expression data, we observed that protein levels were consistently higher in the *yap1* mutant compared with the WT strain, being rapidly reduced after cadmium exposure (Figure 3.1 B and C).

We next examined the growth phenotype of the double mutant *yap1fet4* in the presence of cadmium (Figure 3.1 D and E). If repression of *FET4* by Yap1 is required to prevent cadmium toxicity, one would expect the double mutant to be more resistant than the single *yap1* mutant to this metal. As anticipated, the

double mutant *yap1fet4* grew better than the *yap1* strain in the presence of cadmium (Figure 3.1 D and E). In addition, reintroduction of *FET4* into the *yap1fet4* mutant restores cadmium sensitivity (Figure 3.1 F). Accordingly, intracellular cadmium levels are increased 30% in the *yap1* mutant when compared with the WT, whereas in the *fet4* and *yap1fet4* mutants, these values are 50 – 60% lower than in the *yap1* mutant (Figure 3.1 G). Moreover, supplementation of the growth medium with iron attenuates the *yap1* mutant sensitivity, suggesting the involvement of an iron transporter in this process (Figure 3.1 E). To test this, we also measured cadmium contents when cells were simultaneously treated with iron (Figure 3.1 G). Contrary to our expectations, we noticed that in the presence of iron, cadmium uptake increases. In an attempt to understand this apparently contradictory finding, we revisited our data on the genome-wide transcriptional analysis of *S. cerevisiae* exposed to high iron conditions [29] and searched for genes whose expression was induced by high iron and that may play a role in cadmium detoxification. We found that metallothionein genes *CUP1-1* and *CUP1-2* were up-regulated in response to high iron (Table S1 in Ref. [29]). Cup1-1 overexpression confers resistance to cadmium ions [32], and by qRT-PCR we found that *CUP1* genes are highly expressed when cells are treated simultaneously with cadmium and iron (Figure 3.1H). This finding strongly suggests that iron attenuates cadmium toxicity by inducing *CUP1* expression, which possibly binds and sequesters cadmium. Altogether, these data shed light on a novel Yap1-mediated mechanism of cadmium stress tolerance involving the Fet4 low affinity iron transporter.

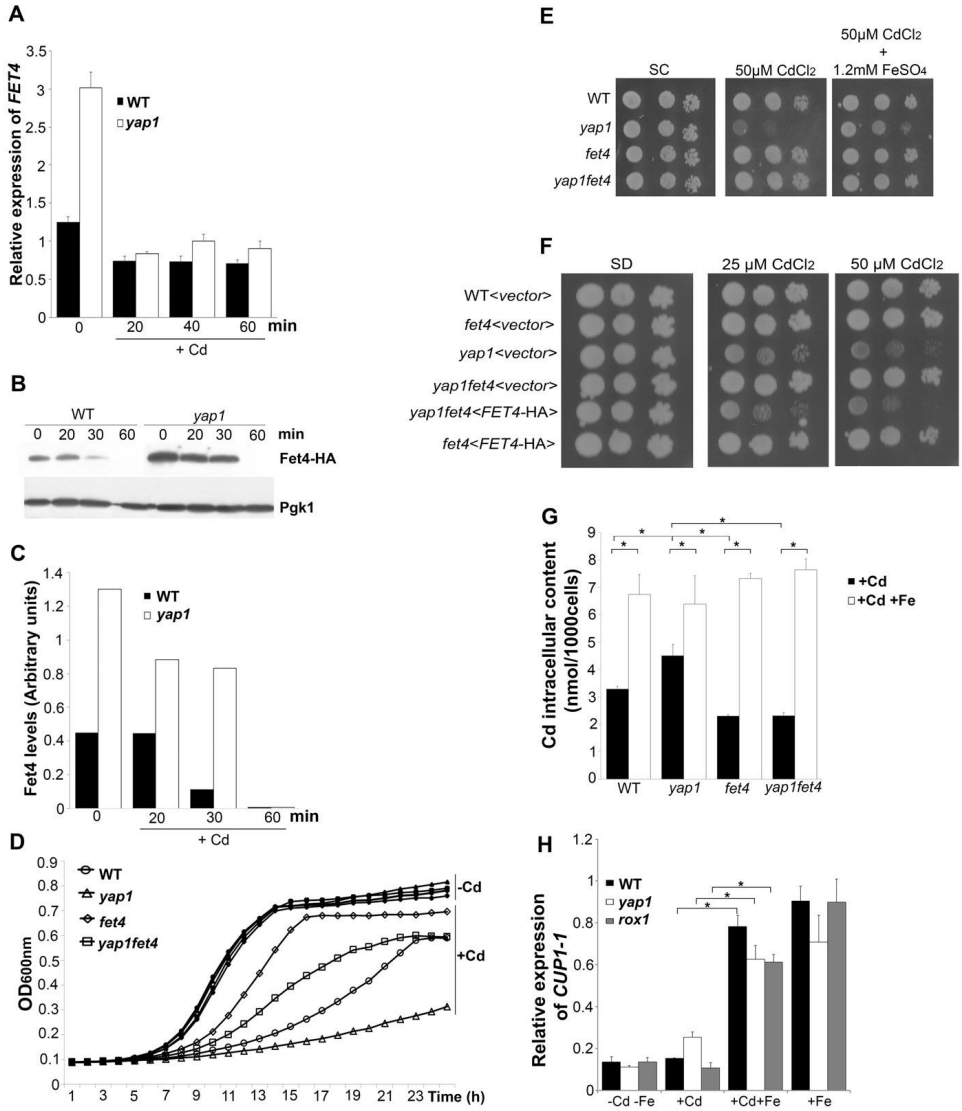

Figure 3. 1 Yap1 mediates *FET4* repression. (A) WT and *yap1* strains were challenged with 25 μM $CdCl_2$ and harvested at the indicated time points. The expression of *FET4* was monitored by qRT-PCR. **(B)** the WT strain and the *yap1* mutant were transformed with a plasmid containing *FET4*-HA, cultures were treated with 25 μM $CdCl_2$, and Fet4 expression was analysed by Western blot with an anti-HA antibody. **(C)** Fet4 protein levels were normalized to Pgk1, from (B) **(D)** Growth exhibited by the WT, *yap1*, *fet4*, and *yap1fet4* strains in SC medium was recorded over a period of 24 h. The open and closed symbols represent strains grown in media containing or not

25 μM $CdCl_2$, respectively. **(E)** Growth sensitivity exhibited by the WT, *yap1*, *fet4*, and *yap1fet4* strains in SC plates containing 50 μM $CdCl_2$ and supplemented with 1.2 mM FeSO*4*. **(F)** exponentially growing WT, *yap1*, *fet4*, and *yap1fet4* cells were transformed with the *FET4*-HA plasmid (<*FET4*-HA>) or with the empty vector (<vector>), harvested, serially diluted, and spotted onto SD plates or SD plates supplemented with 25 or 50 μM $CdCl_2$. **(G)** cadmium content of WT, *yap1*, *fet4*, and *yap1fet4* strains were determined by ICP-AES, after treatment with 25 μM $CdCl_2$ (+Cd) or after treatment with 25 μM $CdCl_2$ and 1.2 mM of $FeSO_4$ (+Cd+Fe) for 6 h. **(H)** WT, *yap1* and *rox1* strains were grown in medium left untreated (-Cd-Fe) or treated with 25 μM $CdCl_2$ (+Cd); with 25 μM $CdCl_2$ and 1.2 mM of $FeSO_4$ (+Cd +Fe) or 1.2 mM of $FeSO_4$ (+Fe). *CUP1* expression levels were assessed by qRT-PCR after 15 min of treatment. In this figure, all values are the means of at least biological triplicates (n=3) ± s.d.. Significance of differences was calculated with the t test. *$p<0.05$.

3.2 Yap1 is a direct regulator of the repressor *ROX1*

The promoter region of *FET4* does not contain any canonical YREs [33], a fact which strongly suggests that the regulation of *FET4* by Yap1 involves another regulator. Strikingly, our previous microarray data indicate Yap1 as a positive regulator of *ROX1* gene (see Chapter II). Rox1 is a repressor of anaerobic genes and also represses *FET4* expression under oxygenated conditions [34, 35]. Remarkably, microarray data equally revealed that all the genes up-regulated in the *yap1* mutant are also known Rox1 targets (see Chapter II and Table 3.3). Furthermore, using YEASTRACT [36], we verified that the promoter region of *ROX1* possesses two YRE sites, located at -414 and -897 bp upstream the ATG codon (Figure 3.2 A).

In line with these data, we bring forward the hypothesis that Yap1 might repress *FET4 via ROX1* induction. As a first approach to test this, a plasmid including the promoter region of *ROX1* fused to the *lacZ* reporter gene (p*ROX1-lacZ*) was generated and used to transform the WT and *yap1* mutant strains. We

observed that β-galactosidase activity was higher in the WT compared with the mutant strain, even in the absence of cadmium (Figure 3.2 B). A slight but significant increase of the β-galactosidase activity was noticed in both strains upon cadmium stress. These results were further confirmed by the analysis of the levels of *ROX1* transcripts in both strains, in either the absence or the presence of cadmium, by qRT-PCR (Figure 3.3 B).

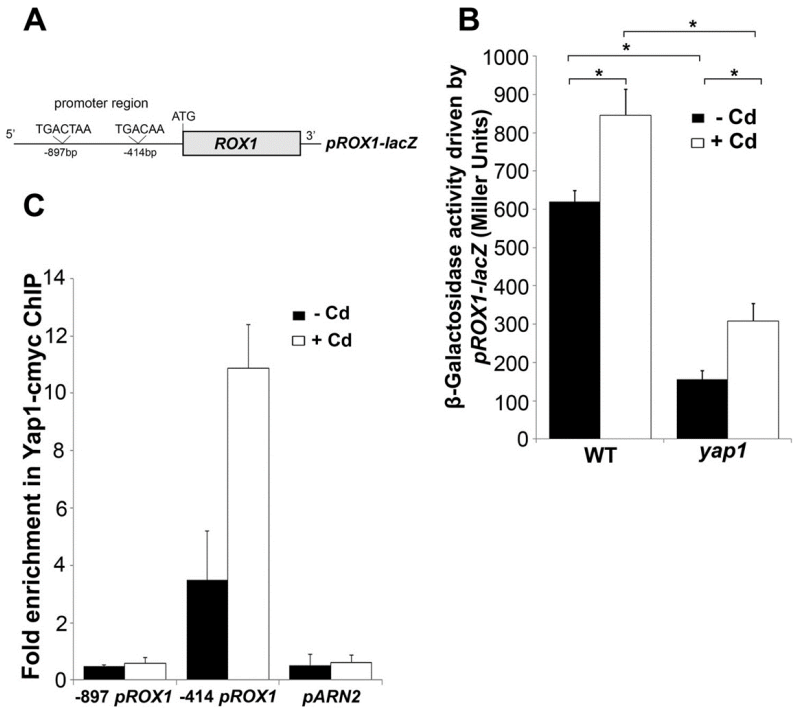

Figure 3. 2 Yap1 is a direct regulator of *ROX1*. **(A)** Schematic representation of the *ROX1* promoter region containing the two YREs sites located at -897 and -414 bp upstream the ATG. **(B)** WT and *yap1* mutant strains were transformed with the pROX1-lacZ plasmid. Cells were grown exponentially in SD medium and challenged with 25 μM $CdCl_2$ for 15 min. β-Galactosidase activity was assayed as detailed under "Experimental Procedures." The values are the means of biological decaplicates ± s.d.. Significance of differences was calculated with the t test. *, p>0.05. **(C)** *yap1* mutant cells transformed with a plasmid containing a c-Myc-tagged version of Yap1 were grown exponentially in SD medium and treated with 25 μM $CdCl_2$ for 10 min. ChIP analyses combined with quantitative PCR were used to determine the fold enrichment

of each YRE. The promoter region of *ARN2* was used as a negative control, because it does not possess YREs. The values are the means of at least biological triplicates ± s.d..

We next examined whether Yap1 is a direct regulator of *ROX1* by carrying out ChIP analyses. A Yap1 c-Myc-tagged version was used to test whether each of the YRE sites found in the *ROX1* promoter region (Figure 3.2 A) was recognized by this factor. As depicted in Figure 3.2 C, after immunoprecipitation of the chromatin bound to Yap1 c-Myc, an enrichment of *ROX1* promoter harboring the YRE located at -414 bp was observed. No enrichment was noticed in the *ROX1* region comprising the YRE located at -897 bp or in the promoter region of the *ARN2* gene (used as a negative control). The increased enrichment in the 414-bp YRE-containing sequence after cadmium treatment correlates well with Yap1 nuclear accumulation kinetics [26]. Although the above data clearly indicate Yap1 as a direct regulator of *ROX1*, we observed that *rox1* strain is more sensitive to cadmium than *yap1* (Figure 3.3 A). This finding suggests that Yap1 cannot be the only regulator of *ROX1*. In agreement, *ROX1* transcripts are not fully dependent on Yap1 (Figure 3.3 B), and *FET4* mRNA (Figure 3.3 C), Fet4 protein levels (Figure 3.3 D and E), and cadmium intracellular contents (Figure 3.3 F) are higher in the *rox1* mutant than in the *yap1* strain. The results here described clearly show that, although partially, Yap1 directly regulates *ROX1* expression.

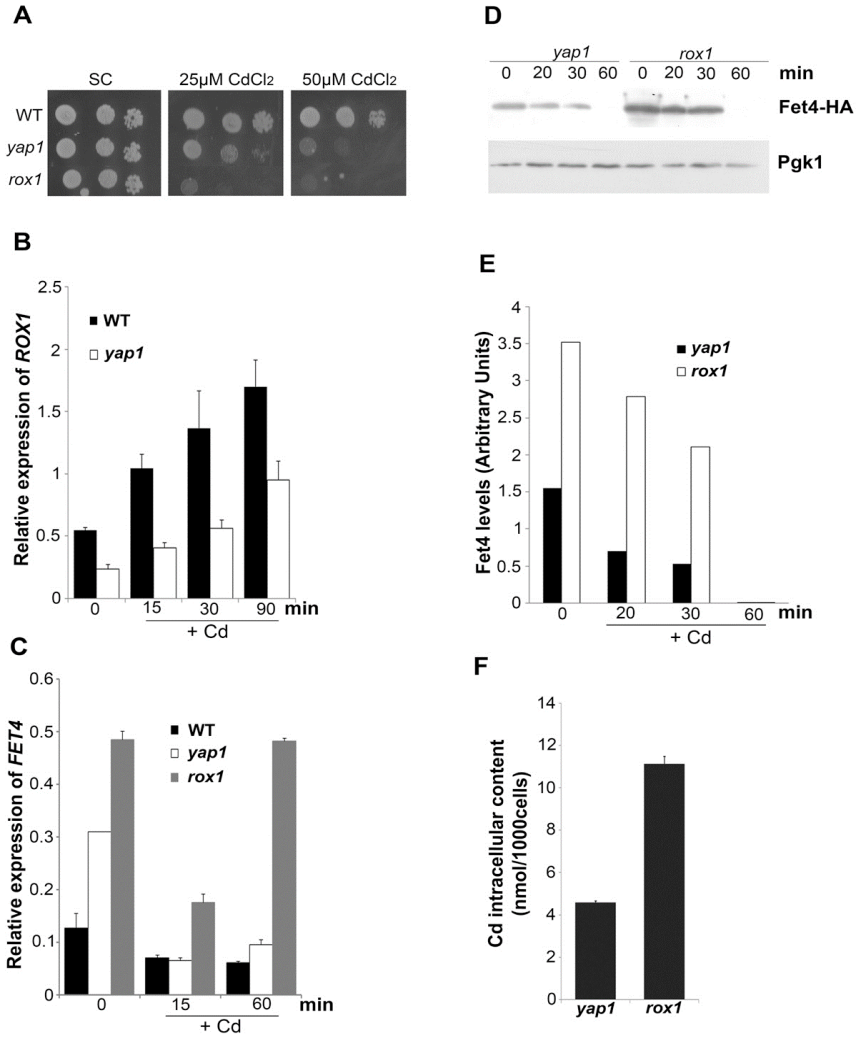

Figure 3. 3 Yap1 partially regulates *ROX1*. **(A)** Growth sensitivity exhibited by WT, *yap1*, and *rox1* strains in SC plates containing 25 or 50 μM $CdCl_2$. **(B)** *ROX1* expression in the WT and *yap1* mutant was analysed by qRT-PCR at the indicated time points, after treatment with 25 μM $CdCl_2$. **(C)** *FET4* expression in the WT and *yap1* and *rox1* mutants was analysed by qRT-PCR, at the indicated time points after treatment with 25 μM $CdCl_2$. **(D)** *yap1* and *rox1* mutants were transformed with *FET4*-HA plasmid and treated with 25 μM $CdCl_2$. Fet4 protein levels were analysed by Western blot, at the indicated time points. **(E)** Fet4 protein levels were normalized to Pgk1, from D. **(F)** cadmium intracellular content in yap1 and rox1 mutant strains was determined by ICP-AES, after treatment with 25 μM $CdCl_2$ for 6 h. The values are the means of at least biological triplicates ± s.d..

Table 3. 3 Rox1 target genes that appear up-regulated in *yap1* mutant cells.

Systematic name	Gene name	Fold Change	Description
YLR034C	*SMF3*	1.3	Member of the Nramp family of the metal transport proteins
YMR319c	*FET4*	4.5	Low affinity Fe(II) transporter of the plasma membrane
YER014w	*HEM14*	1.5	Protoporphyrinogen oxidase
YKL008c	*LAC1*	1.6	Ceramide synthase component
YHR007c	*ERG11*	1.7	Lanosterol 14-α-demethylase
YDR297w	*SUR2*	1.7	Sphinganine C4-hydroxylase
YDR004w	*HEM13*	2.2	Coproporphyrinogen III oxidase
YIL11w	*COX5B*	1.7	Subunit Vb of cytochrome c oxidase
YBR085w	*AAC3*	2.2	Mitochondrial inner membrane ADP/ATP translocator
YEL047c	NA	1.6	Soluble fumarate reductase
YDR518w	*EUG1*	1.4	Disulfide isomerase of the endoplasmatic reticulum lumen
YHR179w	*OYE2*	1.4	Conserved NADPH oxidoredutase
YAL028w	*FRT2*	1.5	Tail-anchored endoplasmic reticulum membrane protein
YNR014w	NA	1.4	Putative protein of unknown function

3. 3 Rox1 mediates Yap1 repression of *FET4*

To confirm whether Yap1 regulates *FET4* expression *via* Rox1, we cloned *ROX1* gene and mutated the functional YRE located at -414 bp (Figures 3.2 C and 3.4 A). The resulting constructs (*p-ROX1* and *MUTp-ROX1*) were next used to transform the double mutant strain *yap1rox1* and the single mutant strain *rox1*. *FET4* expression was thereafter assayed by qRT-PCR (Figure 3.4 B).

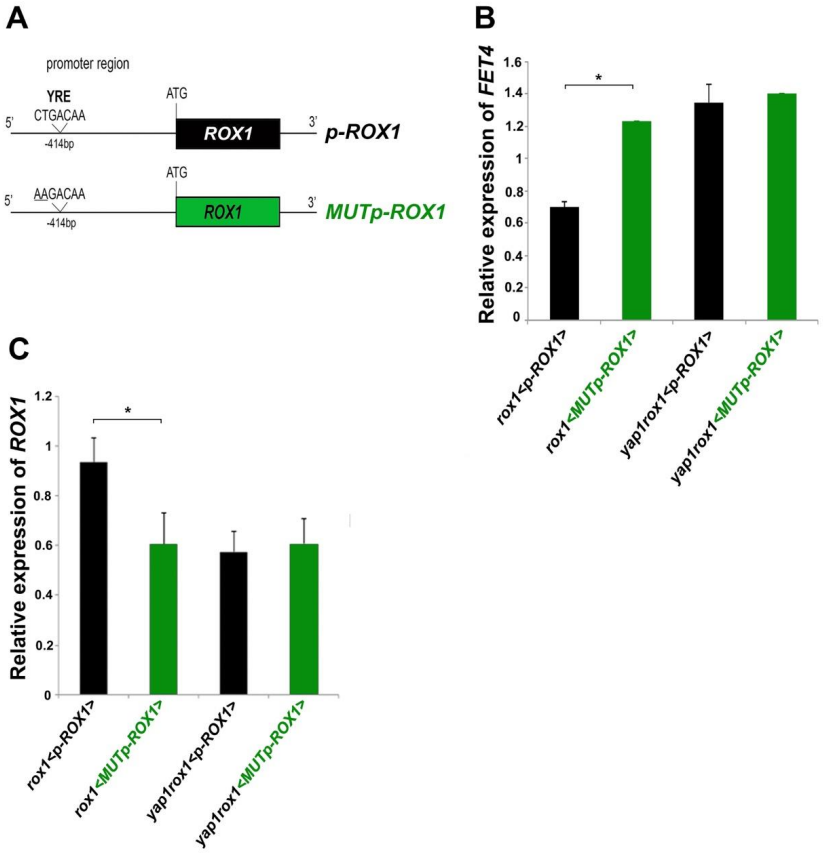

Figure 3. 4 Rox1 mediates Yap1 repression of *FET4*. (A) Schematic representation of the constructs p-*ROX1* and *MUTp-ROX1*. *MUTp-ROX1* possesses two mutations in the functional YRE (CTGACAA to AAGACAA). **(B)** and **(C)** *FET4* (B) and *ROX1* (C) expression in *rox1* and *yap1rox1* mutant strains transformed with *p-ROX1* (black; <*p-ROX1*>) or *MUTp-ROX1* (green; <*MUTp-ROX1*>) was analyzed by qRT-PCR. The values are the means of at least biological triplicates ± s.d.. Significance of differences was calculated with the t test. *, $p<0.05$.

Supporting our hypothesis, we noticed that the expression of *FET4* was higher in *rox1* cells transformed with *MUTp-ROX1* than with *p-ROX1* (Figure 3.4B). The levels of *FET4* transcripts in the former were close to those exhibited by the double mutant *yap1rox1* transformed with *p-ROX1*. As expected, *ROX1* levels were decreased in *rox1* strain transformed with *MUTp-ROX1* and in the double

mutant transformed with *p-ROX1* (Figure 3.4 C). These data support that Yap1 mediates the repression of *FET4* via Rox1.

3.4 The Exoribonuclease 5'-3' Xrn1 alleviates the *FET4* derepression observed in the *yap1* mutant

Xrn1 is an exoribonuclease responsible for the degradation of mRNAs from the 5' to the 3' end. This protein is conserved in all eukaryotes and is involved in the normal mRNA decay [37]. We have recently shown that arsenate stress triggers Xrn1-mediated degradation of *FET3* transcripts [38].

In an attempt to determine whether Xrn1 is also involved in the decrease of *FET4* mRNAs levels observed in the *yap1* mutant after cadmium treatment (see Figures 3.3 C and 3.1 A), we first constructed the double mutant *yap1xrn1* and assayed its cadmium sensitivity. We observed that the double mutant is more sensitive to cadmium than the *yap1* or *xrn1* single mutants (Figure 3.5 A). We next monitored *FET4* mRNA levels in these strains and it was no longer evident, in both *xrn1* and *yap1xrn1* mutants, the accentuated drop of *FET4* levels after stress exposure (Figure 3.5 B). To assess whether the higher sensitivity of the *yap1xrn1* mutant to cadmium was due to higher levels of *FET4* expression, we constructed the triple mutant *yap1xrn1fet4* and observed that it is more tolerant to cadmium compared with the double mutant (Figure 3.5, C and D). Overall, these data strongly suggest that a post-transcriptional mechanism involving Xrn1 may counteract the derepression of *FET4* observed in the *yap1* mutant after cadmium insult.

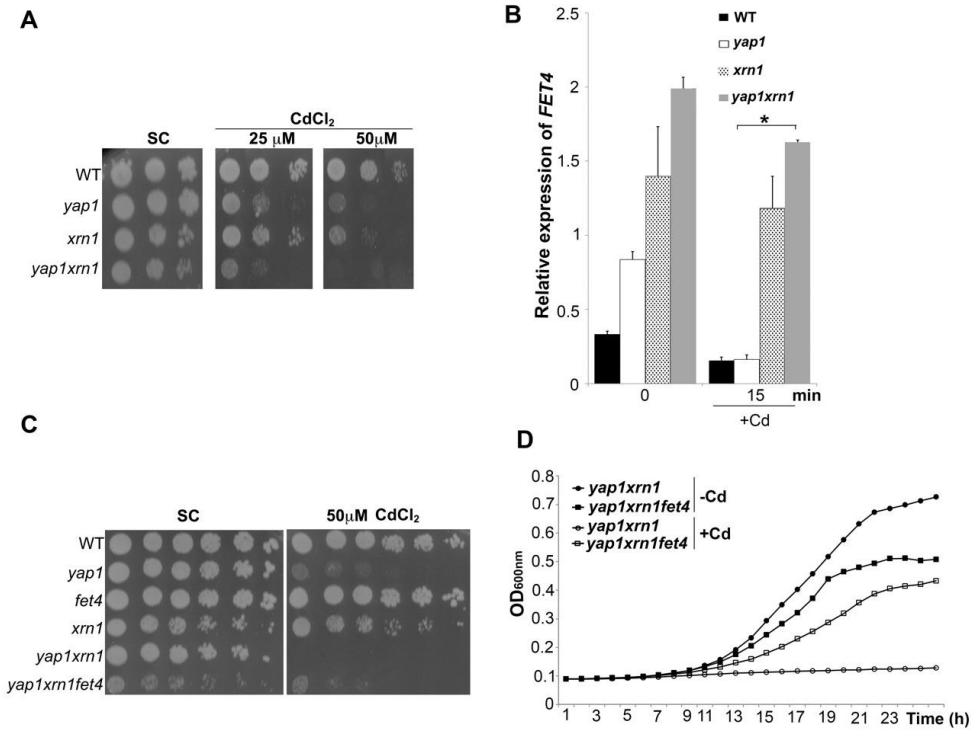

Figure 3. 5 The exoribonuclease Xrn1 controls *FET4* transcript levels and has a role in cadmium tolerance. (A) Growth sensitivity exhibited by WT, *yap1*, *xrn1*, and *yap1xrn1* strains in SC plates containing 25 and 50 μM $CdCl_2$. **(B)** *FET4* expression in WT, *yap1*, *xrn1*, and *yap1xrn1* strains was assessed by qRT-PCR at the indicated time points, after treatment with 25 μM $CdCl_2$. The values are the means of at least biological triplicates ± s.d. *, $p<0.05$. **(C)** deletion of *FET4* from the *yap1xrn1* background partially renders cells more tolerant to cadmium. The indicated strains were spotted onto SC plates supplemented or not with 50 μM $CdCl_2$. **(D)** this effect is also observed when cultures are grown in liquid SC medium with a lower cadmium concentration (25 μM $CdCl_2$).

3.5 Degradation of *FET4* by Xrn1 appears to be stress-specific

To understand whether *FET4* degradation mediated by Xrn1 was stress-specific, we examined and compared the levels of *FET4* and *FET3* in the mutant *xrn1* after treatment with cadmium or arsenate. In the absence of stress, *FET3*

and *FET4* mRNA levels are dependent on Xrn1 (Figure 3.6, A and B, time point 0). As we have previously described [38] and herein confirmed by qRT-PCR, the drop of *FET3* mRNA levels in response to arsenate is dependent on Xrn1 (Figure 3.6 A). Contrary to *FET3*, *FET4* mRNA levels are no longer dependent on Xrn1 after arsenate treatment (Figure 3.6B). In response to cadmium, however, *FET4* mRNA levels continue to be strongly dependent on Xrn1 (Figure 3.6 C), whereas *FET3* transcripts become insensitive to this exoribonuclease (Figure 3.6 D).

Some of the mRNAs degraded by Xrn1 are first cleaved by Rnt1, a double-stranded RNA endonuclease [39]. Rnt1 specifically recognizes particular RNA hairpins and therefore recognizes its targets [39]. Although *FET4* transcripts do not contain such hairpin structures and are not affected by Rnt1 under normal growth conditions [40], we could not certainly rule out the possibility that a different scenario occurs in response to cadmium. Indeed, if Rnt1 specifically recognizes *FET4* transcripts in the presence of cadmium, one would expect *FET4* mRNA levels to be highly dependent on Xrn1. To test this, we constructed the single and the double mutants *rnt1* and *yap1rnt1* and examined their growth phenotype in the presence of cadmium, as well as *FET4* gene expression by qRT-PCR. We observed that cells lacking Rnt1 are not sensitive to cadmium toxicity and deletion of *RNT1* gene from the *yap1* background does not aggravate the sensitive growth phenotype exhibited by the *yap1* mutant to cadmium (Figure 3.6 E). In addition, our results indicate that the absence of Rnt1 does not affect *FET4* mRNA levels either under normal (as previously shown in Ref. [40]) or under cadmium stress conditions (Figure 3.6 F). Overall, our results show that the

mechanism by which Xrn1 controls *FET4* transcripts is stress-dependent and does not rely on the endoribonuclease Rnt1.

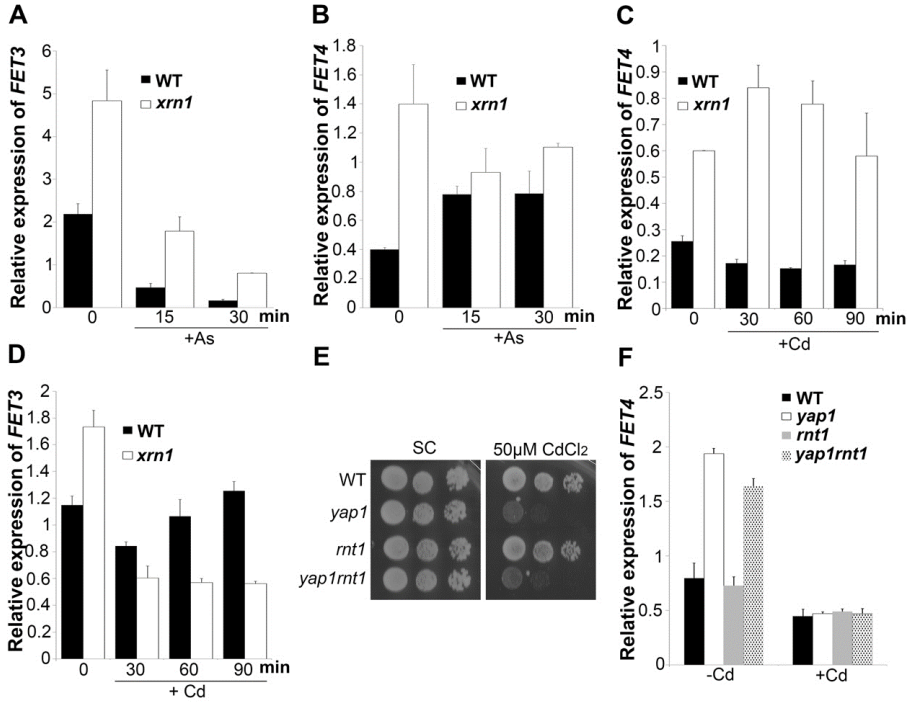

Figure 3. 6 *FET4* transcripts dependence on Xrn1 is stress-specific. (A–D) *FET3* (A) and *FET4* (B) expression in WT and *xrn1* strains were assessed by qRT-PCR at the indicated time points, after treatment with 1 mM AsV (+As) or and after treatment (**C** and **D**) with 25 µM $CdCl_2$ (+Cd). The values are the means of at least biological triplicates ± s.d.. **(E)** growth sensitivity exhibited by WT, *yap1*, *rnt1*, and *yap1rnt1* strains spotted on SC plates supplemented or not with 50 µM $CdCl_2$. **(F)** *FET4* expression levels in WT, *yap1*, *rnt1*, and *yap1rnt1* strains were examined by qRT-PCR after treatment with 25 µM $CdCl_2$ (+Cd) for 15 min. The values are the means of at least biological triplicates ± s.d..

3.6 Iron homeostasis is perturbed by cadmium treatment in the mutant strains *yap1* and *rox1*

Remarkably, we found that *FET4* transcript levels in the *rox1* mutant tend to

increase after prolonged cadmium treatment (Figure 3.3 C). The fact that cadmium induces iron deficiency [34], together with the knowledge that the iron sensing transcription factor, Aft1, controls *FET4* expression in response to depletion of cellular iron levels [41], led us to examine whether iron homeostasis could be over affected in *rox1* strain after extended cadmium treatment. To this end, we have first evaluated iron contents in WT, *yap1*, *rox1*, *fet4*, and *yap1fet4* strains, after and before cadmium stress (Figure 3.7A). In the absence of treatment, iron levels did not differ among the strains. In the presence of cadmium, however, iron levels were significantly increased in the *yap1* and *rox1* mutants, as compared with the WT and control conditions (no cadmium). Among the tested strains, *yap1* was the one exhibiting the highest iron content after cadmium addition (Figure 3.7 A). Because deletion of *FET4* from the *yap1* background restored iron contents to control levels, we conclude that the derepression of *FET4* observed in this strain (Figure 3.1, A–C) was mediating iron increase.

We next monitored Aft1 activity in WT, *yap1* and *rox1* strains by evaluating the expression of *CTH2* and *ARN2*, two target genes of Aft1 [42, 43]. After prolonged cadmium exposure (60 min), the expression of these genes was increased in all the strains, but in a more pronounced way in the *rox1* mutant (Figure 3.7 B and C), suggesting that Aft1 was more active in this strain. If this was the case, then the increase of *FET4* levels observed in *rox1* cells after prolonged cadmium treatment (Figure 3.3 C) could be ascribed to Aft1 activity. To test this hypothesis, we have deleted the two Aft1 *consensus* sites [34] from the

promoter region of the construct *pFET4-HA*. The resulting plasmid (*MUTp-FET4-HA*; Figure 3.7 D) was used to transform WT and *rox1* cells, and Fet4 protein levels were assessed after extended cadmium treatment. Because Fet4-HA protein levels were rapidly reduced after cadmium exposure (Figure 3.3, D and E), we performed these experiments in the presence of PMSF to block the activity of vacuolar proteases (and as such, the vacuolar degradation pathway) and in the presence of MG132, a drug which blocks the proteolytic activity of the proteasome complex. Our results indicate that Fet4 degradation induced by cadmium is not dependent on the proteasome (data not shown) but rather on the vacuolar degradation pathway (Figure 3.7 E). Moreover, our data clearly show that, in the presence of both PMSF and cadmium, deletion of Aft1 *consensus* sites from *FET4* gene compromises Fet4 protein levels in the *rox1* but not in the WT strain, indicating that in the *rox1* strain *FET4* gene is up-regulated by Aft1 when cells are exposed to prolonged cadmium treatment.

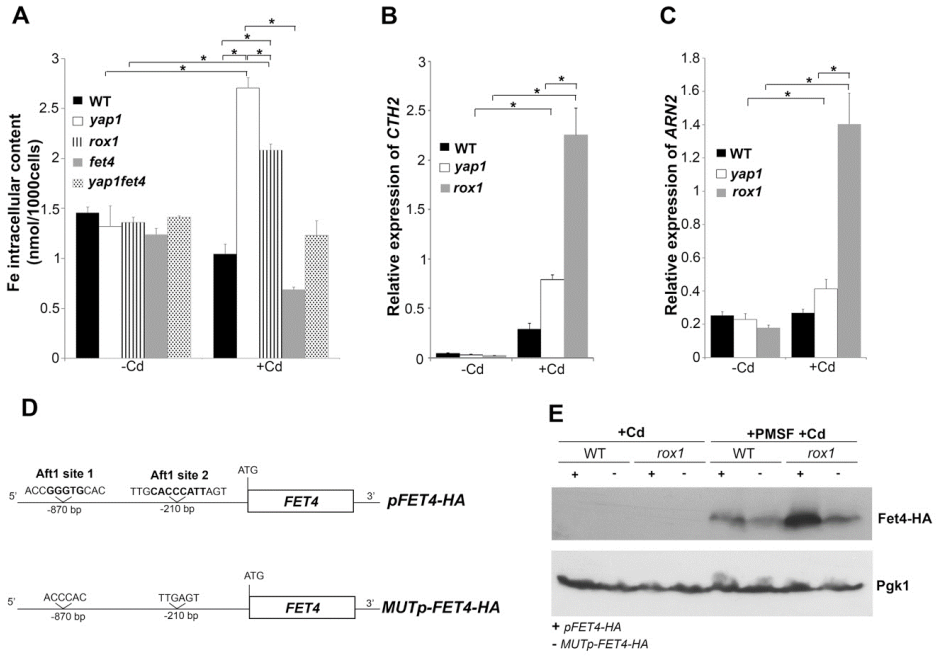

Figure 3. 7 Iron homeostasis is perturbed in the *yap1* and *rox1* mutants. A. Iron intracellular contents in WT, *yap1*, *rox1*, *fet4*, and *yap1fet4* strains were determined by ICP-AES in unstressed cultures (-Cd) and after culture treatment with 25 µM $CdCl_2$ (+Cd) for 6 h. **(B)** and **(C)** *CTH2* (B) and *ARN2* (C) expression in WT, *yap1*, and *rox1* strains was analyzed by qRT-PCR after treatment with 25 µM $CdCl_2$ for 60 min (+Cd). The values are the means of at least biological triplicates ± s.d. Significance of differences was calculated with the t test. *, $p<0.05$. **(D)** Schematic representation of the constructs *pFET4-HA* and *MUTp-FET4-HA*. In the *MUTp-FET4-HA* construct, both Aft1 sites (1 and 2) were deleted. **(E)** WT and *rox1* mutants transformed with *pFET4-HA* or *MUTp-FET4-HA* constructs were treated with Cd for 1 h (+Cd) or treated with 1.2 mM of PMSF for 90 min and then induced with 25 µM $CdCl_2$ for 1 h (+PMSF+Cd). Fet4 protein levels were analyzed by Western blot. Pgk1 was used as a loading control.

4. Discussion

In *S. cerevisiae*, eight stress-responsive transcription factors, Yap1 to Yap8, orchestrate the regulation of gene expression in response to a plethora of

environmental cues (reviewed in Ref. [44]). Yap1, the master regulator of oxidative stress, plays a pivotal role in cell tolerance against metal toxicity, mainly by inducing genes coding for proteins involved in (i) vacuolar metal sequestration, (ii) metal reduction and extrusion, or (iii) detoxification of reactive oxygen species generated by metal-catalyzed Fenton-type chemistry.

In this work, we have identified a new line of action of Yap1 towards cadmium toxicity. We showed that the negative regulation of the low affinity iron transporter gene, *FET4*, mediated by Yap1 (Figure 3.1), is important for yeast resistance to cadmium. Indeed, the *yap1* mutant accumulates higher cadmium levels compared with the WT strain, whereas the deletion of *FET4* gene from the *yap1* background restores cadmium tolerance (Figure 3.1). Other authors have also reported an increase of cadmium levels in *yap1* cells, but did not clarify the underlying mechanism [15].

Another set of data clearly indicates that repression of *FET4* by Yap1 is exerted *via* Rox1, an aerobic repressor of hypoxic genes, previously implicated in cadmium toxicity through a mechanism involving the repression of *FET4* [34]. We have in fact shown Yap1 direct regulation of *ROX1* expression through the recognition of an YRE located at 414 bp upstream of its ATG codon (Figure 3.2) as well as this *consensus* relevance for Rox1-mediated *FET4* repression (Figure 3.4). The mutant *yap1*, however, is more tolerant to cadmium than the *rox1* strain (Figure 3.3). Accordingly, *ROX1* expression is not fully dependent on Yap1 and *FET4* mRNA, and protein levels are therefore consistently higher in the *rox1* mutant than in the *yap1* strain (Figure 3.3). This observation is in agreement with

the fact that Rox1 is also regulated by Hap1 [45], a heme-dependent transcription factor [46]. Moreover, *ROX1* transcript levels and the expression of Rox1 target genes were reported to be only moderately decreased in the *hap1* mutant, suggesting the presence of another regulator [45, 47].

The observed drastic decrease of *FET4* transcripts after cadmium addition to the medium, suggests that *yap1* and *rox1* mutant cells tend to counteract *FET4* derepression (Figure 3.3 C). We further showed that the 5'-3' exoribonuclease Xrn1 is mediating this reduction (Figure 3.5). Although *in vitro* Xrn1 shows little specificity to particular mRNAs, *in vivo* this is not the case. Jones *et al.* [48] have proposed that binding of a specific RNA sequence by ncRNAs and/or RNA-binding proteins may recruit the 5'-3' degradation complex. As such, it is tempting to speculate that cadmium can promote the binding of such an element to *FET4* transcripts leading to their specific degradation. Moreover, it now seems likely that a translational/post-translational regulation of Fet4 activity occurs in response to cadmium, because in the WT strain, the protein levels decrease after cadmium insult (Figure 3.1 B), whereas the mRNA levels do not vary (Figure 3.1A), and the blocking of the vacuolar degradation pathway restores protein levels (Figure 3.7 E).

The intriguing finding that *FET4* mRNA levels in the *rox1* mutant tend to increase over time after the initial cadmium-induced depression (Figure 3.3 C) may result from the combinatorial control of this gene by several transcription factors [34, 49]. Aft1, the major regulator of the iron depletion response, also controls *FET4* expression [41]. Cadmium stress induces iron starvation [34],

implying that differences in the cadmium status of both mutants (*rox1* and *yap1*) may differently activate Aft1. After cadmium treatment, the *yap1* strain accumulates lower cadmium levels compared with the *rox1* strain (Figure 3.3 F), and accordingly, *rox1* cells appear to be more iron-starved because their iron content is lower (Figure 3.7 A), and Aft1 is more active (Figure 3.7 B, C, and *E*).

The fact that *ROX1* regulation mediated by Yap1 occurs under normal growth conditions (Figure 3.2) raises the question whether this regulation could serve a broader purpose, in addition to hindering cellular cadmium uptake *via* the repression of *FET4*. In line with this possibility, Liu and Barrientos [50] have recently reported that reactive oxygen species induce the expression of hypoxic genes in a Rox1-independent manner, although the levels of *ROX1* transcripts are strongly increased upon oxidative stress. Induction of *ROX1* expression was, however, shown to be Yap1-independent. The discrepancy between the data of Liu and Barrientos [50], and our own data are likely due to differences in the quantitative analysis of *ROX1* transcripts (relative expression *versus* fold change). Here, we clearly show that Yap1 up-regulates *ROX1* gene (Figure 3.2), and this may act as a compensatory mechanism of Rox1-defective repression of hypoxic genes, under oxidative environments. Interestingly, controlled hypoxia is often used as a treatment to overcome the catastrophic effects observed after intoxication by ingestion of paraquat, a potent superoxide generator that accumulates in lungs [51, 52]. In this case, hypoxia can be used to mitigate reactive oxygen species. As such, it is reasonable to hypothesize that Yap1 regulation of hypoxic genes through Rox1 may be relevant to overcome oxidative

stress in a scenario where Yap1 activity is impaired and intracellular reactive oxygen species accumulate.

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Chapter IV

ASSOCIATION BETWEEN IRON LOADING AND FLUCONAZOLE TOLERANCE IN *CANDIDA GLABRATA*

The author of this dissertation had a major contribution in this work, namely in the planning of the experimental work, in the execution and analysis of the experiments.

Abstract

In humans, *Candida* species are commensal organisms and opportunistic fungal pathogens. Among them, *Candida glabrata* deserves particular attention, being regarded as a serious clinical threat due to its inherent tolerance to antifungals. Interestingly, growing clinical evidences suggest an association between increased incidence of fungal infections and elevated iron levels, which occurs in patients with iron-loading diseases, such as hemochromatosis. Preliminary results from our laboratory indicate a negative correlation between antifungal (fluconazole) efficacy and iron loading conditions in the yeasts *Saccharomyces cerevisiae* and *C. glabrata*. We observed that when yeast cells were grown in media supplemented with high iron concentrations, the fungistatic activity of fluconazole was significantly decreased. Interestingly, this effect was no longer observed in *S. cerevisiae*, when the yeast iron detoxification pathway was impaired, by disrupting the *CCC1* gene. In *C. glabrata*, however, deletion of the corresponding orthologous gene, *CgCCC1*, did not counteract the attenuated efficacy of the antifungal induced by high iron. Therefore, we put forward the idea that another as yet unidentified players might be mediating iron-loading detoxification in *C. glabrata*. As a first approach to address this hypothesis we performed a phenotypic screening and found several new promising candidate genes whose products appear to play a role in iron-loading detoxification and/or fluconazole tolerance.

1. Introduction

The incidence of invasive fungal infections (FIs) has been increasing in the last decades, becoming an important medical concern, especially among immunocompromised and elderly patients [1-3]. Invasive FIs are associated with high morbidity and mortality rates, longer hospitals stays and, consequently, increased associated health care costs.

Antifungal agents available for the treatment of invasive FIs can be divided in four classes: azoles, echinocandins, polyenes, and pyrimidine analogs [4]. Azoles are by far the most commonly used drugs to fight fungal infections [4-8]. In particular, fluconazole, a triazole, is widely used due to its broad fungal target spectrum and safety profile, having minimal interactions with other drugs and few reported side effects [4-8]. The target of azole drugs is Erg11, a key enzyme of the ergosterol biosynthetic pathway. Erg11 is a cytochrome P450-dependent enzyme 14 α-lanosterol demethylase, which converts lanosterol in ergosterol, a component of the fungal cell membrane [9]. The inhibition of this pathway leads to the accumulation of toxic sterol species which are unable to successfully replace ergosterol, leading to cell growth inhibition [4, 10, 11].

Candida spp occupy a respectable position in the rank of agents causing hospital-acquired infections and are one of the most common causes of bloodstream infections [12]. Bloodstream infections caused by *Candida*, known as candidemias, are considered a serious problem in the Intensive Care Units (ICU). In Portugal, among ICU patients, *Candida albicans* is the most frequent source of *Candida* infections, being followed by *Candida parapsilosis* and

Candida glabrata [13]. However, more than half of the deaths associated with candidemia are due to *C. glabrata* infections [13].

As mentioned in Chapter I, decreased availability of iron (Fe) is part of the host defense strategy for coping with infection. Nonetheless, there are diseases (hemochromatosis, hepatic disease, acute leukemia) and several medical conditions (chemotherapy, repeated blood transfusions), which induce Fe loading in the host, shutting off the nutritional immunity [14, 15]. Indeed, there are several reports correlating increased Fe levels with higher incidence of fungal infections [14-17]. In the yeast *Saccharomyces cerevisiae*, the major player of Fe overload detoxification is Ccc1, a vacuolar transporter that mediates Fe accumulation in the vacuole. In the absence of *CCC1* gene, yeast cells cannot survive in a high-Fe milieu [18]. In our laboratory, during the course of an ongoing project, we found Ccc1 protein levels increased after *S. cerevisiae* treatment with fluconazole (Amaral *et al.* unpublished work). This preliminary result raised the question whether yeast adaptation to Fe overload is important for cells to deal with the antifungal drug. The results presented in this Chapter clearly indicate that (i) Fe alleviates the fluconazole antifungal activity against *S. cerevisiae* and *C. glabrata*, (ii) disruption of *CCC1* counteracts this effect in *S. cerevisiae*, but (iii) a similar mechanism does not seem to be operational in *C. glabrata*. Indeed, although *CgCCC1* gene is important for Fe-loading adaptation in *C. glabrata*, its deletion does not fully compromise the yeast growth under high Fe conditions. This result suggests other yet unidentified players, which are orchestrating Fe detoxification in *C. glabrata*. To further explore this hypothesis,

we performed a phenotypic screening of 619 *C. glabrata* mutant strains [19] designed with the aim of (i) identifying genes involved in *C. glabrata* Fe detoxification and (ii) shed light into the mechanism underlying the lost of fluconazole antifungal efficacy when yeast faces an Fe-loading milieu.

2. Material and Methods

2.1 Strains and growth conditions

S. cerevisiae and *C. glabrata* strains used in this work were grown in liquid synthetic complete medium (SC: 1.7 g/L Difco yeast nitrogen base without ammonium sulfate, 0.79 g/L complete supplement mixture (CSM; MP Biomedicals), 5 g/L ammonium sulfate, supplemented with 2% glucose). For solid media, 1.5 g/100ml Difco agar granulated was added to the liquid medium.

2.2 Spot assays

S. cerevisiae wild-type (*Sc*_WT) and *Sc_ccc1* mutant cells (Table 4.1) at OD_{600} of 0.4 were sequentially diluted and 5 µl of culture were spotted onto SC agar media plates (approximately 5×10^3 to 10 cells) containing the indicated concentrations of $FeSO_4$, Fluconazole (Fluc) or both conditions. *C. glabrata* WT and *Cg_ccc1* mutant cells (Table 4.2) at OD_{600} of 1 were 10-fold sequentially diluted and 5 µl of culture were spotted onto SC agar media containing the indicated concentrations of $FeSO_4$, Fluconazole (Fluc) or both conditions. The assays were made in triplicate. Cultures were grown for 24 h and/or 48 h at 37ºC for *C. glabrata* and 30ºC for *S. cerevisiae*.

Table 4. 1 *S. cerevisiae* strains used in this study.

Strain	Genotype	Source
BY 4742	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0*	EUROSCARF
BY 4742 *ccc1*	MATα *his3Δ1 leu2Δ0 lys2Δ0 uraΔ0 ccc1Δ::kan*	EUROSCARF

Table 4. 2 *C. glabrata* strains used in this study.

Strain	Genotype	Source
HTL	*his3Δ*::FRT *trp1Δ*::FRT *leu2Δ*::FRT (made from ATCC 2001)	[19]
HTL *ccc1*	*his3Δ*::FRT *trp1Δ*::FRT *leu2Δ*::FRT *ccc1Δ*::*NAT1*	[19]

2.3 Immunoblot Assay

S. cerevisiae WT cells containing the plasmid p*CTH2LacZ* [20] were grown until OD_{600} 0.4-0.5 and exposed to and 8 μg/ml of fluconazole. Cells were harvested after 15, 30 and 60 minutes. Total proteins were extracted from cell cultures using cell lysis buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM KCl, 10% glycerol, 0.1% NP40) supplemented with protease inhibitors (Roche). Protein concentrations were determined using the Bradford assay and 80–100 mg of protein were resolved in a 10% SDS-PAGE, and transferred to a nitrocellulose membrane. Protein levels were detected using anti-β-galactosidase (Sigma). Pgk1 was used as loading control. Immunoblots were repeated at least twice.

2.4 Measurement of intracellular iron

*Sc*_Wt, *Sc_aft1* and *Cg*_WT strains were grown until exponential phase in SC medium and left untreated or treated with 8 μg/ml of fluconazole for *S. cerevisiae* strains and 32 μg/ml for *C. glabrata*. Cells were harvested after 15 h of induction with the antifungal drug, collected by centrifugation, and washed once with 10 mM EDTA solution and twice with metal-free water. The total Fe content was measured by inductively coupled plasma atomic emission spectroscopy (ICP-

AES) at REQUIMTE, Faculdade de Ciências e Tecnologia - UNL. Data was normalized against OD_{600}.

2.5 Susceptibility testing of *S. cerevisiae* and *C. glabrata* in broth microdilution assays

Minimal inhibitory concentration (MIC) was determined in accordance with the CLSI standard reference method for broth dilution antifungal susceptibility testing in yeasts M27-A3 [21]. Inocula of *S. cerevisiae* and *C. glabrata* were prepared from cultures growing overnight on SC medium, posteriorly adjusted to a final concentration of cells ranging from 0.5×10^3 to 2.5×10^3 CFU/ml. The concentrations of $FeSO_4$ used ranged from 30 to 0.9375 mM. The MIC plates were incubated at 37°C for *C. glabrata* cells and 30°C for *S. cerevisiae* cells during 24 h. A volume of 5 µl from each well was spotted onto an YPD plate in order to monitor cell viability. The recovery plate was incubated for 1 day at 37°C for *C. glabrata* and 30°C for *S. cerevisiae*. MIC endpoints were determined as described in CLSI standard reference method for broth dilution antifungal susceptibility testing in yeasts M27-A3 [21]. The growth in wells containing $FeSO_4$ was compared with that in growth-control well (no $FeSO_4$) [21].

2.6 *Candida glabrata* library phenotypic profile screening

C. glabrata WT and mutant cells [19] were grown until OD_{600} of 1, 10-fold sequentially diluted and 5 µl of culture were spotted onto SC agar media without drug or containing 10 mM of $FeSO_4$, 2 mM $FeSO_4$, 32 µg/ml of fluconazole

(Fluc), and 2 mM of $FeSO_4$ plus 32 μg/ml of Fluc. This assay was performed once. Cultures were grown for 48 h at 37°C and growth was recorded after 24 and 48 h. Genes were clustered into functional groups according to the Munich Information Center for Protein Sequences database - MIPS (http://mips.helmholtz-muenchen.de/proj/funcatDB/).

3. Results

3.1 Iron alleviates the antifungal activity of fluconazole against the yeasts *S. cerevisiae* and *C. glabrata*

Fluconazole is the most widely used antifungal drug worldwide. In our laboratory, we observed an increase in fluconazole tolerance of *S. cerevisiae* WT (*Sc*_WT), when cultures were grown in high Fe milieu. As depicted in Figure 4.1 A, *S. cerevisiae* cells are pronouncedly sensitive to 10 μg/ml of fluconazole, becoming tolerant to the antifungal drug when co-cultured with 5 mM of $FeSO_4$. Fluconazole action relies on the inhibition of the enzyme Erg11, which converts lanosterol to ergosterol. This inhibition occurs after fluconazole binding to the Fe center of the porphyrin group of the enzyme [4]. In this context, we first tested whether fluconazole treatment could be depleting the intracellular Fe pool. First, the *pCTH2-LacZ* construct [20] was used to transform *Sc*_WT cells and β-galactosidase expression under the control of *CTH2* promoter was monitored by Western blot. When *S. cerevisiae* cells experience Fe depletion, *CTH2* is induced by the transcription factor Aft1, the major regulator of the Fe deprivation

response [20, 22]. After exposing cells to fluconazole, β-galactosidase protein levels slightly increased after 60 min (Figure 4.1 B), suggesting a possible induction of the Fe deprivation response by the drug. We next monitored the growth of *Sc*_WT and *aft1* mutant (*Sc_aft1*) strains, after treatment with fluconazole (Figure 4.1 C). If fluconazole induces the Fe deprivation response, one would expect to observe a decrease in *Sc_aft1* growth when compared with that of *Sc*_WT. As depicted in Figure 4.1 C, while both strains present similar growth under normal physiological conditions, after fluconazole exposure the *Sc_aft1* strain grows better than the corresponding isogenic strain, *Sc*_WT.

To further investigate the above intriguing and apparently contradictory results, the intracellular Fe content of *Sc*_WT and *Sc_aft1* strains was measured by ICP-AES. As depicted in Figure 4.1 D the total Fe levels of *Sc*_WT strain increase in the presence of fluconazole. This observation suggests that cells are prompted to uptake Fe from the media when exposed to the drug, corroborating the increased expression of *CTH2* (Figure 4.1 B). Fe levels of *Sc_aft1* do not vary after fluconazole treatment, indicating that the observed uptake of Fe is dependent on Aft1.

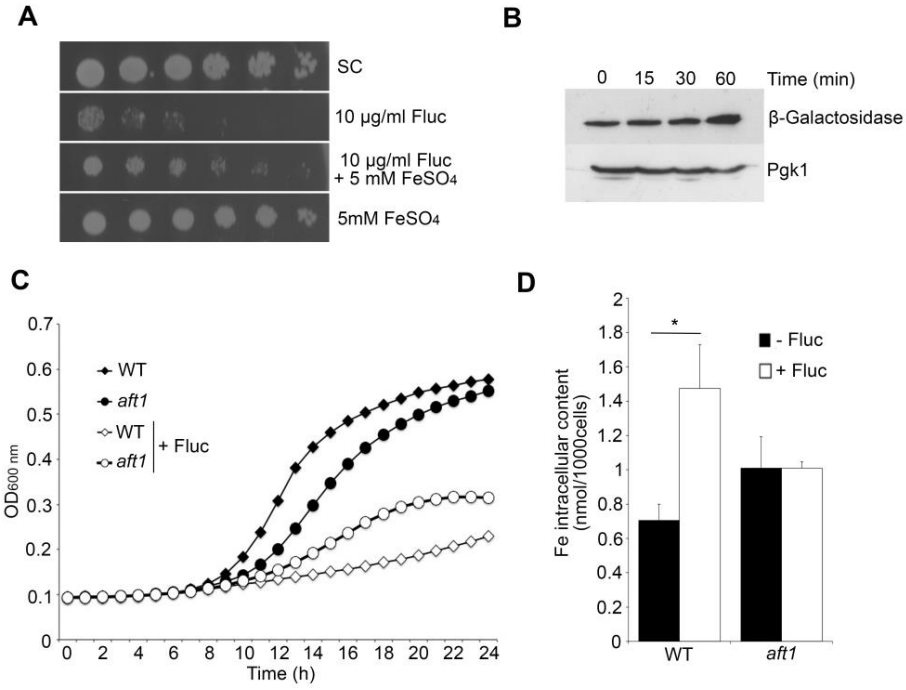

Figure 4. 1 *S. cerevisiae* fluconazole tolerance increases in iron-loading conditions. (A) Growth sensitivity exhibited by *S. cerevisiae* wild-type (WT) cells in SC plates containing 5 mM of $FeSO_4$, 10 µg/ml fluconazole (Fluc) or both. Growth was recorded after 2 days. **(B)** *Sc*_WT strain was transformed with *pCTH2-LacZ*, cultures were treated with the indicated concentrations of Fluc, harvested at the indicated time points and β-Galactosidase expression was analyzed by Western blot. **(C)** Growth of *Sc*_WT and *Sc_aft1* (*aft1*) strains in SC media was recorded over 24 h. Values are means of the biological triplicates. **(D)** Intracellular Fe content of *Sc*_WT and *Sc_aft1* was determined by ICP-AES, with or without addition of 8 µg/ml of Fluc for 15 h. Values are means of the biological triplicates ± s.d.. Significance of differences was calculated with the *t* test. *, $p<0.05$.

The natural tolerance to azoles and the close phylogenetic relation to *S. cerevisiae* [23] impelled us to further test if Fe surplus also increased fluconazole tolerance in *C. glabrata*. Similarly to what was observed in *S. cerevisiae*, also *C. glabrata* are less susceptible to fluconazole under Fe-loading conditions (Figure 4.2 A). To confirm if fluconazole exposure leads to an increased Fe uptake by *C.*

glabrata, the levels of intracellular Fe were assessed. As shown in Figure 4.2 B, the Fe content of *Cg*_WT does not significantly change after fluconazole exposure.

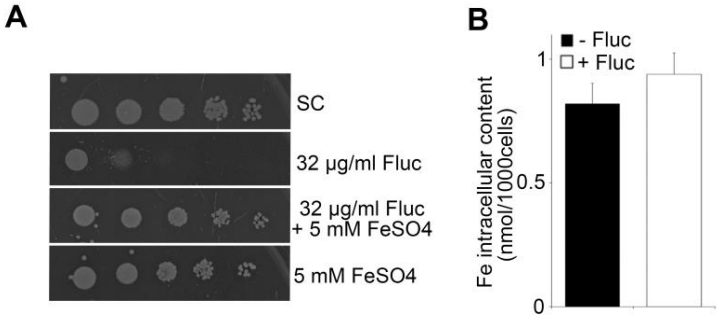

Figure 4. 2 *Candida glabrata* fluconazole tolerance increases under iron-loading conditions. (A) Growth sensitivity exhibited by *Cg*_WT in SC plates containing 5 mM of $FeSO_4$, 32 µg/ml fluconazole (Fluc) and both. **(B)** Intracellular Fe content of *Cg*_WT was determined by ICP-AES, with or without addition of 32 µg/ml of Fluc for 15 h. Values are means of the biological triplicates ± s.d..

3.2. The impairment of iron-loading detoxification pathway acts synergistically with fluconazole in *S. cerevisiae*.

In *S. cerevisiae*, the Ccc1 vacuolar transporter is the major player in Fe overload adaptation, conveying Fe accumulation in this organelle [18]. Remarkably, in our laboratory we observed an induction of Ccc1 protein levels by the antifungal fluconazole (Amaral *et al*, unpublished results). This finding prompted us to monitor fluconazole tolerance of *Sc_ccc1* strain under Fe replete and Fe-loading conditions. We chose $FeSO_4$ and fluconazole concentrations, which did not strongly affect cellular growth, when added individually to the medium (Figure 4.3 A). Interestingly we observed that when cells were exposed

to both conditions simultaneously, *Sc_ccc1* growth was notably affected, whereas *Sc*_WT growth was not.

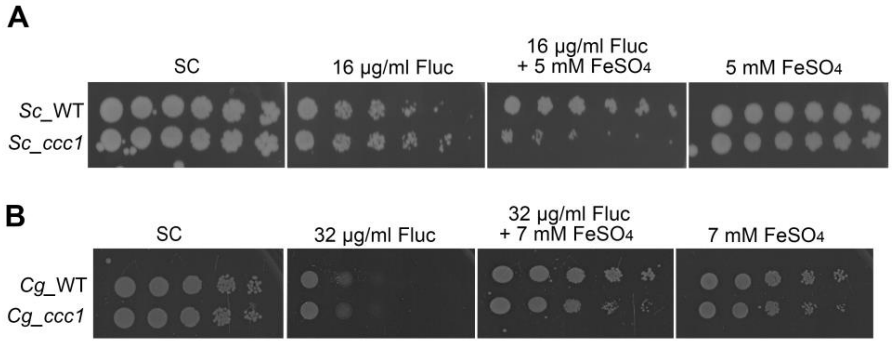

Figure 4. 3 Deletion of the *CCC1* gene promotes a synergistic effect between high iron and fluconazole in *S. cerevisiae*, but not in *C. glabrata*. **(A)** Growth sensitivity of *Sc*_WT and *Sc_ccc1* cells grown in SC plates containing the indicated concentrations of $FeSO_4$ and fluconazole (Fluc). Growth was recorded after 4 days. **(B)** Growth sensitivity of *Cg*_WT and *Cg_ccc1* cells grown in SC plates containing the indicated concentrations of $FeSO_4$ and fluconazole (Fluc).

The above synergism was not observed in *C. glabrata,* since no significant growth differences between *Cg*_WT and *Cg_ccc1* were detected, after simultaneous exposure to high Fe and fluconazole (Figure 4.3 B). This result led us to investigate in more detail the role of *CgCCC1* in Fe overload adaptation.

3.3. In the yeast *C. glabrata*, *CgCcc1* is not the only player involved in the adaptation to iron surplus

As a first approach to evaluate the contribution of Ccc1 to *C. glabrata* adaptation to Fe overload, we compared the growth of *C. glabrata* WT and mutant *ccc1* strains with those of *S. cerevisiae*, in media supplemented with growing Fe concentrations (Figure 4.4 A and B). We observed that while WT strains tolerate similar Fe concentrations, *Sc_ccc1* mutant is much more sensitive

than *Cg_ccc1*. MIC values were next determined according to the CLSI standard reference method for broth dilution susceptibility testing of yeasts M27-A3 [21]. As depicted in Figure 4.4 C, *Sc_ccc1* and *Cg_ccc1* MIC values for $FeSO_4$ are 1.875 mM and 7.5 mM, respectively. In addition, *Cg*_WT seems to be more resistant to high Fe (MIC is higher than 30 mM) than *Sc*_WT (MIC is 15 mM). The increased resistance of *C. glabrata* to high Fe, even in the absence of *CCC1* gene, strongly suggests the involvement of other players in *C. glabrata* adaptation to Fe surplus.

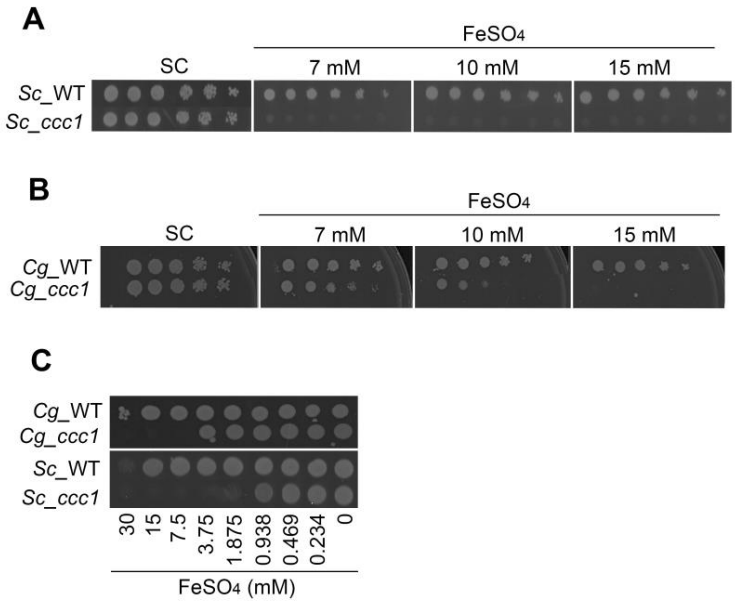

Figure 4. 4 *Ccc1* is not the only player in iron loading response in *C. glabrata*. **(A)** Growth sensitivity of *Sc*_WT and *Sc_ccc1* cells grown in SC plates containing the indicated $FeSO_4$ concentrations **(B)** Growth sensitivity of *Cg*_WT and *Cg_ccc1* cells grown in SC plates containing the indicated $FeSO_4$. **(C)** MICs were determined as described under *Material and Methods*. Growth was recorded after 24 h of incubation.

3.4. Phenotypic screening to identify *C. glabrata* genes involved in iron-loading detoxification and/or increased fluconazole tolerance in an iron rich environment

C. glabrata is one of the species found in the normal microbiota of the gastrointestinal cavity [24]. As such, this fungus should be adapted to drastic fluctuation in Fe bioavailability. In this sense, is not surprising that Fe detoxification pathway does not solely rely on Ccc1. Aiming at (i) identifying the other interveners in Fe detoxification and (ii) understanding *C. glabrata* increased fluconazole tolerance in Fe-loading conditions, we performed a phenotypic screening of the *C. glabrata* mutant collection available at Professor Patrick Van Dijck laboratory and developed by Schwarzmüller *et al* [19].

We have first determined Fe concentrations to be used. To identify novel Fe-loading detoxification players, we used 10 mM of $FeSO_4$, since the *Cgccc1* mutant only shows a significant growth defect for concentrations above it (Figure 4.4 B). To identify genes whose products have a role in the lack of fluconazole efficacy promoted by Fe loading, we used 2 mM of $FeSO_4$, because, as depicted in Figure 4.5, a concentration equal or higher is necessary to impair fluconazole activity. Finally, the phenotypic screening was performed by growing *C. glabrata* cells in SC agar plates supplemented with (i) 10 mM of $FeSO_4$, (ii) 32 μg/ml of fluconazole, (iii) 2 mM of $FeSO_4$ and (iv) 2 mM of $FeSO_4$ plus 32 μg/ml of fluconazole.

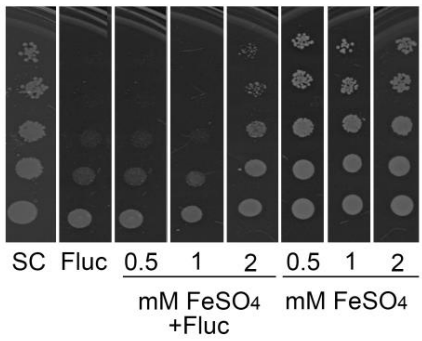

Figure 4. 5 The minimal $FeSO_4$ concentration to impair fluconazole activity in *C. glabrata* is 2 mM. Growth sensitivity of *Cg*WT cells grown in SC plates containing the indicated $FeSO_4$ and fluconazole (Fluc) concentrations.

The phenotypic screening of *C. glabrata* mutant collection allowed us to group the corresponding genes into the following categories: (i) genes involved in Fe overload adaptation, (ii) genes possibly implicated in the Fe alleviation of fluconazole toxicity, (iii) genes participating in fluconazole tolerance, and (iv) genes involved in the iron-loading response whose deletion inhibits growth recovery, after treatment with fluconazole, in a high iron milieu.

3.4.1 Genes involved in iron overload adaptation

In an attempt to unveil the participants in the Fe-loading response of *C. glabrata*, the mutant strains of the collection were grown in media containing 2 mM and 10 mM of $FeSO_4$. As shown in Figure 4.6, twelve mutants were found to be sensitive to Fe surplus and the function of the corresponding genes are listed in Table 4.3. Four mutant strains (*rox1, spt8*, *yps1*, and *vps15*) showed a slight sensitivity to 10 mM of $FeSO_4$, more pronounced at 24 than at 48 h (Figure 4.6). Eight mutants (*yfh1*, *atm1*, *mnn10*, *tpn1*, *ace2*, *met31*, *ktr2*, and *cbk1*) showed

deficient growth after 24 h under normal physiological conditions (Figure 4.6 A). This observation has been previously reported for *yfh1* [19, 25, 26], *atm1* [19, 25], *mnn10* [19], *tpn1* [19], *ktr2* [19], and *cbk1* [19] mutants. *tpn1* and *met31* mutants showed a slight sensitivity to 2 mM of $FeSO_4$ after 48 h (Figure 4.6 B). Notwithstanding, the eight strains mentioned above (*yfh1*, *atm1*, *mnn10*, *tpn1*, *ace2*, *met31*, *ktr2*, and *cbk1*) show a pronounced sensitivity to 10 mM of $FeSO_4$ after 24 and 48 h (Figure 4.6). Among these genes, four have been already implicated in Fe-loading adaptation, namely *ysp1* [27], *mnn10* [25], *cbk1* [25], and *tpn1* [25].

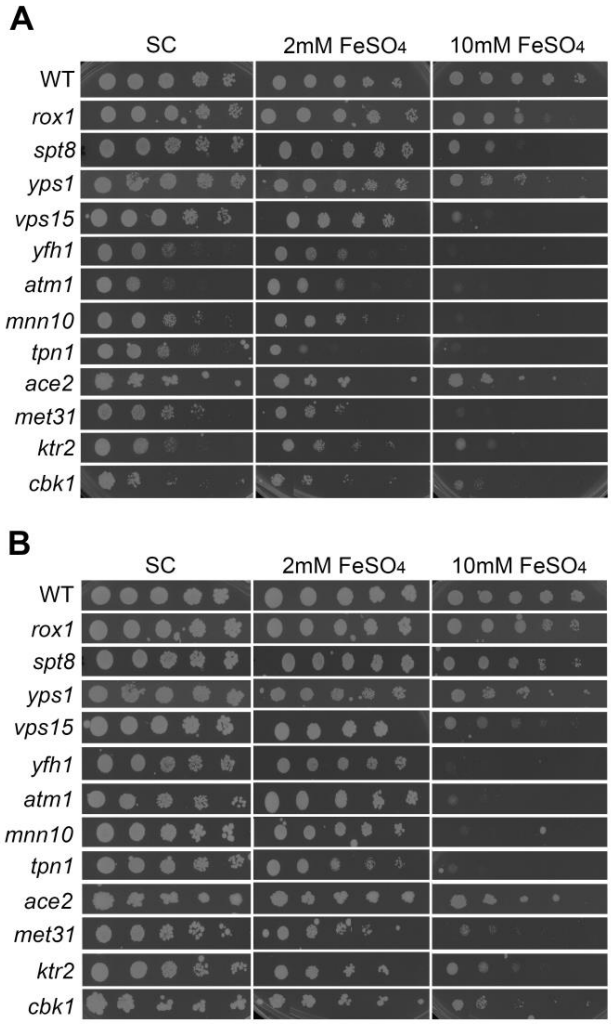

Figure 4. 6 *C. glabrata* mutant strains whose genes are required to iron overload adaptation. WT and the indicated mutant strains were grown in SC plates supplemented or not with 2 and 10 mM of $FeSO_4$. Growth was recorded after **(A)** 24 h and **(B)** 48 h at 37ºC.

Table 4.3 List of genes involved in the adaptation to iron excess.

Systematic name	Gene	Function
CAGL0D05434g	*ROX1*	Heme-dependent repressor of hypoxic genes*
CAGL0F01837g	*SPT8*	Subunit of the SAGA transcriptional regulatory complex
CAGL0M04191g	*YPS1*	Yapsin family aspartic protease; predicted GPI-anchor
CAGL0H08437g	*VPS15*	Ser/Thr kinase activity, ubiquitin binding activity
CAGL0M05643g	*YFH1*	Ferroxidase activity, Fe chaperone and role in cellular iron homeostasis
CAGL0M13739g	*ATM1*	Transmembrane movement activity of substances, mitochondrial inner membrane localization
CAGL0K11231g	*MNN10*	α-1,6-mannosyltransferase activity and role in ascopore formation
CAGL0L06138g	*TPN1*	Vitamin transporter, plasma membrane localization
CAGL0M04323g	*ACE2*	Putative transcription factor, null mutation leads to hypervirulence in immunocompromised mice
CAGL0L12562g	*MET31*	Zinc-finger DNA-binding transcription factor, targets promoters of sulfur metabolic genes*
CAGL0M05841g	*KTR2*	Mannosyltransferase involved in N-linked protein glycosylation*
CAGL0J06072g	*CBK1*	Ser/Thr kinase of the RAM signaling pathway

*Description of the function of the *S. cerevisiae* orthologue (*Saccharomyces cerevisiae* database)

3.4.2 Genes possibly implicated in the iron alleviation of fluconazole toxicity

In order to identify genes important for the increased fluconazole tolerance of *C. glabrata* when exposed to a high Fe milieu, the strains of the *C. glabrata* mutant collection were exposed to 2 mM of $FeSO_4$, 32 μg/ml of fluconazole and Fe and fluconazole together. Fe alleviation of fluconazole antifungal activity was not observed in four strains lacking the genes *SEC13*, *LEM3*, *INP53* and *RGT2*,

(Table 4.4 and Figure 4.7). This result was more evident after 24 h (Figure 4.7 A), but also visible after 48 h of growth (Figure 4.7 B).

Table 4.4 List of genes possibly involved in fluconazole tolerance in iron loading conditions.

Systematic name	Gene	Function
CAGL0J08778g	*SEC13*	Structural component of the outer layer coat protein complex II (COPII) – vesicle ER-to-Golgi transport*
CAGL0D02442g	*LEM3*	Glycoprotein involved in the membrane translocation of phospholipids and alkylphosphocholine drugs
CAGL0B04631g	*INP53*	Polyphosphatidylinositol phosphatase
CAGL0I03872g	*RGT2*	Low-affinity glucose sensor localized at the plasma membrane

*Description of the function of the *S. cerevisiae* orthologue (*Saccharomyces cerevisiae* database).

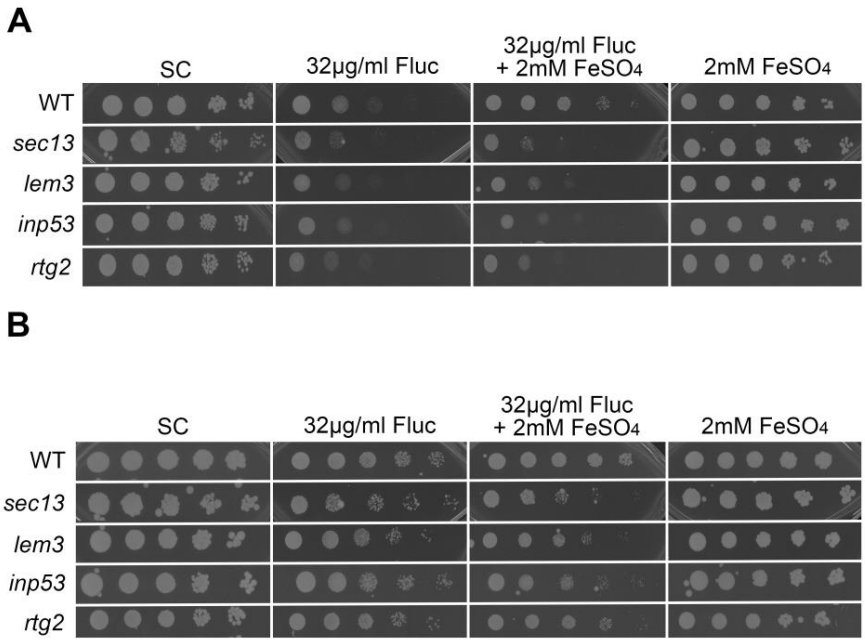

Figure 4.7 *C. glabrata* mutant strains whose genes are possibly involved in fluconazole tolerance in iron surplus conditions. Growth sensitivity exhibited by WT, *sec13*, *lem3*, *inp53*, and *rtg2* strains in SC plates, containing 32 µg/ml of Fluconazole (Fluc), 2 mM of $FeSO_4$ or both. Growth was recorded after **(A)** 24 h and **(B)** 48 h at 37ºC.

The *INP53* gene has been previously implicated in *C. glabrata* tolerance to fluconazole [28], whereas, as far as we are concerned, *SEC13*, *LEM3*, and *RGT2* have never been associated with this drug. Although, *inp53* has been described to exhibit decreased fluconazole tolerance [28], in the experimental conditions aforementioned we did not observed a similar phenotype (Figure 4.7).

3.4.3 Genes participating in fluconazole tolerance

During the phenotypic screening analysis we found 118 mutant strains with increased sensitivity to fluconazole which after Fe supplementation (i) became tolerant to the antifungal drug (109 strains) or (ii) remained sensitive to the drug (9 strains). Additionally, we identified 12 mutant strains with increased tolerance to fluconazole.

The 109 genes whose absence leads to increased fluconazole sensitivity (Table S4) belong to different functional categories (Figure 4.8). The most represented category is "transcription" (33 out of 109 genes), followed by "cell cycle and DNA processing" (20 out of 109 genes), and "metabolism" (15 out of 109 genes). Of those, *SLG1* and *ZCF4* have been previously reported to be involved in fluconazole tolerance [19].

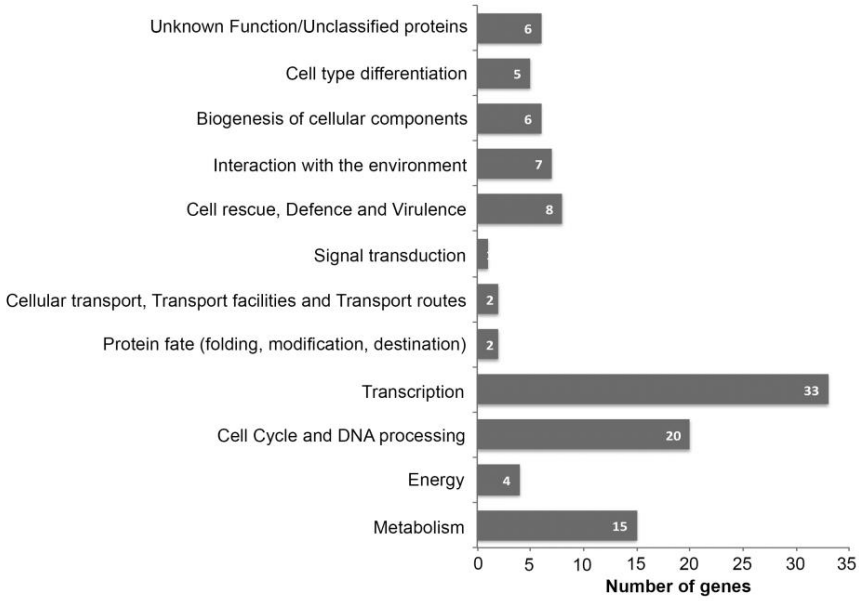

Figure 4. 8 Functional categories of genes whose deletion leads to higher fluconazole sensitivity, which is attenuated by iron supplementation. Genes were sorted into functional categories according to the MIPS database.

The 9 genes whose absence leads to fluconazole sensitivity and impaired growth recovery by high Fe conditions are listed in Table 4.5. Interestingly, *cna1* and *cnb1* mutants were previously shown to be sensitive to fluconazole, and the corresponding genes were described to be important for the virulence of *C. glabrata* [29, 30]. The *pdr1* and *cdr1* mutants are also part of this group. *PDR1* codes for a transcription factor involved in the regulation of drug resistance genes, containing pleiotropic drug response elements (PDRE) in the promoter region [31, 32]. *CDR1* codes for an ABC transporter involved in azole resistance and is regulated by Pdr1 [31, 33]. Schwarzmüller *et al* described *ypk1* mutant as

slightly sensitive to fluconazole [19], whereas we found it to be strongly sensitive to the drug (Figure 4.9).

Table 4.5 List of genes whose deletion leads to increased fluconazole sensitivity, which is not alleviated by iron supplementation.

Systematic name	Gene	Function
CAGL0L11110g	*CNA1*	Catalytic subunit of calcineurin
CAGL0L00605g	*CNB1*	Regulatory subunit of calcineurin
CAGL0A00451g	*PDR1*	Zn finger TF, activator of drug resistance genes
CAGL0M01760g	*CDR1*	ABC multidrug transporter, involved in azoles resistance
CAGL0K06193g	*ADA2*	Transcription coactivator. Component of the ADA and SAGA transcriptional adaptor *
CAGL0E06028g	*ALG5*	Dolichyl-phosphate glucosyltransferase localized in the ER*
CAGL0C03399g	*POP2*	RNase of the DEED superfamily*
CAGL0K03399g	*YPK1*	Serine/threonine kinase
CAGL0G02827g	*SLM1*	Phosphoinositide PI4,5P(2) binding protein, forms a complex with Slm2*

*Description of the function of the *S. cerevisiae* orthologue (*Saccharomyces cerevisiae* database); ER- Endoplasmic Reticulum.

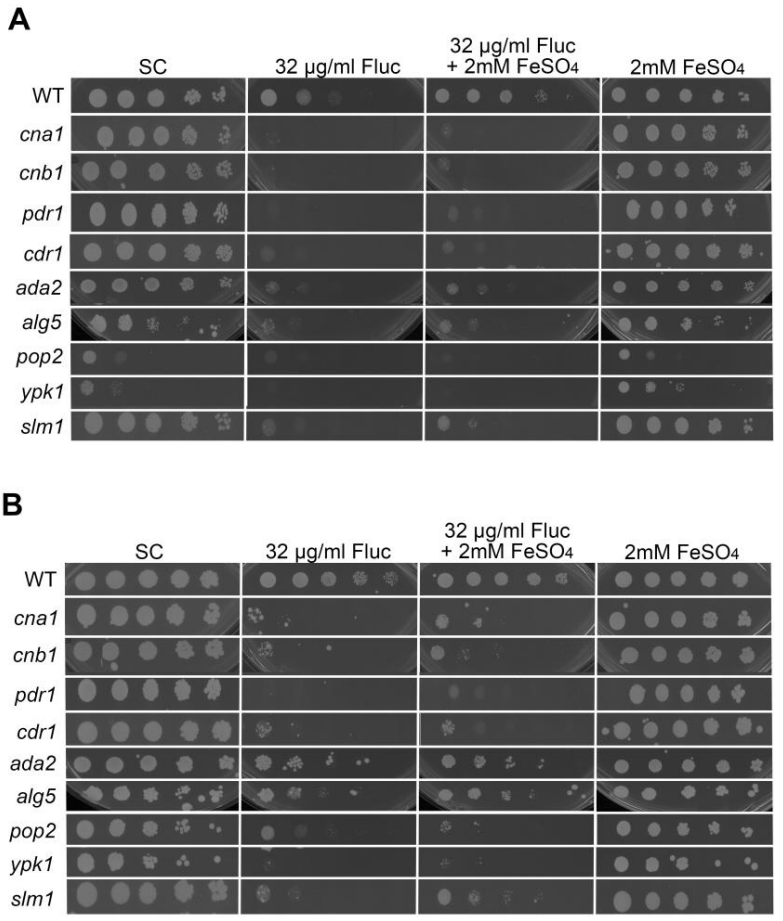

Figure 4.9 *C. glabrata* mutant strains with increased sensitivity to fluconazole, which do not become tolerant to the antifungal drug after iron supplementation. Growth sensitivity exhibited by WT and the indicated mutant strains in SC plates containing 32 µg/ml of Fluconazole (Fluc), 2 mM of $FeSO_4$ or both. Growth was recorded after **(A)** 24 h and **(B)** 48 h at 37ºC.

Finally, we found twelve genes (Table 4.6) whose products have an inhibitory effect on fluconazole tolerance, since their absence lead to a better growth of the corresponding mutant strains in the presence of the antifungal (Figure 4.10). Among these mutants, as far as we are concerned, only *crz1* has been previously described to be more tolerant to fluconazole [29].

Table 4.6 List of genes whose deletion lead to increased fluconazole tolerance.

Systematic name	Gene	Function
CAGL0M07183g	*MDM31*	Mitochondrial protein with possible role in phospholipid metabolism*
CAGL0M05929g	*FMP18*	Constituent of the TIM23 complex (Translocase of the Inner mitochondrial Membrane)*
CAGL0K05797g	*EMI1*	Protein has role in ascospore formation and mitochondrion organization
CAGL0L08910g	*AEP3*	Peripheral mitochondrial inner membrane protein*
CAGL0J03366g	*GET2*	Protein with anchor activity, role in protein insertion into ER membrane*
CAGL0L01463g	*PRM6*	Potassium transporter that mediates K^+ influx
CAGL0K04367g	*MUT1*	High affinity methionine permease; involved in cysteine uptake
CAGL0L12650g	*SSN3*	Cyclin-dependent protein kinase; involved in the phosphorylation of RNA polymerase II*
CAGL0G02409g	*SRP40*	Nuclear protein; role in pre-ribosome assembly or transport
CAGL0M08910g	*SNF1*	Putative Ser/Thr protein kinase required for trehalose utilization
CAGL0M06831g	*CRZ1*	Transcription factor involved in the regulation of genes belonging to the calcineurin pathway
CAGL0C03608g	*TUP1*	Protein of unknown function

*Description of the function of the *S. cerevisiae* orthologue (*Saccharomyces cerevisiae* database); TF- Transcription Factor.

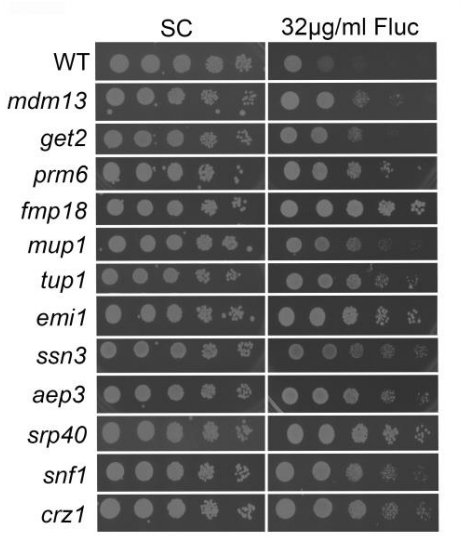

Figure 4. 10 *C. glabrata* mutant strains with increased tolerance to fluconazole. Growth exhibited by WT and the indicated mutant strains in SC plates containing 32 µg/ml of fluconazole (Fluc). Growth was recorded after 24 h at 37ºC.

3.4.4 Genes involved in the iron-loading response whose deletion inhibits growth recovery after treatment with fluconazole, in a high iron milieu.

In the course of the phenotypic screening, we found that the deletion of *TPN1*, one of the genes involved in the adaptation to Fe loading (Figure 4.6) inhibits growth recovery in medium treated with fluconazole and supplemented with Fe (Figure 4.11). A similar phenotype was assigned to a *S. cerevisiae* strain lacking the *CCC1* gene (Figure 4.3). Although conveyed by different players, the synergism between inadaptation to Fe loading and fluconazole may also be operationl in the yeast *C. glabrata*.

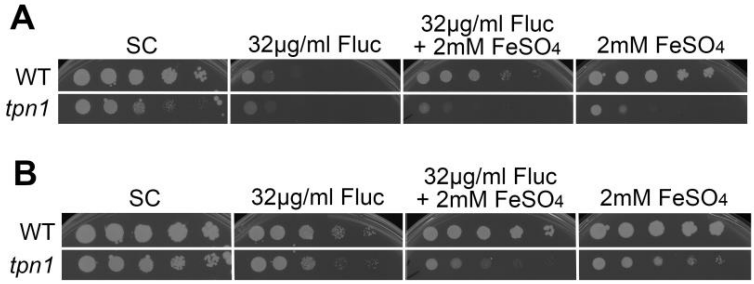

Figure 4.11 In the *C. glabrata* mutant strain *tpn1* a possible synergistic effect between iron loading and fluconazole is observable. Growth exhibited by WT and *tnp1* mutant in SC plates containing 32 µg/ml of fluconazole (Fluc), 2 mM of $FeSO_4$ or both. Growth was recorded after **(A)** 24 h and **(B)** 48 h, at 37°C.

4. Discussion

In this chapter we demonstrated that a high iron milieu favors *S. cerevisiae* and *C. glabrata* tolerance to fluconazole (Figure 4.1 A and 4.2 A). However, increased tolerance to the drug could not be ascribed to fluconazole-induced Fe depletion. Indeed, although fluconazole treatment triggers the expression of *CTH2* gene and the intracellular accumulation of Fe, clearly indicating the activation of the Fe depletion response in the *Sc*_WT strain, suppression of this response (as it happens in the mutant *Sc_aft1*) appears to confer resistance to the drug (Figure 4.1 C). This result led us to speculate if the higher fluconazole sensitivity of *Sc*_WT compared with *Sc_aft1* (Figure 4.1 C) may be due to differences in the Fe content of the strains, further supporting the negative effect of Fe in fluconazole toxicity. In *C. glabrata*, intracellular Fe levels do not vary after fluconazole treatment (Figure 4.2 B), reinforcing the idea that the

mechanisms underlying fluconazole tolerance in a Fe surplus environment is not related to drug-altered cellular Fe requirements. The tolerance to the antifungal promoted by Fe seems to be dose dependent. Concentrations of $FeSO_4$ below 2 mM do not appear to influence fluconazole tolerance in *C. glabrata* (Figure 4.5). It is possible that cells only sense Fe loading at or above this concentration and only then trigger the transcription of genes involved in the gain of tolerance to the antifungal drug. At this stage of the work, it is tempting to speculate if the mechanism involved in *Cg*_WT fluconazole tolerance under Fe surplus conditions may contribute to the exacerbation of fungal infections in individuals suffering from Fe-loading diseases.

Preliminary results from our laboratory, showing an increased expression of Ccc1 after fluconazole treatment (Amaral *et al*, unpublished results), prompted us to assess the tolerance of *Sc_ccc1* mutant to this antifungal. We observed a synergism between the impairment of Fe overload detoxification (*Sc_ccc1* mutant) and fluconazole (Figure 4.3 A). The lack of *CCC1* gene leads to increased cytosolic Fe levels [34], as such it is possible that the intracellular Fe availability of *Sc_ccc1* enhances fluconazole antifungal activity. In *C. glabrata*, however, *CCC1* deletion does not concur with fluconazole toxicity, under Fe-loading conditions (Figure 4.3 B). *C. glabrata* is part of the normal microbiota population of the gastrointestinal cavity, and therefore this fungus should be adapted to drastic fluctuations of Fe bioavailability. This could explain the lack of synergism between inefficient high Fe detoxification and fluconazole activity. Moreover, this fact together with the disparity between Fe-loading sensitivities

displayed by the two mutant species (Figure 4.4), strongly suggests other yet unidentified players involved in Fe-loading adaptation in *C. glabrata*.

In order to identify the mechanism governing Fe-induced fluconazole tolerance as well as the players involved in Fe-loading adaptation in *C. glabrata*, we performed a phenotypic screening using the *C. glabrata* mutant collection available at Professor Patrick Van Dijck laboratory and developed by Schwarzmüller *et al* [19].

We identified four genes (*SEC13*, *LEM3*, *INP53*, and *RTG2*) involved in WT increased tolerance to fluconazole under Fe-loading conditions (Figure 4.7). In *S. cerevisiae*, *SEC13* codes for one component of the coat protein complex II (COPII) and its deletion leads to a defect in ER vesicle formation [35]. *LEM3* has a putative role in phospholipid membrane translocation [36]. The absence of *SEC13* and *LEM3* alters the protein content and the fluidity of the plasma membrane, respectively, compromising its integrity, which could explain their involvement in fluconazole tolerance. Interestingly, mutations in the *INP53* gene, a polyphosphatidylinositol phosphatase, were described in a *C. glabrata* clinical isolate [37]. *RGT2* is a low-affinity glucose sensor important for the activation of glucose transporters in glucose-replete conditions [38]. *C. albicans* cells grown in glucose-containing media showed increased tolerance to azoles [39]. Therefore, deletion of *RGT2* might impair this glucose-mediated mechanism of azole resistance.

Although *C. glabrata* adaptation to Fe overload has not been comprehensively addressed, dispersed studies have identified few players [25-27, 40]. The work of

Merhej *et al* indicates some degree of conservation between the Fe-loading adaptation response of *S. cerevisiae* and *C. glabrata* [40-43]. In both yeasts, the transcription factor Yap5 up-regulates *CCC1* and *GRX4* genes in response to high Fe [40-43]. Our phenotypic screening unraveled twelve other players possibly involved in *C. glabrata* adaptation to Fe-loading conditions (Figure 4.6). Among them are *YPS1* and *VPS15* genes, which had been implicated in *C. glabrata* adaptation to several stresses, being important for cell wall integrity and proper vacuole function [27, 44, 45]. Interestingly the *ROX1* gene also appears to have a role in Fe surplus adaptation. As mentioned in previous chapters, Rox1 is a repressor of *FET4*, which encodes a low affinity Fe transporter [46]. If the regulatory pathway of Rox1 is maintained in *C. glabrata*, than the absence of *ROX1* gene would de-repress *FET4* expression and, consequently, enhance Fe uptake, which would explain the sensitivity of the *rox1* mutant to high Fe (Figure 4.6 A). The mitochondrial genes *ATM1* and *YFH1* appear to have a role in the *C. glabrata* adaptation to Fe-loading conditions (Figure 4.6). *ATM1* codes for a mitochondrial inner membrane ABC transporter involved in the export of iron-sulfur (Fe-S) cluster precursors to the cytosol and *S. cerevisiae* cells lacking this gene retain toxic Fe levels in the mitochondria, which contributes to cell death [47]. Yfh1 is the yeast homolog of the human frataxin protein whose deletion raises intracellular Fe [26]. Accumulation of Fe in these mutants could explain their growth sensitivity under Fe surplus conditions. Our screening also revealed two mannosylation enzymes encoding genes, *MNN10* and *KTR2* as fulfilling a role in Fe-loading response. It is possible that Fe-surplus causes cell wall injury,

which would justify the near absence of growth of the corresponding mutants when exposed to these conditions (Figure 4.6).

A total of 109 mutant strains were more sensitive to fluconazole than the WT, but as sensitive as this strain under Fe-loading conditions (Figure 4.8 and Table S4). Among the corresponding genes was *GAL11a*, which encodes a subunit of the Mediator complex, a multiprotein transcriptional co-activator complex. Gal11a was demonstrated to be important for Pdr1 mediated gene activation [48]. Among Pdr1 targets are the genes encoding drug efflux pumps related to azole resistance [31-33] and accordingly, the lack of *GAL11a* gene renders cells sensitive to fluconazole (Figure S1 and [49]). Contrarily to *pdr1* mutant, however, in a high Fe milieu, the mutant *gal11a* is no longer susceptible to the antifungal (Figure S1 and Figure 4.9). The different phenotypic response of *pdr1* and *gal11a* mutants led us to propose that other subunits of the mediator complex may specifically interact with Pdr1 in the absence of Gal11a, in Fe surplus conditions. Another set of genes curiously found in this group includes *RLM1*, *UVS1*, and *SLG1* (Figure S2). These three genes are involved in the cell wall integrity pathway, therefore suggesting a link between fluconazole toxicity and cell wall status.

The reduction of fluconazole toxicity after Fe supplementation was counteracted by deletion of 9 genes - *CNA1*, *CNB1*, *PDR1*, *CDR1*, *ADA2*, *ALG5*, *POP2*, *YPK1*, and *SLM1* (Figure 4.9). The *cna1* and *cnb1* mutant strains lack the genes encoding the catalytic and regulatory subunits of calcineurin, respectively. Both genes, *CNA1* and *CNB1* are essential for plasma membrane and cell wall

integrity, thermotolerance, and azole and echinocandin tolerance [29]. Moreover, deletion of *CNA1* gene leads to the repression of genes coding for ATP-binding cassette (ABC) multidrug transporters, such as Cdr1, Cdr2 and Yor1 [29]. Regarding the conditions herein tested, the importance of calcineurin in cell integrity and drug efflux could explain the sensitivity of both mutants (*cna1* and *cnb1*) to fluconazole, as well as the lack of increased tolerance to the antifungal in a high Fe milieu. The knockout strains *cdr1* and *pdr1* were also found in this group (Figure 4.9). Altogether, our results suggest that the inability to extrude fluconazole from the cell in the *pdr1* and *cdr1*, as well as possibly in *cna1* and *cnb1* mutants cannot be compensated by Fe supplementation.

We identified twelve mutants with increased fluconazole tolerance (Figure 4.10). There are many clinical isolates of *C. glabrata* and *C. albicans* strains with increased fluconazole tolerance due to gain-of-function (GOF) mutations in the transcription factors involved in the regulation of drug resistance genes [50-52]. In the case of the mutants found in this study (Table 4.6), the reason why deletion of the corresponding genes diminishes fluconazole susceptibility is not clear. However, we noticed four mitochondrial mutants (*mdm13*, *fmp18*, *emi1*, *aep3*) in this group (Table 4.6). Growing evidences suggest that mitochondrial mutants with altered protein and/or lipid structure of the mitochondrial membranes exhibit azole resistance (reviewed in [53]). In this line of thought, the increased fluconazole tolerance of the above mentioned mitochondrial mutants (Figure 4.10) could be due to their involvement in mitochondrion organization, phospholipid metabolism and protein trafficking [54-56]. Another interesting

mutant within this group is *crz1* (Figure 4.10). Crz1 is a transcription factor involved in the calcineurin signaling pathway. The lack of *CRZ1* gene was previously associated with increased fluconazole tolerance [29]. In fact, it was suggested that Crz1 possibly acts as a negative regulator, repressing genes involved in fluconazole tolerance [29], contrarily to what is observed for *C. albicans* and *Candida dubliniensis* species [57, 58].

Differently from *S. cerevisiae* and *C. albicans*, *C. glabrata* has lost the genes involved in the biosynthesis of thiamine, nicotinic acid and pyridoxine (vitamin B6) [59]. *TPN1* encodes a pyridoxine transporter responsible for the cellular uptake of vitamin B6. The loss of genes involved in the biosynthesis of vitamin B6 could explain the growth deficit of the *tpn1* mutant observed under normal physiological conditions (Figure 4.6 and Figure 4.11), as this vitamin is important for the cellular metabolism [60]. Vitamin B6 has several vitamer forms, although only pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP) can be used in catalysis [61]. Several enzymes which participate in heme biosynthesis and Fe-S cluster biogenesis in mitochondria are PLP-dependent [61]. *C. glabrata* response to Fe depletion is orchestrated by *Cg*Aft1, the orthologue of *Sc*Aft1 [25]. In *S. cerevisiae*, when Fe-S cluster biogenesis is perturbed, *Sc*Aft1 accumulates in the nucleus activating the Fe deprivation response [62]. If such mechanism is preserved in *C. glabrata*, than the absence of PLP (expected in the *tpn1* mutant) will impair Fe-S cluster biogenesis and, consequently, lead to increase Fe uptake. This mechanism could explain the inability of *tpn1* to grow in medium supplemented with 10 mM

$FeSO_4$ (Figure 4.6). Moreover, decreased cellular availability of PLP will also perturb heme biosynthesis [61]. In this context, it is tempting to speculate that decreased biosynthesis of heme hinders Erg11 synthesis, which could explain the slight sensitivity to fluconazole observed in *tpn1* mutant (Figure 4.11B). The greater uptake of Fe together with the impairment of Erg11, expected in the *tpn1*, would justify the observation that in this mutant Fe supplementation does not alleviate fluconazole toxicity (Figure 4.11).

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Chapter V

FINAL DISCUSSION AND PERSPECTIVES

Final Discussion and Perspectives

Saccharomyces cerevisiae has been used for decades as a eukaryotic model organism to study the role of metals in cellular metabolism, as well as the mechanisms underlying both metal toxicity and detoxification (reviewed in [1, 2] and Chapter I). The work presented in Chapters II and III brings to light new mechanisms of cobalt and cadmium detoxification mediated by the transcription factor Yap1. We showed that Yap1 is activated by cobalt and induces the expression of genes involved in oxidative stress response. Yap1 also upregulates the expression of the phosphate high affinity transporter, suggesting the involvement of cellular phosphates in the oxidative stress response (Figure 5.1A). Moreover, in this work, Yap1 emerges as a repressor of the expression of *FET4*, a gene encoding a low affinity iron transporter. Yap1 represses Fet4 *via* the regulation of *ROX1*, a transcription factor involved in the repression of hypoxic genes related to anaerobic adaptation (Figure 5.1 B). The repressive activity of Yap1 is particularly relevant regarding cadmium detoxification. Indeed, this work reveals a new route and first line of action of Yap1 in cadmium detoxification, which is the prevention of metal uptake through the repression of *FET4* expression. Interestingly, we observed that even when Yap1 function is abolished (as it happens in the *yap1* mutant) another cellular response against cadmium toxicity, mediated by the exoribonuclease 5'-3' Xrn1, is put at play, assuring a rapid reduction of *FET4* transcripts (Figure 5.1 B).

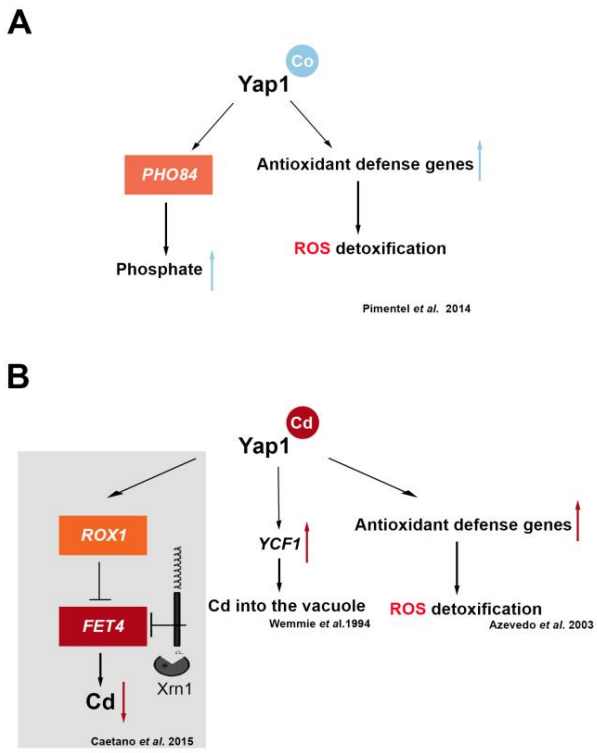

Figure 5. 1 Model for the role of Yap1 in cobalt and cadmium detoxification. (A) Under cobalt surplus conditions Yap1 activates the expression of genes coding cellular antioxidant defenses. Yap1 is also involved in the regulation of *PHO84* gene, coding for a high affinity phosphate transporter. Phosphate levels inside the cell are important to overcome the oxidative stress generated by normal aerobic growth as well as by metals. **(B)** When *S. cerevisiae* cells are exposed to cadmium, Yap1 accumulates in the nucleus leading to the induction of oxidative stress response genes [3]. It also induces the expression of the vacuolar ABC transporter *YCF1*, which is responsible for the accumulation of cadmium in the vacuole [4]. Our work also demonstrated Yap1 direct induction of *ROX1* gene expression, a known repressor of *FET4*, a low affinity iron transporter. This repression leads to decreased expression of *FET4* transporter and consequently avoiding the uptake of cadmium.

In the future it will be worth exploring the apparent specificity of Xrn1 to degrade *FET4* mRNAs upon cadmium exposure. Jones *et al.* proposed a mechanism involving 5'-3' degradation complex recruitment relaying on the binding of regulatory RNA-binding proteins (RBPs) to a specific RNA sequence

[5]. Therefore, it would be interesting to further investigate if cadmium may promote the binding of such an element to *FET4* transcripts leading to their specific degradation.

Because metals are extremely important during infection we next decided to take advantage on the acquired expertise in the field of metal biology and take the challenge to further explore our preliminary results, pointing towards a link between iron bioavailability and antifungal response. In Chapter IV we showed that iron availability interferes with the capacity of *S. cerevisiae* and *C. glabrata* to tolerate fluconazole. By performing a phenotypic screening using *C. glabrata* mutant collection [6] we were able to identify putative players in this process. Furthermore, we found several genes, which together with *CCC1*, mediate iron-loading detoxification in *C. glabrata*. The data so far acquired in Chapter IV set the ground for new lines of research. However, since the *C. glabrata* deletion library used in Chapter IV represents approximately 12% of the genome [6], it will be interesting to firstly perform RNA-seq analysis in order to have a more complete picture of the transcriptional landscape of *C. glabrata* exposed to (i) high iron and (ii) fluconazole plus iron loading. In our opinion, the *TPN1* gene, which encodes the vitamin B6 transporter, is particularly attractive, as its deletion abrogates the decreased fluconazole activity in a context of iron loading. It will be pertinent to study the mechanisms on the basis of this effect and to confirm if our hypothesis that Fe-S biogenesis and heme synthesis might be affected. A BLAST (https://blast.ncbi.nlm.nih.gov) search of the Tpn1 sequence, revealed the

absence of orthologue sequences within the Human genome. This finding together with the plasma membrane localization of Tpn1, highlight this transporter as a good candidate for drug design. Moreover, it will be interesting to construct the double mutant *tpn1ccc1* and verify if the synergism between impaired adaptation to iron-loading conditions and fluconazole converts fluconazole activity from fungistatic to fungicidal. Finally, it will also be worth exploring if iron loading is as well involved in increased tolerance to other antifungal drug classes used to treat invasive fungal infections (pyrimidine analogues, polyenes and echinocandins) and if this effect in specific to *C. glabrata* or is extended to other fungal species.

References

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Supplemental material

Table S 1 Genes dependent on Yap1 under normal growth conditions. DNA microarrays analysis was conducted by comparing RNAs isolated from the *yap1* mutant vs. wild-type (WT) strains, as previously described [20]. SAM analysis was performed using the algorithm implemented in MeV from TM4 software [21]. The array design, spotting protocol, raw data and pre-processed data from all hybridizations were submitted to the ArrayExpress Database (E-MEXP-3874). Searches for putative Yap binding sites were carried out using the Yeastract database [22]. Gene clustering was performed according to the Munich Information Center for Protein Sequences database (MIPS) functional catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB/) (Chapter II).

Sistematic name	Gene	Fold change	YRE*	Function
Oxidative stress response				
YJL101C	*GSH1*	-1.78	-914	γ-glutamylcysteine synthetase, catalyzes the first step in glutathione (GSH) biosynthesis
YGR209C	*TRX2*	-2.23	-181; -210	Cytoplasmic thioredoxin isoenzyme, is part of the thioredoxin system
YJR104C	*SOD1*	-2.29	-142	Cytosolic copper-zinc superoxide dismutase
YML028W	*TSA1*	-2.76	-180	Thioredoxin peroxidase, acts as both a ribosome-associated and free cytoplasmic antioxidant
YLR109W	*AHP1*	-3.11	-313	Thiol-specific peroxiredoxin, reduces hydroperoxides to protect against oxidative damage
YBR244W	*GPX2*	-11.43	-919; -458	Phospholipid hydroperoxide glutathione peroxidase, protects cells from phospholipid hydroperoxides and nonphospholipid peroxides
Response to stress				
YML131W	NA	-1.56	-500	Protein of unknown function
YOL052C-A	*DDR2*	-1.69	-	Multi-stress response protein
YLR346C	NA	-1.76	-338	Putative protein of unknown function found in mitochondria
YNL134C	NA	-2.14	-289; -860; -748	Protein of unknown function
YGR234W	*YHB1*	-2.21	-909; -407; -397	Nitric oxide oxidoreductase; flavohemoglobin involved in nitric oxide detoxification

Systematic name	Gene	Fold change	YRE*	Function
Response to stress				
YDR533C	*HSP31*	-2.22	-261	Possible chaperone and cysteine protease with similarity to *E. coli* Hsp31
YOL151W	*GRE2*	-3.15	-777	3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase (D-lactaldehyde dehydrogenase)
Iron homeostasis				
YMR319C	*FET4*	2.29	-	Low-affinity Fe(II) transporter of the plasma membrane
YKR052C	*MRS4*	-1.54	-403	Iron transporter that mediates Fe^{2+} transport across the inner mitochondrial membrane
YOR382W	*FIT2*	-1.60	-699; -164	Involved in the retention of siderophore-iron in the cell wall
YOR383C	*FIT3*	-1.89	-126	Involved in the retention of siderophore-iron in the cell wall
YOR226C	*ISU2*	-1.91	-438	Required for synthesis of mitochondrial and cytosolic iron-sulfur proteins
Phosphate transport				
YML123C	*PHO84*	-2.71	-252	High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter
YHR136C	*SPL2*	**	-	Protein with similarity to cyclin-dependent kinase inhibitors, down regulates low-affinity phosphate transport during phosphate limitation
Heme biosynthetic pathway				
YBR085W	*AAC3*	2.15	-306	Mitochondrial inner membrane ADP/ATP translocator, exchanges cytosolic ADP for mitochondrially synthesized ATP; expressed under anaerobic conditions
YDR044W	*HEM13*	2.47	-855	Coproporphyrinogen III oxidase, an oxygen requiring enzyme that catalyses the sixth step in the heme biosynthetic pathway
Respiration				
YJR048W	*CYC1*	-3.35	-	Cytochrome c, isoform 1

Systematic name	Gene	Fold change	YRE*	Function
Others				
YNL338W	NA	-1.58	-598; -789	Dubious open reading frame unlikely to encode a protein
YBR054W	*YRO2*	-1.65	-538; -181	Putative protein of unknown function
YGL089C	*MF(ALPHA)2*	-1.81	-323; -394	Mating pheromone alpha-factor, made by alpha cells
YPR065W	*ROX1*	-2.19	-414; -897	Heme-dependent repressor of hypoxic genes
YLR108C	NA	-3.41	-343; -169	Protein of unknown function

* Base pairs upstream of the ATG codon

**Confirmed by qRT-PCR as dependent on Yap1 under normal growth conditions (Figure 1.3)

Table S 2 Genes dependent on Yap1 under Co exposure. DNA microarrays analysis was conducted by comparing RNAs isolated from the *yap1* mutant vs. wild-type (WT) strains grown in the presence of 2 mM of $CoSO_4$ for 60 min, as previously described [20]. SAM analysis was performed using the algorithm implemented in MeV from TM4 software [21]. The array design, spotting protocol, raw data and pre-processed data from all hybridizations were submitted to the ArrayExpress Database (E-MEXP-3874). Searches for putative Yap binding sites were carried out using the YEASTRACT database [22]. Gene clustering was performed according to the Munich Information Center for Protein Sequences database (MIPS) functional catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB/) (Chapter II).

Sistematic name	Gene	Fold change	YRE*	Function
Oxidative stress				
YDR353W	*TRR1*	-1.54	-185; -341	Cytoplasmic thioredoxin reductase; key regulatory enzyme that determines the redox state of the thioredoxin system
YLR109W	*AHP1*	-1.73	-313	Thiol-specific peroxiredoxin, reduces hydroperoxides to protect against oxidative damage
YKL086W	*SRX1*	-1.80	-144; -255; -294	Sulfiredoxin, contributes to oxidative stress resistance
YJL101C	*GSH1*	-1.98	-914	γ-glutamylcysteine synthetase, catalyzes the first step in glutathione (GSH) biosynthesis
YJR104C	*SOD1*	-2.42	-142	Cytosolic copper-zinc superoxide dismutase
YGR209C	*TRX2*	-2.43	-181; -210	Cytoplasmic thioredoxin isoenzyme, is part of the thioredoxin system
YML028W	*TSA1*	-2.93	-180	Thioredoxin peroxidase, acts as both a ribosome-associated and free cytoplasmic antioxidant
YBR244W	*GPX2*	-11.91	-919; -458	Phospholipid hydroperoxide glutathione peroxidase, protects cells from phospholipid hydroperoxides and nonphospholipid peroxides

Systematic name	Gene	Fold change	YRE*	Function
Response to stress				
YAL028W	*FRT2*	1.51	-	Tail-anchored ER membrane protein of unknown function. Interacts with its homolog Frt1p
YML058W-A	*HUG1*	1.83	-315	Protein involved in the Mec1p-mediated checkpoint pathway
YLR346C	NA	-1.77	-338	Putative protein of unknown function found in mitochondria
YDR533C	*HSP31*	-1.81	-261	Possible chaperone and cysteine protease with similarity to *E. coli* Hsp31
YGR234W	*YHB1*	-2.16	-909; -407; -397	Nitric oxide oxidoreductase; flavohemoglobin involved in nitric oxide detoxification
YML131W	NA	-2.20	-500	Protein of unknown function
YNL134C	NA	-2.80	-289; -860; -748	Protein of unknown function
YOL151W	*GRE2*	-2.85	-777	3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase (D-lactaldehyde dehydrogenase)
Iron Homeostasis				
YMR319C	*FET4*	4.54	-	Low-affinity Fe(II) transporter of the plasma membrane
YOR383C	*FIT3*	-1.50	126	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall
YKR052C	*MRS4*	-1.61	403	Iron transporter that mediates Fe^{2+} transport across the inner mitochondrial membrane
YOR226C	*ISU2*	-2.19	438	Required for synthesis of mitochondrial and cytosolic iron-sulfur proteins

Systematic name	Gene	Fold change	YRE*	Function
Phosphate transport				
YHR136C	*SPL2*	-1.57	-	Protein with similarity to cyclin-dependent kinase inhibitors, down regulates low-affinity phosphate transport during phosphate limitation
YML123C	*PHO84*	-2.86	-252	High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter
Lipid metabolism				
YDR297W	*SUR2*	1.72	-230	Sphinganine C4-hydroxylase, catalyses the conversion of sphinganine to phytosphingosine in sphingolipid biosyntheis
YKL008C	*LAC1*	1.57	-	Ceramide synthase component, involved in synthesis of ceramide
YOR049C	*RSB1*	-1.63	-608	Suppressor of sphingoid long chain base (LCB)
Heme biosynthetic pathway				
YBR085W	*AAC3*	2.24	-306	Mitochondrial inner membrane ADP/ATP translocator, exchanges cytosolic ADP for mitochondrially synthesized ATP; expressed under anaerobic conditions
YDR044W	*HEM13*	2.21	-855	Coproporphyrinogen III oxidase, an oxygen requiring enzyme that catalyzes the sixth step in the heme biosynthetic pathway
Respiration				
YIL111W	*COX5B*	1.71	-949	Subunit Vb of cytochrome c oxidase
YJR048W	*CYC1*	-2.99	-	Cytochrome c, isoform 1

Sistematic name	Gene	Fold change	YRE*	Function
Others				
YHR007C	*ERG11*	1.68	-858	Lanosterol 14-α-demethylase
YEL047C	*FRD1*	1.60	-201	Soluble fumarate reductase, is required with isoenzyme Osm1p for anaerobic growth
YNL279W	*PRM1*	-1.53	-	Pheromone-regulated multispanning membrane protein involved in membrane fusion during mating
YLR460C	NA	-1.54	-817; -167; -317	Member of the quinone oxidoreductase family
YNL338W	NA	-1.55	-598; -789	Dubious open reading frame unlikely to encode a protein
YNR044W	*AGA1*	-1.58	-	Anchorage subunit of a-agglutinin of a-cells
YJL153C	*INO1*	-1.58	-	Inositol-3-phosphate synthase, involved in synthesis of inositol phosphates and inositol-containing phospholipids
YBL109W	NA	-1.66	-767	Dubious open reading frame unlikely to encode a protein
YPR065W	*ROX1*	-2.43	-414; -897	Heme-dependent repressor of hypoxic genes
YGL089C	*MF(ALPHA)2*	-2.57	-323; -394	Mating pheromone alpha-factor, made by alpha cells
YLR108C	NA	-4.48	-343; -169	Protein of unknown function

* Base pairs upstream of the ATG codon

Table S 3 Genes dependent on Yap1 whose RNA steady-state levels are altered upon cells incubation with 2 mM of $CoSO_4$ for 60min. DNA microarrays analysis was conducted by comparing RNAs isolated from the wild-type (WT) strain grown in the absence and presence of 2 mM of $CoSO_4$ for 60 min, as previously described [20]. SAM analysis was performed using the algorithm implemented in MeV from TM4 software [21]. The array design, spotting protocol, raw data and pre-processed data from all hybridizations were submitted to the ArrayExpress Database (E-MEXP-3874). Gene clustering was performed according to the Munich Information Center for Protein Sequences database (MIPS) functional catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB/) (Chapter II).

Systematic name	Gene	Fold change	Function
Cellular response to Stress			
YER142C	*MAG1*	1.5	3-methyl-adenine DNA glycosylase
YDR001C	*NTH1*	1.6	Neutral trehalase, degrades trehalose
YDR406W	*PDR15*	1.6	Plasma membrane ATP binding cassette (ABC) transporter
YGR086C	*PIL1*	1.5	Primary protein component of eisosomes
YIL113W	*SDP1*	1.5	Stress-inducible dual-specificity MAP kinase phosphatase
YMR175W	*SIP18*	1.6	Phospholipid-binding hydrophilin
YNL007C	*SIS1*	1.6	Type II HSP40 co-chaperone that interacts with the HSP70 protein Ssa1p
YER103W	*SSA4*	2.4	Heat shock protein that is highly induced upon stress
YGR008C	*STF2*	2.7	Protein involved in resistance to dessication stress
YBR126C	*TPS1*	2.1	Synthase subunit of trehalose-6-P synthase/phosphatase complex
YDR074W	*TPS2*	2.3	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex
YML100W	*TSL1*	4.0	Large subunit of trehalose 6-phosphate synthase/phosphatase complex
YKL035W	*UGP1*	1.8	UDP-glucose pyrophosphorylase (UGPase)
YPL230W	*USV1*	1.8	Putative transcription factor containing a C2H2 zinc finger
YHL035C	*VMR1*	2.1	Vacuolar membrane protein
YIL101C	*XBP1*	1.8	Transcriptional repressor

Systematic name	Gene	Fold change	Function
Cellular response to Stress			
YAL060W	*BDH1*	1.5	NAD-dependent (R,R)-butanediol dehydrogenase
YAL061W	*BDH2*	1.8	Putative medium-chain alcohol dehydrogenase with similarity to BDH1
YOR052C	*TMC1*	1.6	AN1-type zinc finger protein of unknown function
YHR048W	*YHK8*	3.0	Presumed antiporter of the major facilitator superfamily
YFR014C	*CMK1*	1.4	Calmodulin-dependent protein kinase
YNL155W	*CUZ1*	1.6	Protein with a role in the ubiquitin-proteasome pathway
Protein folding			
YLR216C	*CPR6*	1.5	Peptidyl-prolyl cis-trans isomerase (cyclophilin)
YLR259C	*HSP60*	1.6	Tetradecameric mitochondrial chaperonin
YCL043C	*PDI1*	1.5	Protein disulfide isomerase
YAL005C	*SSA1*	1.9	ATPase involved in protein folding and NLS-directed nuclear transport
YLL024C	*SSA2*	1.8	ATP-binding protein
YBR169C	*SSE2*	2.0	Member of the heat shock protein 70 (HSP70) family
YOR027W	*STI1*	1.7	Hsp90 cochaperone
Carbohydrate metabolic process			
YGL156W	*AMS1*	1.7	Vacuolar alpha mannosidase
YBR149W	*ARA1*	1.7	NADP+ dependent arabinose dehydrogenase
YPR026W	*ATH1*	1.9	Acid trehalase required for utilization of extracellular trehalose
YNR001C	*CIT1*	1.8	Citrate synthase
YML070W	*DAK1*	1.5	Dihydroxyacetone kinase
YDR516C	*EMI2*	2.0	Non-essential protein of unknown function
YCL040W	*GLK1*	3.1	Glucokinase

Systematic name	Gene	Fold change	Function
Carbohydrate metabolic process			
YDL022W	*GPD1*	1.8	NAD-dependent glycerol-3-phosphate dehydrogenase
YDL021W	*GPM2*	1.7	Homolog of Gpm1p phosphoglycerate mutase
YER062C	*GPP2*	1.6	DL-glycerol-3-phosphate phosphatase involved in glycerol biosynthesis
YHR104W	*GRE3*	1.9	Aldose reductase
YIL155C	*GUT2*	2.0	Mitochondrial glycerol-3-phosphate dehydrogenase
YFR053C	*HXK1*	4.2	Hexokinase isoenzyme 1
YDR001C	*NTH1*	1.6	Neutral trehalase, degrades trehalose
YIL107C	*PFK26*	1.4	6-phosphofructo-2-kinase
YAL017W	*PSK1*	1.5	PAS domain-containing serine/threonine protein kinase
YGL062W	*PYC1*	1.6	Pyruvate carboxylase isoform
YOR347C	*PYK2*	1.5	Pyruvate kinase
YGR248W	*SOL4*	3.5	6-phosphogluconolactonase
YBR117C	*TKL2*	1.6	Transketolase
YBR126C	*TPS1*	2.1	Synthase subunit of trehalose-6-P synthase/phosphatase complex
YDR074W	*TPS2*	2.3	Phosphatase subunit of the trehalose-6-P synthase/phosphatase complex
YML100W	*TSL1*	4.0	Large subunit of trehalose 6-phosphate synthase/phosphatase complex
YEL012W	*UBC8*	1.5	Ubiquitin-conjugating enzyme that regulates gluconeogenesis
YNL241C	*ZWF1*	1.8	Glucose-6-phosphate dehydrogenase (G6PD)
YOR178C	*GAC1*	1.6	Regulatory subunit for Glc7p type-1 protein phosphatase (PP1)
YPR184W	*GDB1*	2.4	Glycogen debranching enzyme
YER054C	*GIP2*	1.9	Putative regulatory subunit of protein phosphatase Glc7p
YEL011W	*GLC3*	2.8	Glycogen branching enzyme, involved in glycogen accumulation

Systematic name	Gene	Fold change	Function
Carbohydrate metabolic process			
YPR160W	*GPH1*	3.0	Glycogen phosphorylase required for the mobilization of glycogen
YFR015C	*GSY1*	2.3	Glycogen synthase
YLR258W	*GSY2*	2.4	Glycogen synthase
YFR017C	*IGD1*	1.7	Cytoplasmic protein that inhibits Gdb1p glycogen debranching activity
YMR105C	*PGM2*	3.8	Phosphoglucomutase
YKL035W	*UGP1*	1.8	UDP-glucose pyrophosphorylase (UGPase)
YBR056W		1.6	Putative glycoside hydrolase of the mitochondrial intermembrane space
YIR007W		1.5	Putative glycosidase
YJR096W		1.6	Putative xylose and arabinose reductase
YLR345W		1.5	Similar to 6-phosphofructo-2-kinase enzymes
Metal homeostasis			
YOR316C	*COT1*	1.6	Vacuolar transporter that mediates zinc transport into the vacuole
YOL101C	*IZH4*	2.1	Membrane protein involved in zinc ion homeostasis
YJR059W	*PTK2*	1.5	Putative serine/threonine protein kinase
YHL040C	*ARN1*	2.7	ARN family transporter for siderophore-iron chelates
YHL047C	*ARN2*	1.6	Transporter
YOL158C	*ENB1*	2.6	Endosomal ferric enterobactin transporter
YMR058W	*FET3*	3.1	Ferro-O2-oxidoreductase
YOR382W	*FIT2*	2.5	Mannoprotein that is incorporated into the cell wall
YOR383C	*FIT3*	2.1	Mannoprotein that is incorporated into the cell wall
YLR214W	*FRE1*	1.5	Ferric reductase and cupric reductase
YKL220C	*FRE2*	3.6	Ferric reductase and cupric reductase

Systematic name	Gene	Fold change	Function
Metal homeostasis			
YER145C	*FTR1*	2.8	High affinity iron permease
YLR205C	*HMX1*	1.9	ER localized heme oxygenase
YPL135W	*ISU1*	1.5	Conserved protein of the mitochondrial matrix
YOR226C	*ISU2*	1.6	Protein required for synthesis of iron-sulfur proteins
YEL065W	*SIT1*	3.0	Ferrioxamine B transporter
YLR136C	*TIS11*	3.9	mRNA-binding protein expressed during iron starvation
Lipid Metabolism			
YJL079C	*PRY1*	2.0	Sterol binding protein involved in the export of acetylated sterols
YKL150W	*MCR1*	1.6	Mitochondrial NADH-cytochrome b5 reductase
YHR190W	*ERG9*	1.7	Farnesyl-diphosphate farnesyl transferase (squalene synthase)
YOL151W	*GRE2*	1.9	3-methylbutanal reductase and NADPH-dependent methylglyoxal reductase
YKR003W	*OSH6*	1.5	Member of an oxysterol-binding protein family
YDR096W	*GIS1*	1.5	Histone demethylase and transcription factor
YGL055W	*OLE1*	2.3	Delta(9) fatty acid desaturase
Cell wall organization or biogenesis			
YKL096W	*CWP1*	1.8	Cell wall mannoprotein that localizes to birth scars of daughter cells
YDL222C	*FMP45*	2.3	Integral membrane protein localized to mitochondria
YJL159W	*HSP150*	1.5	O-mannosylated heat shock protein
YDR077W	*SED1*	1.6	Major stress-induced structural GPI-cell wall glycoprotein
YIR039C	*YPS6*	1.5	Putative GPI-anchored aspartic protease
Amino acid metabolism			
YLR438W	*CAR2*	2.4	L-ornithine transaminase (OTAse)

Systematic name	Gene	Fold change	Function
Amino acid metabolism			
YNL160W	*YGP1*	2.3	Cell wall-related secretory glycoprotein
YNR001C	*CIT1*	1.8	Citrate synthase
YOR136W	*IDH2*	1.5	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase
YMR250W	*GAD1*	2.3	Glutamate decarboxylase
YMR189W	*GCV2*	1.6	P subunit of the mitochondrial glycine decarboxylase complex
YER091C	*MET6*	1.5	Cobalamin-independent methionine synthase
YLR303W	*MET17*	1.5	O-acetyl homoserine-O-acetyl serine sulfhydrylase
YDR380W	*ARO10*	1.4	Phenylpyruvate decarboxylase
YDR019C	*GCV1*	1.8	T subunit of the mitochondrial glycine decarboxylase complex
YGR019W	*UGA1*	1.6	Gamma-aminobutyrate (GABA) transaminase
YIR038C	*GTT1*	1.8	ER associated glutathione S-transferase capable of homodimerization
YKR076W	*ECM4*	2.0	Omega class glutathione transferase
YLR299W	*ECM38*	1.5	Gamma-glutamyltranspeptidase
YJR078W	*BNA2*	1.5	Putative tryptophan 2,3-dioxygenase or indoleamine 2,3-dioxygenase
RNA catabolic process			
YLR270W	*DCS1*	1.9	Non-essential hydrolase involved in mRNA decapping
YOR173W	*DCS2*	3.4	m(7)GpppX pyrophosphatase regulator
YPL123C	*RNY1*	1.8	Vacuolar RNase of the T(2) family
Others of known function			
YBR233W-A	*DAD3*	2.3	Essential subunit of the Dam1 complex (aka DASH complex)
YDR171W	*HSP42*	2.6	Small heat shock protein (sHSP) with chaperone activity
YCL038C	*ATG22*	1.5	Vacuolar integral membrane protein required for efflux of amino acids

Systematic name	Gene	Fold change	Function
Others of known function			
YPL017C	*IRC15*	1.6	Microtubule associated protein
YHL024W	*RIM4*	1.9	Putative RNA-binding protein
YML128C	*MSC1*	3.3	Protein of unknown function
YPL154C	*PEP4*	1.8	Vacuolar aspartyl protease (proteinase A)
YMR169C	*ALD3*	3.8	Cytoplasmic aldehyde dehydrogenase
YGR070W	*ROM1*	1.5	GDP/GTP exchange protein (GEP) for Rho1p
YOR374W	*ALD4*	2.5	Mitochondrial aldehyde dehydrogenase
YNL274C	*GOR1*	2.2	Glyoxylate reductase
YMR110C	*HFD1*	1.6	Hexadecenal dehydrogenase
YDL130W-A	*STF1*	2.1	Protein involved in regulation of the mitochondrial F1F0-ATP synthase
YDL181W	*INH1*	1.5	Protein that inhibits ATP hydrolysis by the F1F0-ATP synthase
YER037W	*PHM8*	1.9	Lysophosphatidic acid (LPA) phosphatase, nucleotidase
YGR244C	*LSC2*	1.5	Beta subunit of succinyl-CoA ligase
YEL039C	*CYC7*	2.1	Cytochrome c isoform 2, expressed under hypoxic conditions
YGL037C	*PNC1*	2.4	Nicotinamidase that converts nicotinamide to nicotinic acid
YKL103C	*APE1*	2.6	Vacuolar aminopeptidase yscI
YOR227W	*HER1*	1.5	Protein of unknown function
YMR251W-A	*HOR7*	1.8	Protein of unknown function
YMR174C	*PAI3*	1.8	Cytoplasmic proteinase A (Pep4p) inhibitor
YCL057W	*PRD1*	1.6	Zinc metalloendopeptidase
YHR087W	*RTC3*	3.8	Protein of unknown function involved in RNA metabolism
YDL204W	*RTN2*	3.3	Reticulon protein

Systematic name	Gene	Fold change	Function
Others of known function			
YBR212W	*NGR1*	1.4	RNA binding protein that negatively regulates growth rate
YNL055C	*POR1*	1.7	Mitochondrial porin (voltage-dependent anion channel)
YHL030W	*ECM29*	1.5	Scaffold protein
YDR379C-A	*SDH6*	1.8	Protein involved in assembly of the succinate dehydrogenase complex
YBR132C	*AGP2*	1.6	Plasma membrane regulator of polyamine and carnitine transport
YOL082W	*ATG19*	1.9	Receptor protein for the cytoplasm-to-vacuole targeting (Cvt) pathway
YOL083W	*ATG34*	1.9	Receptor protein involved in selective autophagy during starvation
YGL121C	*GPG1*	2.2	Proposed gamma subunit of the heterotrimeric G protein
YLR178C	*TFS1*	3.1	Protein that interacts with and inhibits carboxypeptidase Y and Ira2p
YJL164C	*TPK1*	2.1	cAMP-dependent protein kinase catalytic subunit
YMR304W	*UBP15*	1.5	Ubiquitin-specific protease involved in protein deubiquitination
YLR080W	*EMP46*	1.6	Integral membrane component of ER-derived COPII-coated vesicles
YOR298C-A	*MBF1*	1.5	Transcriptional coactivator
YKR046C	*PET10*	1.5	Protein of unknown function that co-purifies with lipid particles
YBR214W	*SDS24*	2.0	Protein involved in cell separation during budding
YHR016C	*YSC84*	1.7	Actin-binding protein
YEL060C	*PRB1*	2.4	Vacuolar proteinase B (yscB)
YNL015W	*PBI2*	1.6	Cytosolic inhibitor of vacuolar proteinase B (PRB1)
YNL279W	*PRM1*	1.6	Pheromone-regulated multispanning membrane protein
YDR533C	*HSP31*	2.3	Methylglyoxalase that converts methylglyoxal to D-lactate
YBR139W		1.6	Putative serine type carboxypeptidase
YMR315W		2.7	Protein with NADP(H) oxidoreductase activity

Systematic name	Gene	Fold change	Function
Others of known function			
YNL200C		1.8	NADHX epimerase
YCR061W		1.7	Protein of unknown function
YGL039W		1.5	Oxidoreductase shown to reduce carbonyl compounds to chiral alcohols
YJL068C		1.5	Esterase that can function as an S-formylglutathione hydrolase
Unkwon function			
YBR052C	*RFS1*	1.6	Protein of unknown function
YDR032C	*PST2*	1.6	Protein with similarity to a family of flavodoxin-like proteins
YHL021C	*AIM17*	2.4	Putative protein of unknown function
YER067W	*RGI1*	2.3	Protein of unknown function
YLR251W	*SYM1*	1.5	Protein required for ethanol metabolism
YKL047W	*ANR2*	1.5	Putative protein of unknown function
YGR146C	*ECL1*	1.6	Protein of unknown function
YDR070C	*FMP16*	3.1	Protein of unknown function
YBR047W	*FMP23*	1.8	Putative protein of unknown function
YJL161W	*FMP33*	2.2	Putative protein of unknown function
YPL222W	*FMP40*	1.5	Putative protein of unknown function
YPL223C	*GRE1*	1.8	Hydrophilin essential in dessication-rehydration process
YPL054W	*LEE1*	1.5	Zinc-finger protein of unknown function
YKL142W	*MRP8*	1.7	Protein of unknown function
YBR230C	*OM14*	1.6	Integral mitochondrial outer membrane protein
YIL136W	*OM45*	2.4	Mitochondrial outer membrane protein of unknown function
YOL084W	*PHM7*	1.5	Protein of unknown function

Systematic name	Gene	Fold change	Function
Unkwon function			
YOR285W	*RDL1*	1.6	Protein of unknown function containing a rhodanese-like domain
YLR327C	*TMA10*	5.5	Protein of unknown function that associates with ribosomes
YDL110C	*TMA17*	1.8	Protein of unknown function that associates with ribosomes
YPL186C	*UIP4*	1.5	Protein that interacts with Ulp1p
YBR054W	*YRO2*	1.5	Protein of unknown function with similarity to archaeal rhodopsins
YJL116C	*NCA3*	1.6	Protein involved in mitochondrion organization
YBR053C		1.7	Putative protein of unknown function
YBR116C		1.6	Dubious open reading frame
YBR137W		1.8	Protein of unknown function
YBR285W		1.5	Putative protein of unknown function
YCL042W		3.8	Putative protein of unknown function
YDL159W-A		2.2	Putative protein of unknown function
YDR476C		1.5	Putative protein of unknown function
YEL073C		1.5	Putative protein of unknown function
YER079W		1.5	Putative protein of unknown function
YER158C		1.5	Protein of unknown function
YFL042C		1.5	Putative protein of unknown function
YGR130C		1.5	Component of the eisosome with unknown function
YHR097C		1.5	Putative protein of unknown function
YIL055C		1.6	Putative protein of unknown function
YJL163C		1.8	Putative protein of unknown function
YJR079W		1.6	Putative protein of unknown function

Systematic name	Gene	Fold change	Function
Unkwon function			
YKL091C		1.5	Putative phosphatidylinositol/phosphatidylcholine transfer protein
YKR011C		1.8	Protein of unknown function
YLR108C		1.7	Protein of unknown function
YLR149C		2.0	Protein of unknown function
YLR252W		1.9	Dubious open reading frame
YML131W		1.7	Protein of unknown function
YMR090W		1.9	Putative protein of unknown function
YMR181C		1.5	Protein of unknown function
YMR196W		2.3	Putative protein of unknown function
YNL134C		2.3	Protein of unknown function
YOL153C		2.1	Hypothetical protein
YOR289W		1.8	Putative protein of unknown function
YFL054C		1.8	Putative channel-like protein
YHR138C		1.7	Protein of unknown function

Table S 4 List of genes whose deletion lead to increased fluconazole sensitivity which become tolerant with iron supplementation (Chapter IV).

Systematic name	Gene	Function
METABOLISM		
Amino acid metabolism		
CAGL0B02651g	*MET32*	Zinc-finger DNA-binding transcriptional activator; involved in transcriptional regulation of the methionine biosynthetic genes*
CAGL0F03025g	*ARO80*	Zinc-finger transcriptional activator of the Zn2Cys6 family; activates transcription of aromatic amino acid catabolic genes in the presence of aromatic amino acids*
CAGL0L09691g	*PUT3*	Transcriptional activator that binds to specific gene recruitment sequences and regulates proline utilization genes, constitutively binds *PUT1* and *PUT2* promoter regions*
Nitrogen, sulfur and selenium metabolism		
CAGL0H07557g	*FZF1*	Transcription factor involved in sulfite metabolism*
CAGL0K07634g	*GAT1*	Transcriptional activator of nitrogen catabolite repression genes
Nucleotide/nucleoside/nucleobase metabolism		
CAGL0D02904g	*PPR1*	Zinc-finger transcriptional activator factor; involved in *de novo* pyrimidine biosynthesis, in response to pyrimidine starvation*
C-compound and carbohydrate metabolism		
CAGL0L06842g	*THI3*	Transcription factor binding activity and role in positive regulation of thiamine biosynthetic process
CAGL0F09229g	*TOG1*	Transcriptional activator of oleate genes; regulates genes involved in fatty acid utilization*
CAGL0A01628g	*MIG1*	Transcriptional regulatory protein involved in glucose repression
CAGL0A03696g	*GAL83*	Protein with AMP-activated protein kinase activity, receptor signaling complex scaffold activity
CAGL0C02519g	*MIG3*	Transcriptional regulator with major role in catabolite repression and ethanol response; involved in response to toxic agents*

Systematic name	Gene	Function
C-compound and carbohydrate metabolism		
CAGL0E04884g	*ADR1*	Carbon source-responsive zinc-finger transcription factor; required for transcription of the glucose-repressed gene *ADH2*, peroxisomal protein genes, and genes required for ethanol, glycerol, and fatty acid utilization*
CAGL0E06116g	*RGM1*	Putative zinc-finger transcription factor; overproduction impairs cell growth and induces expression of genes involved in monosaccharide catabolism and aldehyde metabolism*
CAGL0M00594g	*ZMS1*	Zinc-finger protein involved in the transcriptional control of both nuclear and mitochondrial genes, many of which specify products required for glycerol-based growth, respiration, and other functions*
Lipid, fatty acid and isoprenoid metabolism		
CAGL0L09383g	*SUT2*	Zn2Cys6 family transcription factor; positively regulates sterol uptake under anaerobic conditions*
ENERGY		
Regulation of glycolysis and gluconeogenesis		
CAGL0E05566g	*TYE7*	Serine-rich protein that binds E-boxes of glycolytic genes and contributes to their activation*
CAGL0F07315g	*SNF4*	Protein part of the activating γ-subunit of the AMP-activated Snf1 kinase complex; involved in the activation of glucose-repressed genes, represses glucose-induced genes*
CAGL0G05379g	*GCR2*	Transcriptional activator of genes involved in glycolysis*
CAGL0K12078g	*NRG1*	Transcriptional repressor; recruits the Cyc8-Tup1 complex to promoters and mediates glucose repression*
CELL CYCLE AND DNA PROCESSING		
DNA processing		
CAGL0J04356g	*APN2*	Class II abasic (AP) endonuclease involved in repair of DNA damage*

Systematic name	Gene	Function
DNA processing		
CAGL0A03322g	*HOS2*	Histone deacetylase and subunit of Set3 and Rpd3L complexes; required for gene activation *via* specific deacetylation of lysines in H3 and H4 histone tails*
CAGL0B03575g	*HAT2*	Subunit of the Hat1-Hat2 histone acetyltransferase complex; required for high affinity binding of the complex to free histone H4*
CAGL0B01441g	*RPD3*	Histone deacetylase, component of both the Rpd3S and Rpd3L complexes; regulates transcription, silencing, autophagy and other processes by influencing chromatin remodeling*
CAGL0C00297g	*EZL1*	Histone methyltransferase with a role in transcriptional elongation*
CAGL0D02926g	*BRE2*	Subunit of COMPASS complex, which is involved in silencing at telomeres*
CAGL0E02805g	*HIR1*	Subunit of the HIR complex; HIR is a nucleosome assembly complex involved in regulation of histone gene transcription*
CAGL0J06974g	*HOS3*	Trichostatin A-insensitive homodimeric histone deacetylase (HDAC)*
CAGL0L08162g	*SNT1*	Subunit of the Set3C deacetylase complex*
CAGL0L02123g	*SNF5*	Subunit of the SWI/SNF chromatin remodeling complex and is involved in transcriptional regulation*
CAGL0L08668g	*HST2*	Cytoplasmic NAD^+-dependent protein deacetylase whose targets are primarily cytoplasmic proteins*
CAGL0L11770g	*CHD1*	Chromatin remodeler that regulates various aspects of transcription*
Cell cycle		
CAGL0B00462g	*KAR4*	Transcription factor required for response to pheromones, also required during meiosis*
CAGL0D01012g	*MBP1*	Transcription factor; involved in regulation of cell cycle progression from G1 to S phase*
CAGL0G08646g	*POG1*	Nuclear chromatin-associated protein with possible role in cell cycle regulation*

Systematic name	Gene	Function
Cell cycle		
CAGL0G08866g	*FKH1*	Forkhead family transcription factor; rate-limiting replication origin activator; involved in the expression of G2/M phase genes*
CAGL0G07249g	*YHP1*	Homeobox transcriptional repressor; binds to Mcm1 and to early cell cycle boxes (ECBs) in the promoters of cell cycle-regulated genes expressed in M/G1 phase*
CAGL0J10120g	*FKH2*	Forkhead family transcription factor positively regulates transcriptional elongation and has major role in expression of G2/M phase genes*
CAGL0K04257g	*RME1*	Zinc finger protein involved in control of meiosis*
Cell cycle		
CAGL0L13090g	*NDT80*	Meiosis-specific transcription factor*
TRANSCRIPTION		
RNA synthesis		
CAGL0H06215g	*GAL1a*	Component of the transcriptional Mediator complex that provides interfaces between RNA polymerase II and upstream activator proteins; one of the two Gal11 homologs of *S. cerevisiae* Gal11 present in *C. glabrata*
CAGL0F00803g	*GAL11b*	Component of the transcriptional Mediator complex that provides interfaces between RNA polymerase II and upstream activator proteins; one of the two Gal11 homologs of *S. cerevisiae* Gal11 present in *C. glabrata*
CAGL0H02959g	*TOS8*	Homeodomain-containing protein and putative transcription factor which has a role in filamentous growth and nuclear chromatin localization
CAGL0H06677g	*IMP2*	Transcriptional activator involved in maintenance of ion homeostasis*
CAGL0H07711g	*RTF1*	Regulates gene expression by directing co-transcriptional histone modification and influences transcription and chromatin structure*

Systematic name	Gene	Function
RNA synthesis		
CAGL0B01188g	*TOS4*	Putative transcription factor, contains Forkhead Associated domain and binds chromatin*
CAGL0B03421g	*HAP1*	Zinc-finger transcription factor involved in the complex regulation of gene expression in response to levels of heme and oxygen; localizes to the mitochondrion as well as to the nucleus*
CAGL0E05434g	*TEA1*	Ty1 enhancer activator involved in Ty enhancer-mediated transcription*
CAGL0G08107g	*RPH1*	Histone demethylase and transcription factor*
CAGL0H06347g	*DEP1*	Role in histone deacetylation and negative regulation of chromatin silencing at rDNA
CAGL0H04367g	*WAR1*	Homodimeric Zn2Cys6 zinc finger transcription factor*
CAGL0K04697g	*SPT4*	Predicted transcription factor; relative distribution to the nucleus increases upon DNA replication stress*
CAGL0K06985g	*ERT1*	Transcriptional regulator; involved in regulation of gluconeogenesis and fermentable carbon utilization*
CAGL0K08756g	*AP5*	Basic leucine zipper (bZIP) iron-sensing transcription factor*
CAGL0K06413g	*STP1*	Transcription factor involved in the transcription activation of amino acid permease genes and may have a role in tRNA processing*
CAGL0L12980g	*SET1*	Histone methyltransferase, subunit of the COMPASS complex; required in transcriptional silencing near telomeres and at silent mating type loci*
CAGL0L03674g	*GSM1*	Putative zinc cluster protein of unknown function; proposed to be involved in the regulation of energy metabolism*
CAGL0M04983g	*MBF1*	Transcriptional co-activator and bridges the DNA-binding region of Gcn4p and TATA-binding protein Spt15p*
CAGL0M00286g	*HIR3*	Subunit of the HIR complex, a nucleosome assembly complex involved in regulation of histone gene transcription*

Systematic name	Gene	Function
RNA synthesis		
CAGL0I00968g	*HIR2*	Subunit of HIR nucleosome assembly complex and involved in the regulation of histone gene transcription*
CAGL0M01540g	*SPT3*	Subunit of the SAGA and SAGA-like transcriptional regulatory complexes*
CAGL0G03421g	*FLO8*	Transcription factor required for flocculation*
CAGL0H07843g	*HAP2*	Transcriptional activator activity
CAGL0K08624g	*HAP4*	Transcriptional activator activity
CAGL0K09900g	*HAP5*	Transcription factor activity
CAGL0A04235g	*SAS3*	Histone acetyltransferase catalytic subunit of NuA3 complex; acetylates histone H3 and is involved in transcriptional silencing*
CAGL0G02739g	*XBP1*	Transcriptional repressor*
CAGL0B04895g	*RFX1*	Major transcriptional repressor of DNA-damage-regulated genes*
CAGL0K03003g	*MOT3*	Transcriptional repressor, activator*
CAGL0H02145g		Ortholog(s) have sequence-specific DNA binding, transcription factor activity, sequence-specific DNA binding activity
CAGL0G09757g		Has domain(s) with predicted DNA binding, RNA polymerase II transcription factor activity, sequence-specific DNA binding, zinc ion binding activity and role in regulation of transcription
CAGL0F02519g		Has domain(s) with predicted DNA binding, RNA polymerase II transcription factor activity, sequence-specific DNA binding, zinc ion binding activity and role in regulation of transcription
PROTEIN FATE		
Protein modification		
CAGL0F02783g	*SIZ1*	SUMO E3 ligase that promotes attachment of small ubiquitin-related modifier sumo (Smt3) to primarily cytoplasmic proteins*

Systematic name	Gene	Function
Protein modification		
CAGL0J10296g	*APJ1*	Chaperone with a role in SUMO-mediated protein degradation*
CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES		
Vesicular transport		
CAGL0I08855g	*KRE11*	Component of transport protein particle (TRAPP) complex II; TRAPPII regulates intra-Golgi and endosome-Golgi traffic; role in cell wall β-glucan biosynthesis and the stress response*
CAGL0L02299g	*VPS1*	Dynamin-like GTPase required for vacuolar sorting and also involved in actin cytoskeleton organization, endocytosis, late Golgi-retention of some proteins, regulation of peroxisome biogenesis*
Cellular signaling		
CAGL0K09944g	*PDE2*	High-affinity cyclic AMP phosphodiesterase; component of the cAMP-dependent protein kinase signaling system, protects the cell from extracellular cAMP*
CELL RESCUE, DEFENSE AND VIRULENCE		
Stress response		
CAGL0L09339g	*HAA1*	Putative transcription factor, involved in regulation of response to acetic acid stress
CAGL0F07909g	*HAL9*	Protein of unknown function and is detected in highly purified mitochondria in high-throughput studies*
CAGL0G03179g	*ASK10*	Regulator activity of a glycerol channel and a role in cellular protein catabolic process, cellular response to oxidative stress, positive regulation of glycerol transport
CAGL0L04400g	*YRR1*	Zn2-Cys6 zinc-finger transcription factor and activates genes involved in multidrug resistance*
CAGL0L06028g	*GTS1*	Protein involved in transcription regulation; possibly involved in cell size, heat tolerance*
CAGL0L07436g	*PHO2*	Homeobox transcription factor; regulatory targets include genes involved in phosphate metabolism*

Systematic name	Gene	Function
Protein modification		
CAGL0L04576g	*YRM1*	Zinc finger transcription factor involved in multidrug resistance*
CAGL0M13189g	*MSN4*	Putative transcription factor similar to S. cerevisiae Msn4p; involved in response to oxidative stress
INTERACTION WITH THE ENVIRNOMENT		
Homeostais of cations		
CAGL0A04455g	*SEF1*	Putative RNA polymerase II transcription factor, involved in regulation of iron acquisition genes; required for growth under iron depletion
CAGL0I04180g	*AMT1*	Metal-activated transcription factor; binds promoters of metallothionein genes; autoregulates its own expression; gene is downregulated in azole-resistant strain
CAGL0J05060g	*ZAP1*	Zinc-regulated transcription factor; binds to zinc-responsive promoters to induce transcription of certain genes in presence of zinc, represses other genes in low zinc*
CAGL0M03597g	*MID1*	Putative calcium transporter; putative regulatory subunit of a plasma membrane gated channel involved in Ca2+ uptake; required for viability upon prolonged fluconazole stress
Cellular sensing and response to external stimulus		
CAGL0G09020g	*TPK2*	cAMP-dependent protein kinase catalytic subunit; promotes vegetative growth in response to nutrients*
CAGL0I05236g	*BCY1*	cAMP dependent protein kinase, regulatory subunit
Chemoperception and response		
CAGL0D02486g	*RDS1*	Putative zinc cluster transcription factor; involved in conferring resistance to cycloheximide*
BIOGENESIS OF CELLULAR COMPONENTS		
Cell wall		
CAGL0F01507g	*SLG1*	Putative sensor of stress-activated signaling; gene is downregulated in azole-resistant strain

Systematic name	Gene	Function
Cell wall		
CAGL0L00583g	*UVS1*	Putative transcription factor; mutation affects transcriptional regulation of genes involved in growth on non-fermentable carbon sources, response to salt stress and cell wall biosynthesis*
CAGL0H05621g	*RLM1*	Putative transcription factor with a predicted role in cell wall integrity
Organization of chromosome structure		
CAGL0F08283g	*GCN5*	H3 histone acetyltransferase activity, lysine-acetylated histone binding activity
CAGL0G00704g	*SWD1*	Subunit of the COMPASS complex; COMPASS methylates histone H3 and is required in telomeric transcriptional silencing*
CAGL0M02585g	*SPP1*	Subunit of COMPASS complex which methylates histone H3 and is required in telomeric transcriptional silencing*
CELL TYPE DIFFERENTIATION		
Fungal cell type differentiation		
CAGL0F04081g	*TEC1*	Transcription factor targeting filamentation genes and Ty1 expression*
CAGL0H03575g	*SPO1*	Meiosis-specific pro-spore protein; required for meiotic spindle pole body duplication and separation*
CAGL0L12782g	*DIG1*	MAP kinase-responsive inhibitor of the Ste12 transcription factor; involved in the regulation of mating-specific genes and the invasive growth pathway*
CAGL0L07480g	*NRG2*	Transcriptional repressor; mediates glucose repression and negatively regulates filamentous growth*
CAGL0M01254g	*STE12*	Putative transcription factor, required for filamentous growth induced by nitrogen starvation and for virulence; functionally complements S. cerevisiae ste12 mutant
UNKNOWN FUNCTION/ UNCLASSIFIED PROTEINS		
CAGL0J01595g	*GLM6*	Gene used for molecular typing of *C. glabrata* strain isolates

Systematic name	Gene	Function
UNKNOWN FUNCTION/ UNCLASSIFIED PROTEINS		
CAGL0K02343g		Protein of unknown function
CAGL0A00583g		Protein of unknown function
CAGL0A01892g		Protein of unknown function
CAGL0K04631g		Protein of unknown function
CAGL0M01870g		Protein of unknown function

* Description of the function of the *S. cerevisiae* orthologue (*Saccharomyces cerevisiae* database).

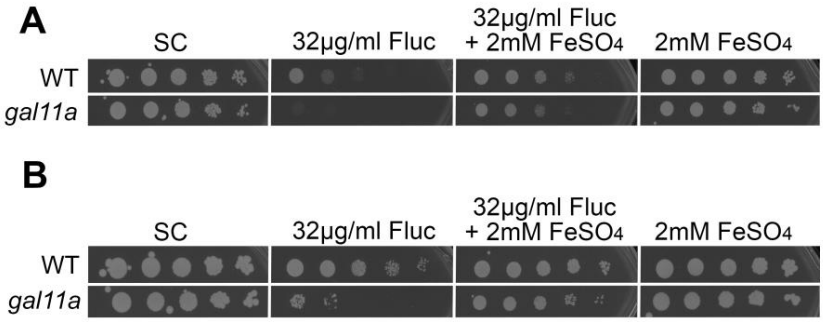

Figure S 1 *C. glabrata* mutant strains of the mediator complex with increased fluconazole sensitivity that become tolerant to the antifungal in a high iron milieu. Growth sensitivity exhibited by WT and *gal11a* strains in SC plates with no drug or with 32 µg/ml of Fluconazole (Fluc), 2 mM of $FeSO_4$, and both simultaneously. Growth was recorded after (A) 24 h and (B) 48 h at 37ºC.

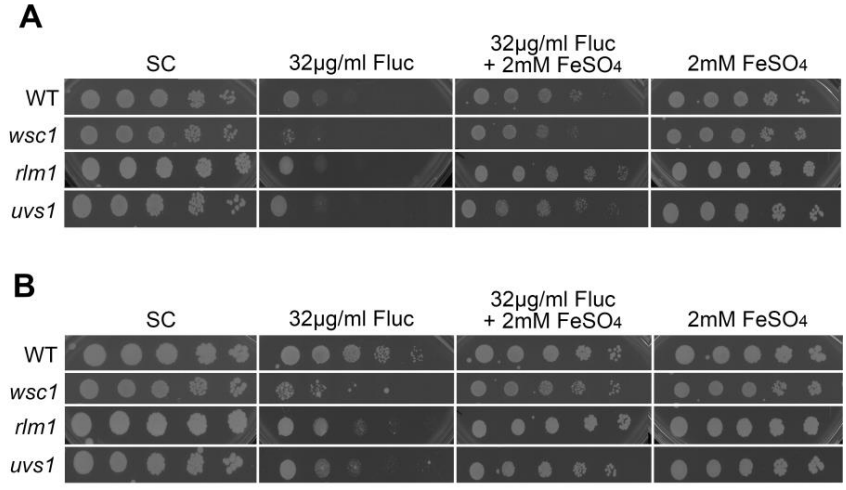

Figure S 2 *C. glabrata* mutant strains involved in cell wall integrity with increased fluconazole sensitivity which become tolerant to the antifungal in a high iron milieu. Growth sensitivity exhibited by WT and the indicated mutant strains in SC plates with no drug or with 32 µg/ml of Fluconazole (Fluc), 2 mM of $FeSO_4$, and both simultaneously.

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