Yeast as a model system to study genetic and post-translational regulation of metabolic pathways in mammals

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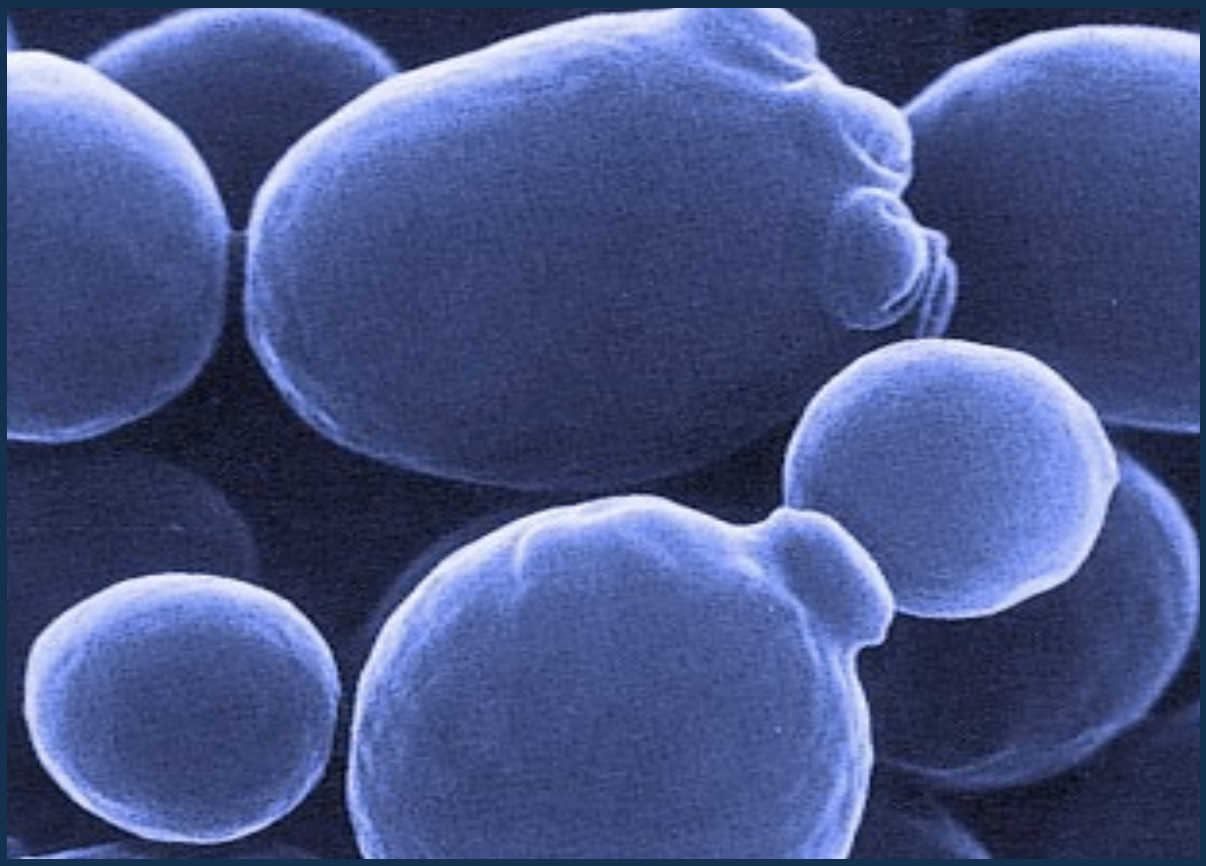
2. **Human Heat Shock Transcription Factor 1 regulation via post-translational modification in Yeast.**

Liliana S. Batista Nascimento



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
June, 2012

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Knowledge Creation

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Esta tese é dedicada à memória
do meu querido avô João

This thesis is dedicated to
the memory of my loved grandfather João

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List of Publications

Scientific articles in international peer reviewed journals

- Ferreira RT, Silva ARC, Pimentel C, **Batista-Nascimento L**, Rodrigues-Pousada C, Menezes, RA, 2012 Arsenic stress elicits cytosolic Ca^{2+}-burst and Crz1 activation in *Saccharomyces cerevisiae*. MICROBIOLOGY (*in press*).

- **Batista-Nascimento L**, Pimentel C, Menezes R, Rodrigues-Pousada C, 2012 Iron and Neurodegeneration: from cellular homeostasis to disease. Oxidative Medicine and Cellular Longevity Journal.

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Foreword

This dissertation represents the research developed during my graduation course.

Initially I spent some time in the laboratory of Professor Dennis J. Thiele, Department of Pharmacology and Cancer Biology conducting studies related to the importance of key repressive phosphorylation events in the regulation of human Heat Shock Transcription Factor 1 (HSF1). The results were published in PLos ONE in a paper that I co-authored and are described in Chapter 2.

At the laboratory of Professor Claudina Rodrigues-Pousada I have carried out an extensive transcriptomic analysis to address the response of *Saccharomyces cerevisiae* to arsenic stress. This work shows for the first time that arsenic, a toxic metalloid also used as a chemotherapy drug used to treat a specific type of acute promyelocytic leukemia (APL), disrupts the cellular iron homeostasis. This disruption also seems to take place in mammalian cells, which if confirmed could be a critical discovery for clinical application. This study is described on Chapter 1 and will soon be published.

Although these projects seem to be unrelated, both represent two different ways of studying stress response, one through the effects of cellular arsenic toxicity and the other through the understanding of the mechanism of human HSF1 regulation, the major stress-activated transcription factor. Moreover in both studies we employed the best understood eukaryotic organism, *Saccharomyces cerevisiae*, which in the last decade has been used as a model system to gain insights about the

mechanisms occurring in many human diseases, in particular, neurodegenerative disorders. This subject is addressed in more detail in two reviews that I co-author, presented in the appendix section of this dissertation.

Abbreviations

ALS - Amyotrophic Lateral Sclerosis

AP-1 – Activator Protein 1

APL – Acute Promyelocytic Leukemia

AQP- aquaglyceroporins

As – Arsenic

ATO – Arsenic Trioxide

BCA - Bicinchoninic Acid

BPS - Bathophenanthrolinedisulfonate

CBP - CREB-binding protein

CCS – Copper Chaperone SOD1

CREB - cAMP response element-binding

DABCO - 1,4-diazabicyclo[2.2.2]octane

DAPI - 4',6-diamidino-2-phenylindole

DBD – DNA Binding Domain

DFX - Desferroxamine

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl Sulfoxide

DNA – deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic Acid

EGS - Ethylene Glycol Succinimidyl succinate

ER – Endoplasmic Reticulum

ERK - Extracellular signal-regulated kinases

FAC - Ferric Ammonium Citrate

FBS - Fetal Bovine Serum

Fe - Iron

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GEO - Gene Expression Omnibus

GFP - Green Fluorescent Protein

GSK3 - Glycogen synthase kinase 3

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIS - Histidine

HOG1 – High Osmolarity Glycerol

HS – Heat Shock

HSE – Heat Shock Element

HSF – Heat Shock Factor

HSP – Heat Shock Protein

HSR – Heat Shock Response

ICP - inductively coupled plasma

IRE – Iron Responsive Element

Abbreviations

IRP – Ireon Responsive Protein

JNK - c-Jun N-terminal kinase

MAP - Mitogen-activated Protein

MAPK - Mitogen-activated Protein Kinase

MEF- Mouse Embrionic Fibroblast

MRP – Multi Drug-resistance Pump

NAD - Nicotinamide adenine dinucleotide

NCBI - National Center for Biotechnology Information

ONPG - Ortho-Nitrophenyl-β-Galactoside

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDSM - Phosphorylation-Dependent Sumoylation Modifications

PKC - Protein kinase C

PM – Plasma Membrane

RD – Regulatory Domain

RNA - ribonucleic acid

ROS – Redox Oxygen Species

RT- PCR – Real Time Polymerase Chain Reaction

SDS - Sodium Dodecyl Sulfate

SDS – PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

STRE- Stress Response Element

SUMO - Small Ubiquitin-like Modifier

TCA – Tricarboxylic Acid Cycle

UTR – Untranslated Region

WHO – World Health Organization

WT – Wild-Type

Yap – Yeast Activator Protein

YPD – Yeast Peptone Dextrose

YRE – Yap Response Element

Abstract

The work presented in this thesis describes the use of yeast *Saccharomyces cerevisiae* as a model system to study two different stress response processes and its extrapolation to higher eukaryotes.

In the Chapter 1 of this work we report the transcriptomic analysis of *S. cerevisiae* response to arsenic, a highly toxic and carcinogenic metalloid. This study shows that arsenic stress interferes with genes involved in iron homeostasis including those encoding proteins that function in iron uptake, incorporation into heme and Fe–S (iron–sulfur) clusters, compartmentalization to and mobilization from intracellular stores and other functions in iron balance. In *S. cerevisiae* iron metabolism is balanced by two distinct iron transport systems, depending on the bioavailability of the metal in the extracellular environment. Interestingly mRNA levels encoding Fet3 and Ftr1, a complex required for high-affinity iron uptake, are drastically decreased upon arsenic exposure. Moreover the *FET3* mRNA decrease is mediated by the major pathway for mRNA decay, the 5'-3' exonuclease Xrn1 and is independent of the formation of ROS. Strikingly, upon addition of arsenic Fet3 does not localize in the plasma membrane but rather resides in the endoplasmic reticulum. Also, the *fet3ftr1* mutant shows increased arsenic resistance over the wild-type, suggesting that Fet3 plays a role in arsenic toxicity. Unexpectedly, arsenic treatment seems to activate the non-reductive iron uptake system. Finally, we provide data suggesting that arsenic-mediated disruption of iron homeostasis also occurs in mammalian cells, an observation that can be relevant for clinical applications.

In the Chapter 2 of this work we report on the repression mechanisms for human Heat Shock Factor 1 (hHSF1) activity when expressed in *S. Cerevisiae*. The heat shock transcription factor plays an important role in the cellular response to proteotoxic stressors. Under normal conditions, HSF1 is maintained as an inactive monomer and lacks the ability to activate gene expression. In response to stress, HSF1 homotrimerizes, accumulates in the nucleus, becomes hyper-phosphorylated, binds DNA and activates gene transcription. Upon passage of the stress, HSF1 becomes hypo-phosphorylated and relocalizes to the cytoplasm becoming re-established as an inactive monomer. While significant advances have been made to understand the mechanisms involved in the HSF1 activation, relatively little is known about the mechanisms underlying repression. HSF1 is a highly conserved protein amongst eukaryotes and previous data have shown that hHSF1 is unable to complement for the loss of the essential yeast HSF, as hHSF1 is maintained in an inactive monomer in yeast. Interestingly, many of the proteins linked to the repression of HSF1 function, including the protein chaperones HSP90 and HSP70, as well as the kinases JNK, ERK and GSK3, are conserved in yeast. We hypothesized that gaining an understanding of the mechanisms by which hHSF1 is maintained in an inactive state in yeast would further our comprehension of HSF1 regulation in metazoans. To explore these mechanisms we used a variety of biochemical and genetic approaches to identify potential inhibitory phosphorylation sites as well as a screen of mutant yeast strains to identify potential hHSF1 repressor proteins. Here we show that when hHSF1 is expressed in yeast it is phosphorylated at S303 by the MAP-kinase Slt2 and that this phosphorylation occurs independent of a priming event previously

believed to be essential for this phosphorylation. In addition, while previous studies have suggested that S303 phosphorylation repressed HSF1-dependent transactivation, we show in both yeast and mammalian cells that S303 phosphorylation also represses HSF1 multimerization. Together, our data support the hypothesis that the yeast can be used to simplify and elucidate complex regulatory mechanisms modulating mammalian HSF1 activity as well as identify novel regulatory aspects controlling HSF1 activity in yeast and mammalian cells.

Taken together, this work suggests that yeast cells can be a powerful experimental tool for investigating aspects as arsenic toxicity and human HSF1 regulation by providing a platform for the understanding of these mechanisms in higher eukaryotes.

Resumo

O trabalho apresentado nesta tese descreve a utilização da levedura *Saccharomyces cerevisiae* como um sistema modelo para estudar dois processos diferentes da resposta ao stress e a sua extrapolação para os eucariotas superiores.

No Capítulo 1 deste trabalho, apresentamos a análise transcriptómica da resposta *S. cerevisiae* ao arsénio, um metalóide altamente tóxico e cancerígeno. Este estudo demonstra que o stress pelo arsénio interfere com os genes envolvidos na homeostase do ferro, em particular, os genes que codificam as proteínas que funcionam na absorção de ferro, incorporação nos clusters de heme Fe-S (ferro-enxofre), na compartimentalização e mobilização das reservas intracelulares entre outras funções. Em *S. cerevisiae* o equilibrio do metabolismo do ferro ocorre atraves de dois sistemas distintos de transporte do ferro, dependendo da biodisponibilidade deste metal no ambiente extracelular. Curiosamente os níveis de mRNA que codificam Fet3 e Ftr1, um complexo de alta afinidade para a absorção de ferro, estão drasticamente diminuidos após exposição ao arsénio. Além disso a diminuição do *FET3* mRNA é mediada pela via principal de degradação do mRNA através da 5'-3 'exonuclease Xrn1 e é independente da formação de ROS. Surpreendentemente, após a adição de arsénio Fet3 não está localizado na membrana plasmática mas sim no retículo endoplasmático. Além disso, o mutante *fet3ftr1* apresenta mais resistência ao arsénio que a estirpe do tipo selvagem, sugerindo que o Fet3 desempenha um papel na toxicidade do arsénio. Curiosamente, o tratamento com arsénio parece ativar a via não-redutora da absorção do ferro. Finalmente, este estudo

apresenta dados que sugerem que a perturbação da homeostase do ferro pelo arsénio também ocorre em células de mamífero, uma observação que pode ser relevante para aplicações clínicas.

No Capítulo 2 deste trabalho descrevemos alguns dos mecanismos de repressão da atividade do factor de transcrição humano "Heat Shock Factor" (HSF1) expresso na *S. cerevisiae*. O HSF1 desempenha um papel muito importante na resposta celular a factores de stress proteotóxicos. Em condições fisiológicas, o HSF1 é mantido como um monómero inactivo e não tem a capacidade para activar a expressão de genes. Após o stress, HSF1 homotrimeriza, desloca-se para o núcleo, torna-se hiperfosforilado, liga-se DNA e activa a transcrição dos genes alvo. Após o stress, HSF1 torna-se hipo-fosforilado e regressa para o citoplasma, voltando à forma de monómero inativo. Embora já tenham sido feitos avanços significativos para compreender os mecanismos envolvidos na ativação do HSF1, relativamente pouco se sabe sobre os mecanismos subjacentes à sua repressão. HSF1 é uma proteína altamente conservada entre os eucariotas e dados anteriores demonstraram que o HSF1 humano (hHSF1) é incapaz de complementar a ausência do HSF, essencial na levedura, visto que o hHSF1 é mantido como um monómero inactivo. Curiosamente, muitas das proteínas envolvidas na repressão da função do HSF1, incluindo as proteínas chaperone Hsp90 e Hsp70, bem como a JNK quinases, ERK e GSK3 estão conservados na levedura. Desta forma a compreensão dos mecanismos pelos quais hHSF1 é mantido num estado inativo na levedura poderá aprofundar a nossa compreensão da regulação do HSF1 em metazoários. Para explorar estes mecanismos utilizou-se uma variedade de abordagens bioquímicas e genéticas para

identificar potenciais sítios inibidores de fosforilação, bem como uma série de mutantes na levedura, com o objectivo de identificar potenciais proteínas repressoras do hHSF1. Neste trabalho mostra-se que quando HSF1 humano é expresso em levedura está fosforilado na S303 pela Slt2 MAP-quinase e que esta fosforilação ocorre independentemente da fosforilação prévia da S307 que até então se acreditava ser essencial. Além disso, enquanto que estudos anteriores sugeriam que a fosforilação na S303 reprime a transactivação dependente do HSF1, nós mostramos em levedura e células de mamíferos que a fosforilação na S303 também reprime a multimerização do HSF1. Em suma, os nossos resultados apoiam a hipótese de que a levedura pode ser usada para simplificar e elucidar mecanismos complexos de regulação da actividade do HSF1 humano, bem como identificar novos aspectos reguladores que controlam a actividade do HSF1 na levedura e nas células de mamíferos.

Finalmente este trabalho sugere que as células de levedura podem constituir uma poderosa ferramenta experimental para investigar aspectos como a toxicidade de arsénio e regulação da actividade do HSF1 humano, fornecendo uma plataforma para a compreensão destes mecanismos em eucariotas superiores.

General Introduction

The Yeast Model System – Saccharomyces cerevisiae

YEAST CELLS - "OUR RELATIVES"

Impressively nature keeps using the same building blocks to construct organisms as different as yeast cells, worms, flies, mice, and humans. It's this simplicity that allows us to study our own genes using the blob-like cells of yeast. Due to their basic cellular mechanisms of replication, recombination, cell division and metabolism conserved in mammals S. cerevisiae is considered the model system for molecular genetic research. Yeast is extremely easy to work with in the lab and the types of experiments we can do with them are endless. They can help conduct scientific research and make bread, beer, and other food stuffs. Can a mouse, fish, or fly do that?

Saccharomyces cerevisiae: An experimental organism

Sixteen years ago, in 1996, it was released the electronic version of the first complete DNA sequence of a eukaryotic genome, the yeast *Saccharomyces cerevisiae [1, 2]*, one of the oldest "domesticated" organisms. Already at the time there was no doubt that this yeast would be a fine "model organism", useful for interpreting and understanding human DNA sequences [3].

The yeast genome contains 6000 genes and at least 60% have statistically robust human homologues or one conserved domain with human genes. Interestingly more than 30% of human disease genes have counterparts in yeast, and about 71 human genes complement yeast mutations (Table 1 in Appendix) [4]. Despite the tremendous achievement in sequencing the entire human genome (3 billion base pairs in human DNA), researchers would not be able to use this "treasure" if they did not have access to the information provided by model organisms like yeast. Indeed, their genomic homology shows the conservation of the fundamental cell biological processes between yeast and mammalian cells. Furthermore yeast cells, just like our cells, have a nucleus containing chromosomes and divide in a similar manner recapitulating fundamental aspects of the eukaryotic biology.

Therefore *S. cerevisiae* as a simple eukaryote with a tractable genome, a short generation time, and a large network of researchers who have generated exquisite research tools, became a popular organism suited to help reveal the function of genes implicated in human biology. Many times the first clue about the function of higher eukaryotic genes has risen from the homology to a yeast gene. *S. cerevisiae*, in particular, has allowed the investigation of many important aspects of eukaryotic

biology, including the process of cell division and genetic transmission, transcriptional regulation, biogenesis and function of cellular organelles, protein targeting and secretion, cytoskeletal dynamics and regulation, and cellular metabolism [5].

Certainly, yeast does not have Parkinson or heart disease, but yet many of the human genes that are malfunctioning in these and other disorders have parallel genes in this model organism, where they can be more easily studied. Therefore many researchers are now developing yeast models of many human diseases. Also, the modern tools available in *S. cerevisiae* make possible to perform systematic analysis of a cellular process or phenotype, such us, genetic screening libraries, transcriptomic, proteomic and metabolomic analysis, chemical genomics and chemical genetics, and phenotypic microarray analysis [6].

Despite yeast cells don't have brains enormous attention has been directed recently to their potential as an experimental model for neurodegenerative diseases, such as Alzheimer, Parkinson and Huntington (see reviews in appendix). Many of the problems in these diseases derive from problems in protein folding and trafficking, which is largely the same in yeast as it is in neurons [7]. Additionally yeast has been extensively used to study cancer and age-related disorders.

Khurana and Lindquist [6] have described that depending on whether a yeast homologue exists or not there are two approaches that can be followed for modeling human disease in yeast. If there is a yeast homologue, the gene can be disrupted or overexpressed to determine the loss or gain of function phenotypes, respectively. In the case that there is not a yeast homologue the human gene can be expressed in yeast and any relevant phenotype that results from this expression can

be analyzed. Thus conditions from cancer to neurodegenerative and prion diseases, as well as biological processes such as cell death and DNA repair are currently studied in yeast cells and then scaled up in more complex animals. Furthermore, screening libraries of yeast varieties and mutants is widely used to find potential gene targets for drug development.

Strikingly according to *McGary K. L. et al* work [8], that describes a new system biology approach for identifying model organisms for human diseases, yeast could also be a powerful model organism for studying angiogenesis, although they don't have blood vessels.

Throughout this dissertation we report the use of the yeast assay system to further understand two types of stress response. The first, regarding how arsenic stress induces a global metabolic reprogramming of the cellular iron homeostasis both in *S. cerevisiae* and mammals and the second concerning the mechanisms that regulate human Heat Shock Factor 1, a stress-activated transcription factor, via post-translational modifications. Given the tools available in *S. cerevisiae,* this is an ideal system to begin to decipher these biological phenomena.

The Response to Stress

All living organisms are subject to changes in the environment, to which they must adapt in order to survive. Yeast cell homeostasis is achieved through the activation of several transcription factors, each one acting singly or in combination to perform specific functions. In the yeast *S. cerevisiae*, cell stress response is determined by the action of three major

types of activators, the partially redundant zinc-finger transcription factors Msn2 and Msn4, the Heat Shock Factor (HSF), and the basic-leucine zipper (b-Zip) transcription factors Yap family (Yeast-specific AP-1 like factors) [9].

The response mediated by the transcription factors Msn2 and Msn4 happens via stress response element (STREs) present on the target gene promoters. The STRE was the first common *cis*-element identified in a set of genes that sense a broad array of stress conditions determined by general stress response. It is estimated that over 186 genes (approximately 3% of the yeast genome) are potentially subject to STRE-mediated regulation [10]. Msn2/4 are controlled by high osmolarity via the HOG signal pathway, which comprises a MAP kinase module. Some of the important aspects of Msn2/Msn4 regulation include nuclear-cytoplasmic shuttling and targeted degradation of these factors at gene promoters during transcriptional activation [11]. The intracellular distribution of Msn2/4 is highly correlated with their phosphorylation state. It has been suggested that cAMP-dependent PKA inhibits nuclear import of Msn2/4, through direct phosphorylation of their nuclear localization signal (NLS). A second mechanism for PKA-mediated regulation of STRE-controlled gene expression involves the Ccr4-Not complex, a global transcriptional regulator that affects genes positively and negatively [12].

HSF mediates the transcriptional response to heat, infection and inflammation, pharmacological agents, and other stresses by binding to the heat shock elements (HSEs), 5'-nGAAn-3', present in its target genes, mainly heat shock proteins (Hsps) and activating their transcription, thereby playing a central role in the cellular homeostatic control

mechanisms [13]. HSF and HSEs are among the most highly conserved transcriptional regulatory elements in nature. Indeed, previous studies have demonstrated that both mammalian HSF1 and HSF2 expressed together can rescue the viability defect of *S. cerevisiae* cells lacking endogenous yeast HSF [14]. HSF is essential for cell viability in *S. cerevisiae,* oogenesis and early development in *Drosophila melanogaster,* extended life span in *Caenorhabditis elegans,* and extraembryonic development and stress resistance in mammals.

In yeast Hsf1 is constitutively bound as a trimer to high affinity HSEs present on several promoters leading to a moderate expression of the heat shock genes. Upon stress the occupancy of specific HSEs in the yeast *HSP82* promoter increases, suggesting that some yeast HSEs are inducibly bound, as observed in higher eukaryotes [15]. Moreover, the identification of genome-wide *Saccharomyces cerevisiae* HSF targets by chromatin immunoprecipitation, combined with DNA microarray analysis, demonstrated global heat-stimulated binding of HSF to many target genes [16]. Furthermore, similar to mammalian HSF1, yeast HSF is activated by multiple stresses, including heat shock, oxidative stress, and glucose starvation [17].

Recent studies have also provided novel insights into the role of HSF in growth, development, disease, and aging and in the complex metabolic reprogramming that occurs in all cells in response to stress [18].

The Yap family has a total of eight members and belongs to the bZIP family of transcription factors that is widely conserved from yeast to human [19]. Functionally, the Yap family is involved in a variety of stress-related programs, including the response to DNA damage and oxidative, osmotic, and toxic metal stresses. For instance Yap1 plays the major role

in oxidative stress, Yap2 in cadmium stress, Yap4 and Yap6 in osmotic stress, and Yap8 in arsenic stress nevertheless there is also evidence of cross-talk between the Yap members. For example, the *yap1yap2* double mutant is more sensitive to oxidative stress than either single mutant alone, as is the *yap1yap8* double mutant to arsenic stress [9]. Moreover at least four Yap proteins (Yap1, Yap2, Yap4 and Yap5) bind most efficiently to the Yap Responsive Element (YRE) TTAC/GTAA. Both Yap1 and Yap8 bind to the sequence GATTTAATAATCA, in which the bases flanking the core sequence (underlined) are essential for Yap8 recognition [20]. So far, only the corresponding binding site for Yap3, Yap6 and Yap7 has not been characterized yet.

The three types of stress control seem to have overlapping, but distinct functions. Some stress proteins encoded by HSE-regulated genes are necessary for growth of yeast under moderate stress, products of STRE-activated genes appear to be important for survival under severe stress and YRE-controlled genes may mainly function during oxidative stress and in the response to toxic conditions, such as caused by heavy metal ions.

While our understanding of these processes in higher eukaryotes remains incomplete, earlier studies have suggested that many of these phenomena may be conserved in yeast.

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Chapter 1

Introduction

Dealing with Arsenic

ARSENIC

Arsenic, a metalloid, has a long history of use in human civilization. The use of arsenical therapeutics dates back to the times of ancient Rome, when Galen and Hippocrates used them to treat ulcers. Arsenic trioxide is now used as a cancer chemotherapeutic against hematopoietic tumors. Acute arsenic poisoning is less common but it has been documented after attempted suicides or murders.

This chapter includes data of a manuscript in preparation:

Batista-Nascimento, L. Thiele, D.J., Rodrigues-Pousada, C. (2012) Arsenic exposure induces iron deficiency and destabilizes the high-affinity iron uptake system.

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 and is the first author of the manuscript.

The presence of nonessential metals like arsenic (As) in the environment is prevalent. Since these metals are highly toxic, they pose a considerable threat to nature and to human health. The main routes of poisoning are through occupational exposure or through ingestion of contaminated food and water. The contamination of drinking water by arsenic is a major health concern because of the large number of contaminated sites and people at risk of exposure. These metals are implicated in a broad spectrum of degenerative conditions in humans, including neurotoxicity, nephrotoxicity, genotoxicity and carcinogenesis. Chronic As exposure induces cardiovascular diseases, neurological disorders and liver injury, and is associated with cancers of the skin, bladder, liver and lung [1]. The mechanism of As toxicity is not fully understood at the molecular level. In general, it may act by targeting signaling or regulatory proteins that control cell proliferation, differentiation and cell cycle regulation. Due to its complex chemistry and ability to form many different compounds As is an intricate element to understand. Arsenic is most commonly found in two oxidation states, AsIII and AsV. The most common inorganic AsIII compounds found are arsenic trioxide, sodium arsenite, and arsenic trichloride. AsV compounds such as arsenic pentoxide, arsenic acid, and arsenates are also quite common. Arsenic is methylated by microorganisms, but inorganic AsIII and AsV compounds are considered the most toxic [2]. A common property of As is its high reactivity with sulphydryl groups. Hence, it can bind to and affect the activity of many proteins. AsIII can inhibit more than 200 enzymes, in events such as DNA repair, methylation of DNA, and increase radical formation and activation of the protooncogene c-myc [3, 4]. In addition, this metal is known to generate oxidative stress in cells and its toxicity has partly been

attributed to its capability to induce formation of reactive oxygen species (ROS). The damage caused by ROS to lipids, proteins and DNA are likely to contribute to As toxicity [3]. Nevertheless, neither the exact details of metal-induced ROS generation nor the full set of toxicity targets is known. Drugs containing arsenicals such as arsenic trioxide (Trisenox®) have been used to treat acute promyelocytic leukaemia (APL) and other haematological and solid cancers [5]. However, the development of resistance threatens the efficacy of medical treatment and hence, there is an increasing demand to identify tolerance mechanisms. Tolerance and detoxification mechanisms often involve extrusion of the toxic ions from the cell, sequestration within internal organelles, chelation by metal-binding proteins, and reduction of uptake. As shown in Figure 1, organisms take up AsV via phosphate transporters (Pho84 and Pho87) and AsIII by aquaglyceroporins (GlpF in E. coli, Fps1 in yeast, and *AQP7/9* in mammals) and hexose permeases (HXT family in yeast, and *GLUT1/4* in mammals). Once inside the cell AsV is reduced to AsIII by the Acr2 (arsenate-reductase) enzyme, with glutathione and glutaredoxin serving as a source of reducing potential [7].

In vertebrates, five sodium/phosphate transporters NaPiIIa, NaPiIIb, NaPiIIc, Pit-1 and Pit-2, which constitute the mammalian phosphate uptake system, were recently identified as arsenic transporters. NaPiIIa, NaPiIIc, Pit-1 and Pit-2 correspond to the low-affinity and NaPiIIb represent the high-affinity AsV transport. Next, AsV is reduced to AsIII by CDC25 phosphatases/arsenate reductases [8].

AsIII is then extruded from *S. cerevisiae* cells by the Acr3 (plasma membrane arsenite-efflux protein), and compartmentalized into the vacuole by Ycf1, a member of the MRP family of the ABC superfamily of

drug-resistance pumps. In mammals, Mrp isoforms, such as Mrp1 and Mrp2, pump AsIII out of cells [7, 9].

Studies with the budding yeast *S. cerevisiae* have demonstrated that in response to arsenic stress cells utilize two b-Zip transcription factors, Yap1 and Yap8, members of the Yap (Yeast AP-1 like) family. Yap8 is the master regulator of this response, mediating the transcriptional activation of *ACR2* and *ACR3* [10, 11] and Yap1 regulates *YCF1* expression and helps to maintain the cellular redox homeostasis [12]. In addition, arsenic can be methylated [13], although this process may increase arsenic toxicity rather than contributing toward detoxification [7]. Moreover, AsIII, the most toxic form of As, triggers increased ROS production in mammals [14], but not to any large extent in wild-type yeast [12].

Nevertheless, AsIII-induced oxidative stress and lipid peroxidation are detected in mutants with impaired AsIII detoxification (*yap8* mutant cells) or oxidative stress defense (*yap1* mutant cells) systems, indicating that AsIII enhances ROS levels also in yeast [12],

Arsenic toxicity has therefore been suggested to be caused by oxidative stress, impaired DNA repair, inhibition of enzyme function and by disturbing the function of proteins that regulate proliferation, cell cycle progression, apoptosis or differentiation [15-19] (Figure 2).

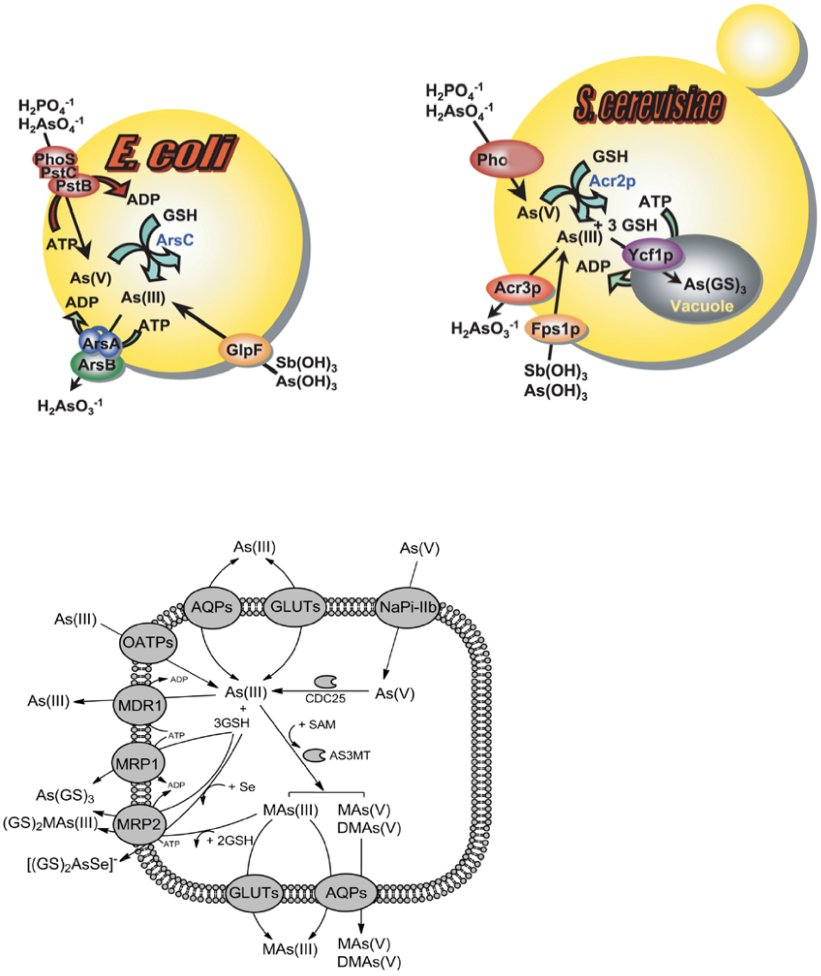

Figure 1: Arsenic detoxification in prokaryotes (*E.coli*) and eukaryotes (*S. cerevisiae* and mammals). AsV is taken up by phosphate and sodium/phosphate transporters, and AsIII is taken up by aquaglyceroporins (GlpF in E. coli, Fps1p in yeast and Aqp7 and Aqp9 in mammals). AsV is reduced to AsIII by the bacterial ArsC, the yeast Acr2p or the mammal CDC25 enzymes. Glutathione and glutaredoxin serve as the source of reducing potential. In *E. coli*, AsIII is extruded from the cells

by ArsB alone or by the ArsAB ATPase and in yeast by Acr3, the plasma membrane AsIII efflux protein, and Ycf1, a member of the MRP family of the ABC superfamily of drug-resistance pumps that transports $As(GS)_3$ into the vacuole. In mammals Mrp isoforms pump $As(GS)_3$ out of cells. Adapted form [6] and [7].

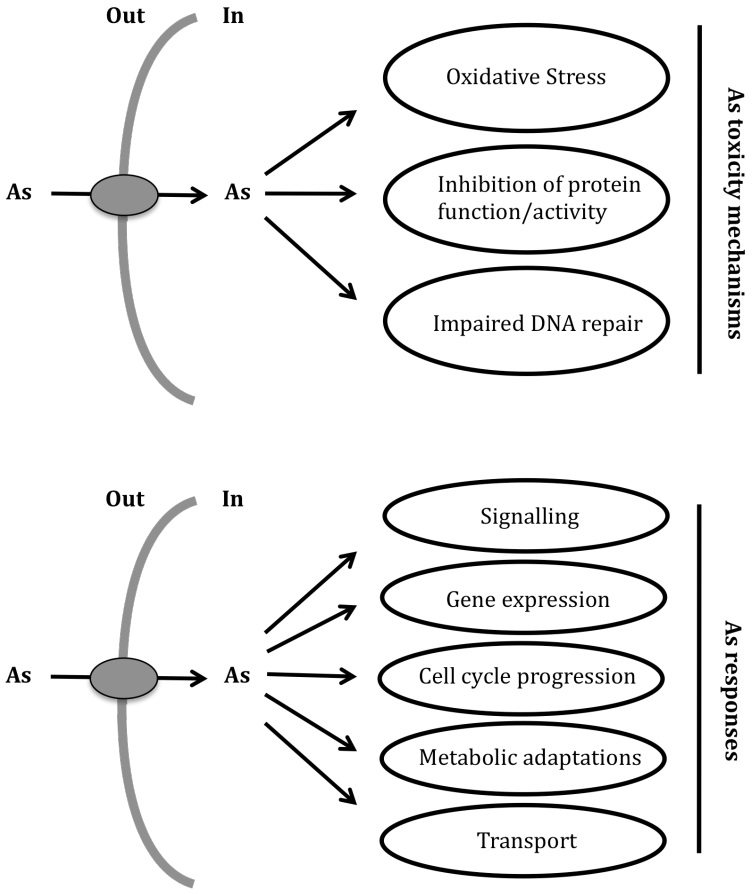

Figure 2: Arsenic response and toxicity mechanisms. Arsenic triggers oxidative stress in cells, interferes with protein function and activity

and/or impair DNA repair mechanisms either directly or indirectly. In response to As exposure, cells arrest cell cycle progression, alter gene expression and metabolism, and adjust transport processes to safeguard cellular and genetic integrity. Adapted from [20].

Since in mammals the proteins responsible for As reduction have not yet been identified, it is likely that additional pathways contribute to the full As tolerance in cells. Our work shows for the first time that arsenic disrupts the cellular iron (Fe) homeostasis.

Many of the genes and biological systems that function in yeast Fe homeostasis are conserved throughout eukaryotes to humans [21]. *S. cerevisiae* expresses three genetically distinct transport systems for Fe, two reductive systems and one non-reductive system (Figure 3). The reductive Fe uptake system consists in a low-affinity pathway defined by Fet4, that can also transport other metals and in a high-affinity pathway that is mediated by a protein complex composed of a multicopper ferroxidase Fet3, the mammalian Ceruloplasmine (Cp) homologue, and a permease Ftr1. The Fet3-Ftr1 complex is specific for Fe and is regulated both transcriptionally and post-transcriptionally by this metal [22-24]. The non-reductive Fe uptake system is mediated by the ARN family (Arn1-4) of membrane permeases that transport siderophore-Fe^{3+} complexes [22-24]. Additionally *Harris et al*, [25] showed for the first time that Fet3, can functionally replace Cp in restoring Fe homeostasis. Moreover cells are able to spare Fe through the regulation of Tis11 homologues, Cth1/2-mediated degradation of mRNAs coding for Fe-binding proteins, thereby facilitating the utilization of limited cellular Fe levels [26, 27]. Since *S.*

cerevisiae, lacks the Fe storage protein, ferritin, during Fe overload this is sequestered into the vacuole by the Ccc1 transporter, which is under the control of the Yap5 transcription factor [28]. On the other hand, Fet5/Fth1 complex mobilizes Fe out of the vacuole for use during Fe limitation [29].

Given the similarities between yeast and mammals unveiling metal toxicity and tolerance mechanisms in yeast may prove of value for identifying similar mechanisms in higher eukaryotes.

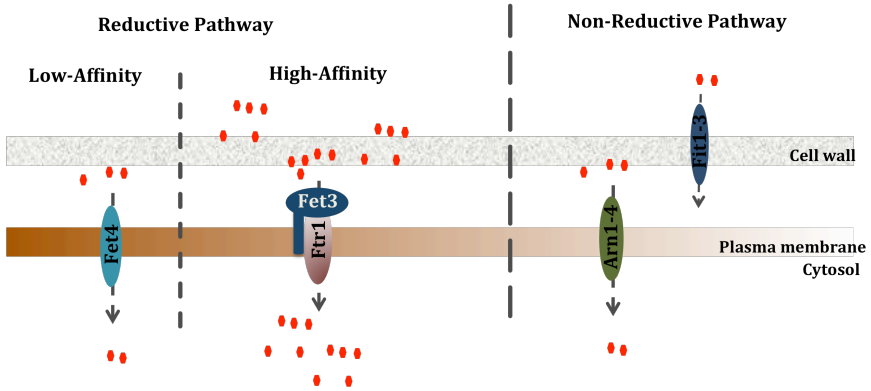

Figure 3: Yeast Iron Uptake Systems. Yeast has distinct transport systems for Fe, the reductive systems and the non-reductive system. The reductive iron uptake pathway consists in a low-affinity system defined by Fet4 and in a high-affinity system mediated by Fet3 and Ftr1. The non-reductive iron uptake is mediated by the ARN family (Arn1-4) membrane permeases that transport siderophore-ferric iron complexes.

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Abstract

Arsenic is a double-edge sword. On the one hand it is powerful carcinogen and on the other it has been used therapeutically in the treatment of acute promyelocytic leukemia. In this study we report the genome-wide response of *Saccharomyces cerevisiae* to arsenic. Our data suggest that this metalloid interferes with the expression of genes involved in iron homeostasis including those encoding proteins that function in iron uptake, trafficking and utilization. Interestingly arsenic activates the iron responsive transcription factor, Aft1, but the mRNA levels encoding Fet3 and Ftr1, a complex required for high-affinity iron uptake, are drastically decreased upon arsenic exposure. Moreover *FET3* mRNA decay is mediated by the 5'-3' exonuclease Xrn1 and independent of the formation of ROS. Strikingly, upon addition of arsenic Fet3 does not localize in the plasma membrane but rather in the endoplasmic reticulum. Also the *fet3ftr1* mutant shows arsenic resistance over the wild-type suggesting that Fet3 plays a role in arsenic toxicity. Unexpectedly, arsenic treatment seems to activate the non-reductive iron uptake system. Finally we provide data suggesting that arsenic disruption of iron homeostasis also occurs in mammalian cells, an observation that can be relevant to clinical applications.

Introduction

Arsenic (As), the 20th most abundant element in the earth's crust [1] is a highly toxic metalloid with respect to human health. Arsenic can induce skin lesions, hemorrhagic gastroenteritis, cardiac arrhythmia, psychiatric disease, and cancer. Its toxicity and carcinogenicity are suggested to be linked to oxidative stress. Although often synonymous of poison, it is one of the oldest drugs in history of mankind, first used to treat cutaneous ulcers, later periodic fever and malaria [2], and currently used as a potent agent against acute promyelocytic leukemia [3]. Due to its paradoxical biological effects, cellular As metabolism has been studied to understand its mechanisms of action. To date, it has never been shown a correlation between As effects and iron (Fe) homeostasis.

Cells require Fe for a wide array of metabolic functions, which include oxygen transport, cellular respiration, the tricarboxylic (TCA) cycle, lipid metabolism, gene regulation, and DNA replication and repair [4], yet Fe is toxic when present in excess. The yeast *S. cerevisiae* expresses three distinct transport pathways for Fe, two reductive systems and a non-reductive one. The reductive pathways consists of a low-affinity uptake system operated by Fet4 that can also transport other metals, and of a high-affinity Fe uptake system that is mediated by a protein complex composed of the multicopper ferroxidase Fet3 and the permease Ftr1. The Fet3-Ftr1 complex is specific for Fe and is regulated both transcriptionally and post-transcriptionally by Fe [5-7]. The non-reductive Fe uptake pathway is mediated by the ARN family (Arn1-4) of membrane permeases that transport siderophore-ferric iron complexes [8, 9]. In *S. cerevisiae*, these Fe uptake systems are largely induced under iron scarce

conditions by the Aft1 transcription factor and its homologue Aft2, as part of the iron regulon [10].

We have here conducted a genome-wide mRNA profiling of the *S. cerevisiae* response to As, which revealed a potent induction of the Fe gene regulon. However, neither *FET3* nor *FTR1* appeared induced by this treatment. We show that lack of the induction of these two genes is in fact the consequence of a degradation of their respective mRNAs by the major pathway for mRNA decay mediated by the 5'-3' exonuclease Xrn1. Moreover our data suggest a role of Fet3 in mediating As toxicity based on the improved As tolerance of the corresponding null mutant. Taken together this work provides a molecular connection between As toxicity and Fe homeostasis, which could be relevant to further understand the toxic and therapeutic effects of As at molecular level.

Material and Methods

Yeast Strains and Growth conditions

The plasmids and yeast strains used in this study are listed in Table 1 in supplemental material. To produce the *aft1* or *aft2* mutants the complete coding region of the *AFT1* and *AFT2* genes were deleted by homologous recombination with *HIS* and KanMX cassettes, respectively. Deletion was confirmed by PCR analysis of genomic DNA using upstream and downstream *AFT1* and *AFT2* specific primers. Spot assays were carried out by spotting 5µl of early exponential phase cultures (A_{600}=0.4) sequentially diluted (approximately 5×10^3 to 10 cells) in medium containing 2mM Na_2HAsO_4 (AsV), 400uM H_2O_2, 100uM Fe_2SO_4 or 10-100uM BPS. Growth was recorded after incubation for 2 days at 30°C.

Table 1: Strains used in this study.

Strain	Genotype	Source
BY4742	MATα his3Δ 1 leu2Δ 0 lys2Δ 0 ura3Δ 0	Euroscarf
aft1	MATα his3Δ 1 leu2Δ 0 lys2Δ 0 ura3Δ 0 *AFT1*::kanMX4	This Study
aft2	MATα his3Δ 1 leu2Δ 0 lys2Δ 0 ura3Δ 0 *AFT2*::kanMX4	This Study
DEY1457	MATα can1 his3Δ 1 leu2Δ trp1Δ ura3Δ ade6Δ	D. Kosman
fet3ftr1	MATα can1 his3Δ 1 leu2Δ trp1Δ ura3Δ ade6Δfet3::HIS3ftr1::TRP1	D. Kosman
YPH499	MATa ura3-52lys2-801_amberade2-101_ochretrp1-Δ63his3-Δ200leu2-Δ1	C.C. Philpott
arn1-arn4	MATa lys2-801_amberade2-101_ochretrp1-Δ63his3-Δ200leu2-Δ1	C.C. Philpott
BY4741	MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0	D.J. Thiele
cth2	MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0 *CTH2*::KanMX4	D.J. Thiele
xrn1	MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0 *XRN1*::KanMX4	D.J. Thiele
ccr4	MATa his3Δ 1 leu2Δ 0 met15Δ 0 ura3Δ 0 *CCR4*::KanMX4	D.J .Thiele

Cell Survival Curves

Logarithmic-phase yeast cells were grown in rich medium and incubated on YPD plates containing different concentrations of AsV at 30°C for 2 days to allow formation of colonies. Data are expressed as percentages of colonies formed compared with control cultures not exposed to AsV.

DNA Microarray Analysis

Wild-type (WT) cells were grown in triplicate in media containing 2mM of AsV for 1 hour, and RNA was extracted, labeled, and hybridized to Affymetrix Yeast Genome S98 arrays. (For further information about cell preparation, synthesis of labeled DNA, hybridization, scanning and data acquisition, and quality control steps, visit the Duke Microarray Core Facility at http://www.genome.duke.edu/cores/microarray/). All data were analyzed by Dr. Jörg Becker from Instituto Gulbenkian de Ciência (IGC) using both Partek® Genomics Suite™ and dChip softwares.

Accession Numbers

Microarray data have been deposit in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE33427.

RNA blot Analysis

Cells were grown overnight in YPD medium until saturation, diluted to OD600nm of 0.1 and allowed to grow until early exponential phase OD600nm of 0.4 - 0.5. Aliquots of these cells were left untreated or were treated with either 2mM of AsV, 100uM of Fe_2SO_4, 100uM of BPS or 400uM H_2O_2. Total yeast RNA was isolated by the hot phenol method [4].

PCR-amplified fragments were gel purified and radiolabeled with ^{32}P-dCTP to be used as probes. *U3*, a small nuclear RNA (SNR17A) was used as loading control.

Real-Time PCR Analysis

RNA was extracted from early log-phase cultures that were either untreated or exposed during 60 min to 2mM AsV. DNA was removed by on-column DNAse I digestion (RNase-Free DNase Set; Qiagen). Total RNA (1µg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). qPCR reactions were performed in the LightCycler 480 Instrument (Roche), using LightCycler 480 Green I Master (Roche) and the oligonucleotides are listed in Table S2. 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 480 Software 1. Actin (*ACT1*) was used as a reference gene. All assays were made in triplicate.

β-Galactosidase assay

WT cells and the isogenic *aft1* mutant were transformed with a β-galactosidase reporter plasmid containing the promoter sequence of *CTH2* previously described [11], pCM64-*CTH2*-FeRE-*CYC1*-LacZ. WT was also co-transformed with the plasmids pEG202LexA-*AFT1* and pSH18-34. Cells were grown in selective media to early log phase in the presence of 2mM AsV, 100uM Fe_2SO_4 or 100µM of BPS. β-galactosidase was measured in triplicate using a 96 well plate by following the degradation of the colorimetric substrate ONPG (o-nitrophenyl-b-D-galactopyraniside) at A_{420} and normalized against total protein concentration.

Measurement of Total Iron

Strains were grown in YPD media with 1mM of AsV for 4 hours, collected by centrifugation and washed twice in 10mM EDTA and once in metal-free water. The total Fe was measured by inductively coupled plasma (ICP) atomic emission spectroscopy at CQFB - Centro de Química Fina e Biotecnologia (Caparica, Portugal) on cells suspended in 0.1N Nitric acid.

Protein Analysis

Protein extracts were generated from cell cultures using cell lysis buffer (50mM HEPES, pH 7.5, 1mM EDTA, 100mM KCl, 10% glycerol, 0.1% NP40) supplemented with protease inhibitors (Roche). Protein concentrations were quantified using the Bradford assay and 80-100µg of protein was resolved by SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare), and immunoblotted with Anti-HA and Anti-cMyc (Roche) Anti-TAP-tag (Life Technologies). Anti-Pgk1 (Life Technologies) was used as loading control.

Fluorescence Microscopy

Microscopy experiments were carried out on live cultures using LEICA DMRA2 Microscope coupled with a CoolSNAP^{TM} HQ Photometrics camera (Roper Scientific). The analysis of fluorescence intensity was done using the MetaMorph software package (MDS Analytical Technologies). Overnight liquid cultures expressing Aft1-GFP or Fet3-GFP were re-inoculated to an optical density A_{600}=0.1 in YPD medium containing 1mM of AsV, or 100uM of BPS. Cells were incubated for 5 minutes at 30°C with 1ug/mL 4'6-diamidino-2-phenylindole (DAPI) to stain yeast nuclei. After washing with phosphate-buffered saline (PBS) cells were resuspended in

DABCO solution (75% (v/v) glycerol, 0.25X PBS and 200mM diazabicycloctane, Sigma–Aldrich) and visualized.

Cells and Media

Mouse embryonic fibroblasts (MEF) cells were maintained in DMEM (Sigma-Aldrich) with 10% of fetal bovine serum. Cells were Fe loaded by addition of FAC (100uM) and Fe depleted by addition of Desferroxamine (DFX) (5uM) and treated with Arsenic Trioxide [12] (5uM) for 24 hours. To extract protein, cells were solubilized in 1.0% of Triton X-100, 150mM NaCl, 10mM EDTA, and 10mM Tris (pH7.4) with a protease inhibitor cocktail (Roche). Protein concentrations were quantified using the Bradford assay, 80-100µg of protein was resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare). Ferritin levels were detected using Mouse Anti-Ferritin (Abcam), Css1 levels using Rabbit Anti-Css (Santa Cruz Biotechnology, Inc), Dmt1 levels using Rabbit Anti-NRAMP22-S (Alpha Diagnostic International), and Rat Anti-Tubulin (Novus Biologicals) was used as a loading control.

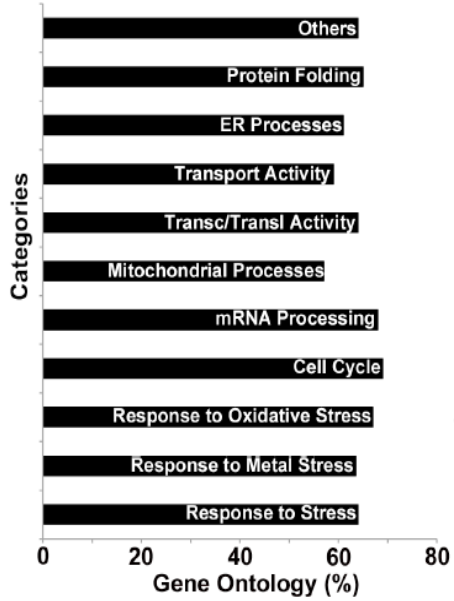
Results

The Saccharomyces cerevisiae Genome-wide Response to Arsenic Exposure

In *S. cerevisiae*, resistance to As involves Yap1 and Yap8, two AP-1 like transcription factors that regulate the expression genes involved in redox homeostasis and As detoxification processes, respectively [13]. However, additional pathways should most likely contribute to cellular As tolerance. To identify novel As tolerance pathways, we analyzed the

genome-wide mRNA profile of yeast cells exposed to As. Fig. 1 summarizes the results of these experiments (see data deposit in http://www.ncbi.nlm.nih.gov/geo/). In total, 1608 genes had their expression significantly altered upon As exposure. Classification of these genes by Gene Ontology showed that the functional categories most affected were related to the response to stress, metals and oxidative stress, cell cycle, mRNA processing, mitochondrial processes, transcriptional and translational activity, transporter activity, endoplasmic reticulum processes, protein folding, among others (Fig.1A). Many of these genes have already been shown to respond to As [14, 15], however genes responsive to metals, such as, zinc, iron (Fe) and copper (Fig. 1B), are here shown for the first time to be affected by As. Within the cellular Fe homeostasis category, 82 genes showed changes of at least 1.2-fold in their expression levels in response to As stress (Fig. 1C and Table S3 in supplemental material). Among these, many of the genes of the Aft1/2 regulon [16-18] were present, such as *CTH2*, the ARN family genes and *FIT3* that encode cell wall mannoproteins involved in siderophore-Fe uptake (Fig. 1D and Table 2). Surprisingly, despite a large induction of the Aft1/2 regulon, the mRNA levels encoding Fet3 and Ftr1 appeared significantly decreased upon As exposure (Fig. 1D) and (Table S3 in the supplemental material).

A


Others
Protein Folding
ER Processes
Transport Activity
Transc/Transl Activity
Mitochondrial Processes
mRNA Processing
Cell Cycle
Response to Oxidative Stress
Response to Metal Stress
Response to Stress
Categories
0
20
40
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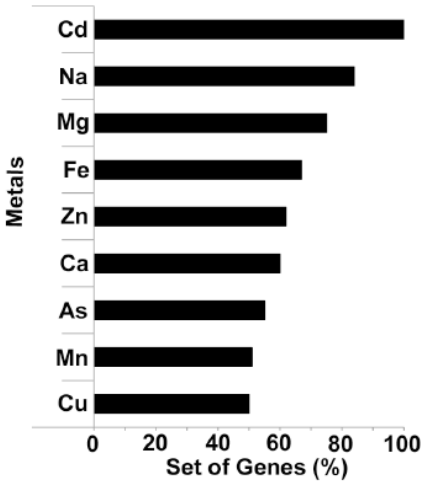
Gene Ontology (%)

B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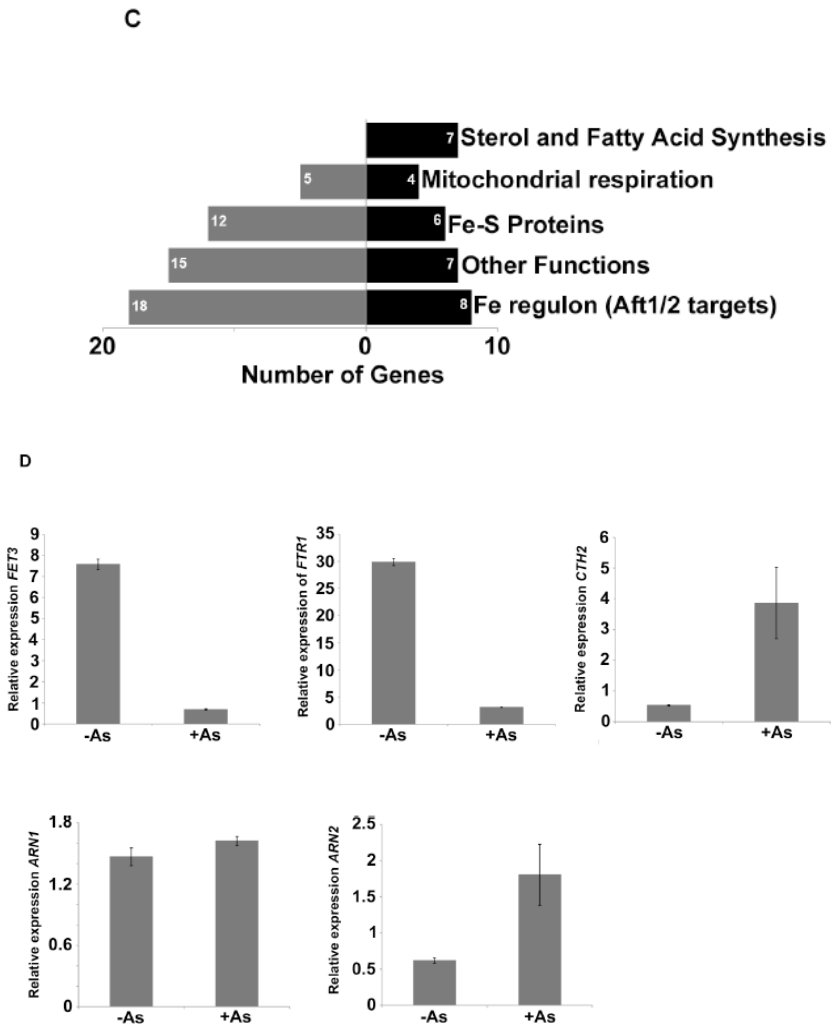


Figure 1: Genome-wide response of *S. cerevisiae* upon arsenic exposure. A. Representation of the results from triplicate Affymetrix DNA microarray studies showing the Gene Ontology of the main categories with at least 15% of the mRNAs changed between WTAsV *vs* WT. B. Representation of the percentage of metal related mRNAs changed in response to arsenate treatment. C. Representation of the total number of mRNAs corresponding to Fe-dependent processes up- or down-regulated in a wild-type strain upon addition of arsenic. D. Microarray validation by RT-PCR of mRNAs representative of iron homeostasis. All assays were made in triplicate.

Table 2: Iron transport systems genes whose mRNA levels were down- or up-regulated in a WT upon As treatment.

Aft1 targets

Iron Transport Systems

Reductive Pathway

GENE	FUNCTION	FOLD
FET3	Multicopper ferroxidase involved in high-affinity Fe uptake	-2.14
FTR1	High-affinity Fe permease, forms a complex with Fet3	-1.55
FET4	Low-affinity Fe transport protein	-2.94

Non-reductive Pathway

GENE	FUNCTION	FOLD
FIT3	Cell wall mannoprotein - siderophore-iron uptake	1.73
ARN1	Ferrirubin, ferrirhodin, and Fe-siderophores transporter	2.17
ARN2	Triacetylfusarinine C transporter	2.07
ARN4	Enterobactin transporter	1.63

The average fold induction of triplicate samples is represented.

An increase of 1.5-fold and a p-value <0.005 were used.

Aft1 is activated by arsenic stress

Except for *FET3* and *FTR1*, the mRNA profiling analysis indicated that As exposure mimics the genomic response to Fe deficiency (23-25,49,50). Yeast cells respond to Fe deprivation by the Aft1/2-dependent transcriptional activation of the Fe regulon that encode proteins involved in Fe uptake, trafficking and utilization [19]. Aft1 is controlled at the level of its nucleocytoplasmic distribution. To confirm that As can activate Aft1/2, we monitored by fluorescence microscopy the cellular localization of an Aft1-GFP fusion in cells exposed to As (Fig. 2A). Indeed As caused the redistribution of Aft1 from a diffuse fluorescence pattern to a punctuated one corresponding to the nucleus, similarly to what was seen in controlled cells treated with the iron chelator BPS. Subcellular localization of a Myc-Aft1 fusion by cellular fractionation similarly showed that in As-treated lysates, Aft1 was almost exclusively found in the nucleus, as also seen with BPS, whereas in the absence of treatment it was equally present in the cytosolic and nuclear fraction (Fig. 2B). Arsenic also induced the expression of the Aft1-dependent gene *CTH2* to levels similar to those triggered by BPS, as monitored using a promoter-LacZ fusion (Fig. 2C). In the *aft1* mutant, *CTH2* induction was abolished, as expected [11]. To question the functional relationship between As and the Aft1/2 regulon, we next analyzed the tolerance to As of *aft1* and *aft2* single mutants by growth in the presence of AsV (2mM). The *aft1* mutant was unable to grow under this condition, while the *aft2* mutant displayed normal growth (Fig. 2D). Interestingly As-induced growth inhibition of *aft1* could be totally reversed by addition of Fe (Fig. 2D), which indicates not only that As activates the Aft1/2-dependent Fe-deprivation response, but also causes a depletion of cellular iron.

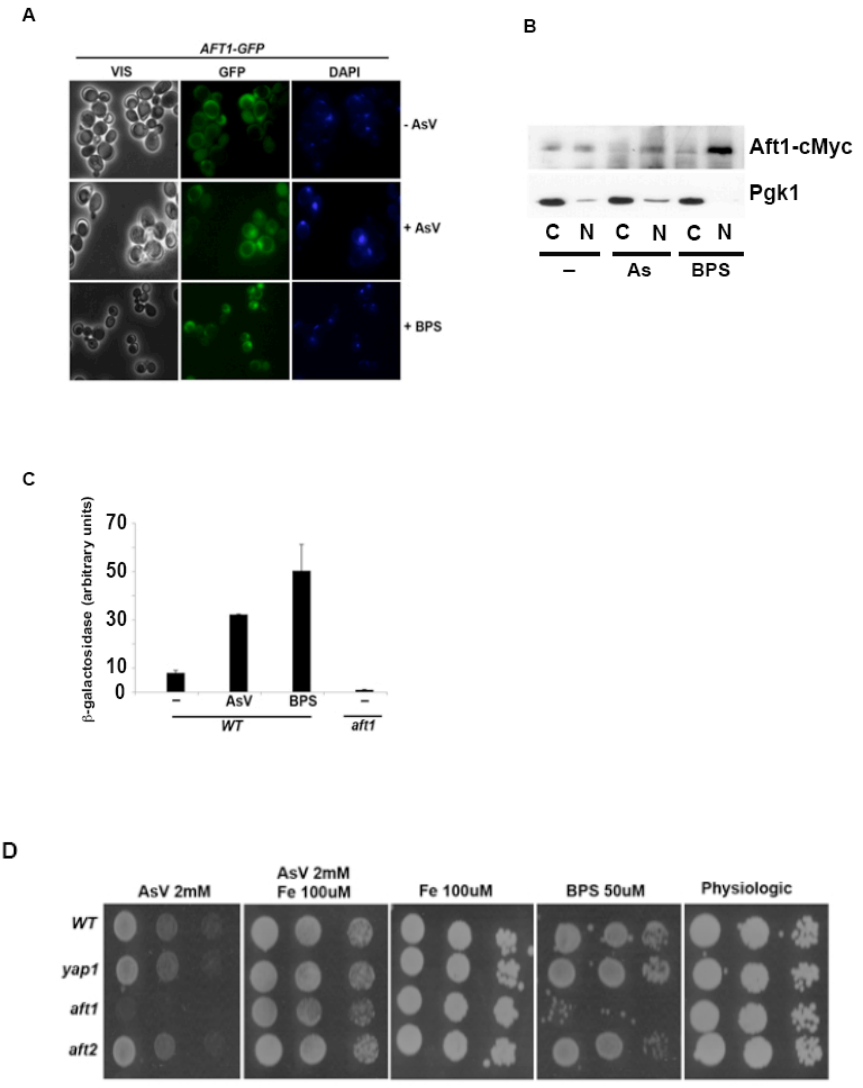


FIGURE 2: Aft1 activation in response to arsenic stress. A. Representation of *AFT1*-GFP fusion expressed diffusely throughout the nucleus and cytoplasm under physiological, while in presence of arsenate Aft1 localizes to the nucleus. B. Nucleus-cytoplasm fractionation. In presence of arsenate Aft1 localizes within the nucleus. Aft1-cMyc in presence of arsenate is found in the nuclear fraction. Cytosolic fraction was identified by incubation with an antibody against Pgk1. C. Arsenate induces the expression of *CTH2-lacZ* reporter construct. Wild-type cells transformed with both plasmids pSH18-34 and pCM64-*CTH2*-FeRE-CYC1-LacZ were grown in media containing BPS 100uM or AsV 2mM and β-

galactosidase activity was measured. Bars represent the average plus standard deviation of three independent experiments. D. *aft1* mutant is sensitive to arsenate and recovers upon supplementation with Fe_2SO_4. *yap1*, *aft1*, *aft2* mutants, and the respective WT were spotted on YPD or YPD plus AsV 2mM, BPS 50uM, or $FeSO_4$ 100uM.

Arsenic triggers degradation of the FET3 mRNA by the 5'-3' exonuclease Xrn1

As shown above, As caused a potent induction of the genes of the Fe regulon, except for *FET3* and *FTR1* that were paradoxically down-regulated (Fig. 1D, Figs. S1 A, B, Table S1). Fet3 is the multicopper oxidase that oxidizes ferrous (Fe^{2+}) to ferric iron (Fe^{3+}), for subsequent cellular uptake by the transmembrane permease Ftr1 [20, 21]. In response to Fe starvation, *S. cerevisiae* induces the expression of the Cth1 and Cth2 mRNA-binding proteins, which promote decay of mRNAs encoding for Fe binding proteins [4]. However, in a *cth2* mutant *FET3* mRNA levels remained undetectable in the presence of As, similar to what was seen in As-treated WT cells (Fig. 3A), in keeping with the notion that *FET3* does not carry any Cth1/2 AREs in its 3' UTR.

Another major route for mRNA degradation in eukaryotic cells begins with poly-A deadenylation by Ccr4, followed by decapping, by Dcp1/2, and 5'-3' exonucleotidic decay by Xrn1 [22, 23]. To test the possible role of this pathway in mediating As-dependent *FET3* mRNA decay we used *ccr4* and *xrn1* mutants. Importantly, the *xrn1* mutation had the effect of increasing the *FET3* mRNAs from the very low, undetectable levels seen in As treated cells to those of untreated wild type cells (Fig. 3B), indicating that As triggers degradation of this messengers by the 5'-3' exonuclease Xrn1. Because oxidative stress has been associated with changes in the stability of the Aft1 regulon mRNAs [24] and As compounds induce

oxidative stress, we also analyzed *FET3* expression levels in the presence of H_2O_2. As shown in Fig. 3C we did not observe any decrease of the *FET3* mRNAs upon addition of H_2O_2, suggesting that *FET3* degradation by Xrn1 is independent of ROS formation. Taken together, these results demonstrate that As triggers *FET3* mRNA degradation by Xrn1.

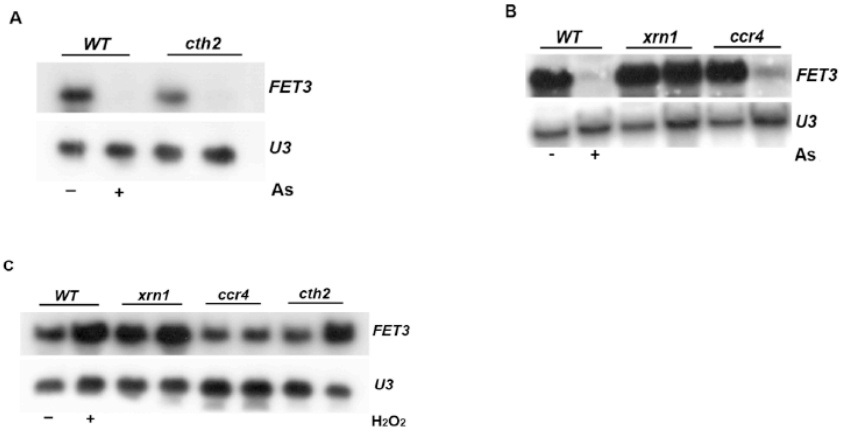
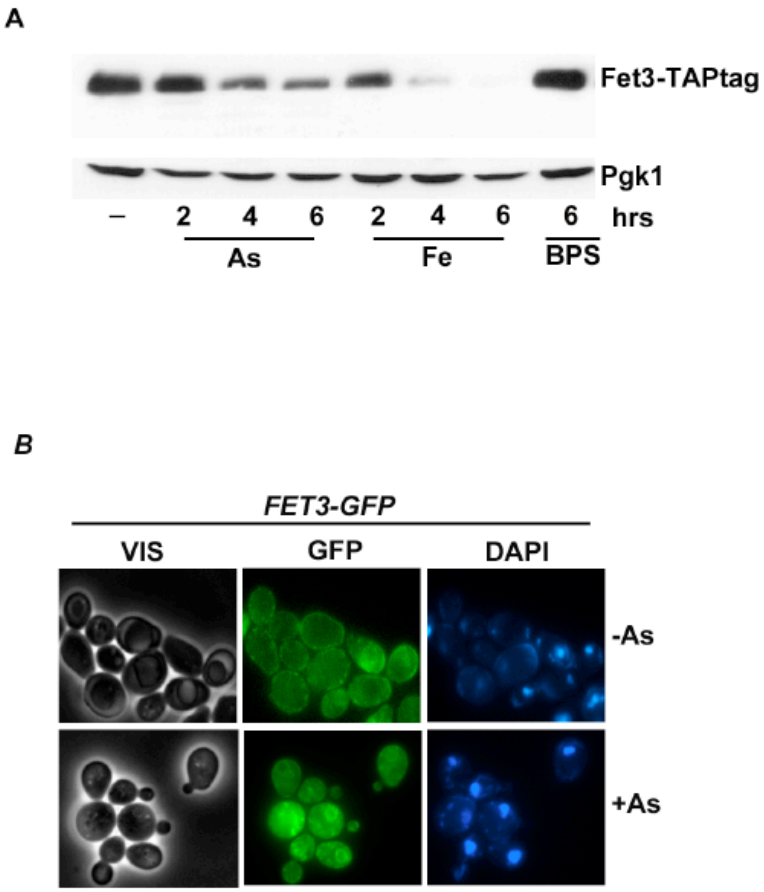


FIGURE 3: Exonuclease Xrn1 mediates *FET3* mRNA degradation upon As addition. *cth2*, *xrn1*, and *ccr4* mutant cells and the correspondent wild-type were grown in media containing AsV 2mM or H_2O_2 400uM for one hour. Cells were harvested, RNA extracted and analyzed for *FET3* expression. *U3* was used as loading control. A. *FET3* mRNA down-regulation is Cth2 independent. B. Upon arsenate addition *FET3* expression levels remain stable only in the absence of *XRN1*. C. *FET3* mRNA degradation is independent of the ROS formation.

Arsenic impairs high affinity Fe uptake by reducing FET3 protein levels and plasma membrane expression

To explore the functional consequences of the decrease of *FET3* mRNA levels of As-treated cells, we first measured Fet3 protein levels. These also appeared decreased in lysates of cells treated for 4 hours by As, but not as much as under Fe repletion (Fe) (Fig. 4A). As control, the Fet3 protein levels observed in Fe-depleted cells (BPS) are shown. Felice et al reported that high Fe levels cause not only the repression of the Aft1 Fe regulon, but also the internalization and degradation of the Fet3/Ftr1 transport system [7]. Thus as another way of checking Fet3 expression, we examined its cellular localization (Fig. 4B). In untreated cells, Fet3 was largely localized at the plasma membrane, while upon 1 hr exposure to As it was essentially concentrated in an internal compartment resembling the endoplasmic reticulum (ER) [25]. To evaluate the functional consequence of loss of Fet3 protein expression, we then examined the effect of As on the Fe total cellular content. A 4 hrs treatment with As at 1mM caused a 70% decrease on the Fe cellular content (Fig. 4C). To further evaluate whether the decrease in Fe was a result of the impairment of the Fet3-Ftr1 Fe reductive pathway, we checked whether addition of various siderophores to the medium could restore the Fe cellular content of As-treated cells by recruitment of the non-reductive pathway of the Arn proteins family of siderophore-Fe transporters, the expression of which is induced rather than repressed by As (Fig. 1D and Table 2). Addition of 5 uM siderophores indeed fully restored the Fe cellular content of As-treated cells, and this effect was dependent upon the presence of Aft1 and Arn1-4, as it was not observed in the

corresponding mutants (Fig. 4C). The data thus indicate that As decreases Fet3 protein levels and expression at the plasma membrane thereby causing defective Fe uptake by the Fet3-Ftr1 Fe reductive pathway and Fe deficiency. Furthermore, they are totally consistent with the induction of the Aft1 regulon and with the effect of exogenous Fe of mitigating As toxicity in *aft1* cells (Fig. 2D).



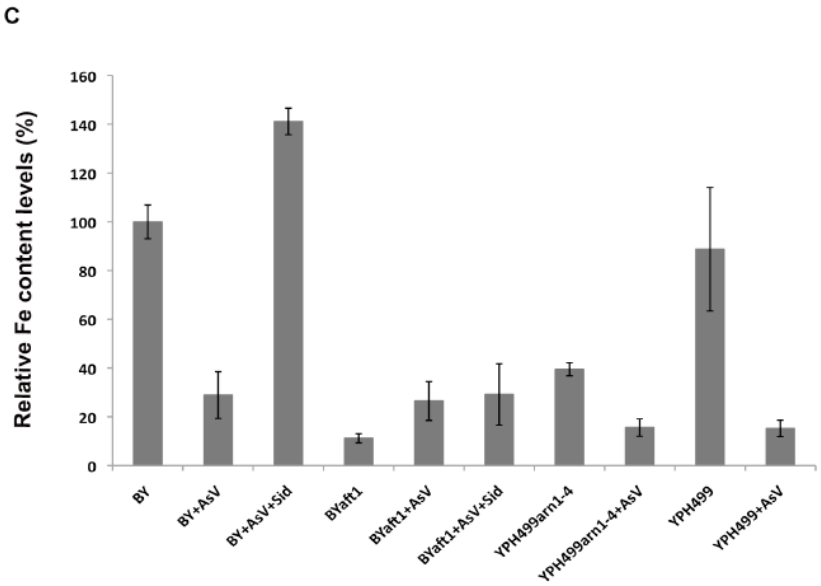


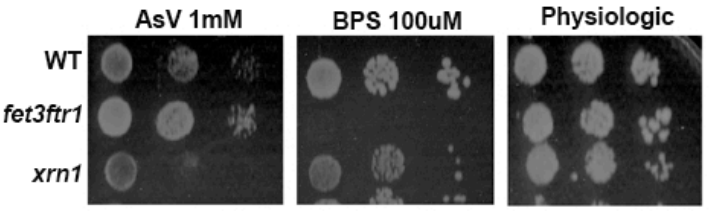
FIGURE 4: Arsenic exposure reduces Fet3 protein levels and mislocalization at the endoplasmic reticulum. A. Fet3 levels decrease after 4hrs of arsenate treatment. Protein was extracted from cells grown in media containing AsV 2mM, Fe_2SO_4 100uM or BPS 100uM for 2, 4 and 6 hours and analyzed by western blot. B. Representation of *FET3*-GFP strain expressed diffusely throughout the plasma membrane while in presence of arsenate localizes mainly to the endoplasmic reticulum. C. Upon arsenate exposure yeast cells redirect iron internalization through the non-reductive transport system. The intracellular iron content levels determined by ICP in the wild-type strain upon exposure of AsV 1mM for 4 hours are remarkably decreased. After addition of siderophores 5uM (ferrichrome, ferrirubin, enterobactin and desferroxamin) the wild-type cells recover the iron uptake. As expected *aft1* mutant and arn1-4 mutant are unable to consume enough iron comparing with the wild-type cells. Values are made relative to 100 in physiological conditions. Bars represent the average plus standard deviation of three independent experiments.

The fet3ftr1 double mutant is resistant to arsenic

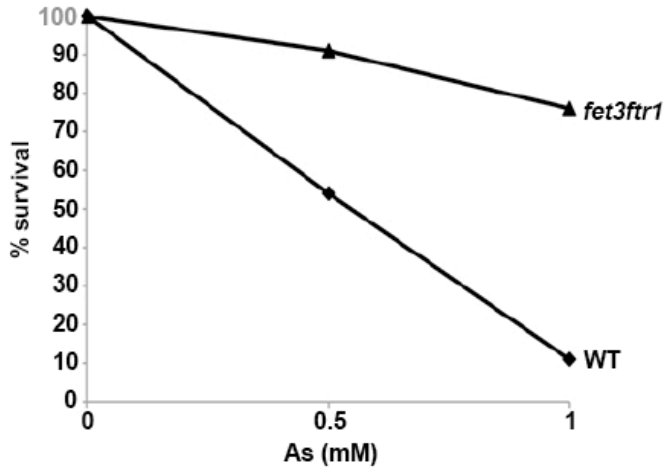
To test the possible relevance of Fet3 in mediating As biological effects, we assayed the As plate tolerance of the *fet3ftr1* double mutant. Surprisingly, this mutant appeared significantly more resistant to As than its WT counterpart, whereas the *xrn1* mutant that stabilizes *FET3* mRNA levels was much less resistant than the WT (Fig. 5A). In a cell survival assay the *fet3ftr1* was also significantly more resistant to As-induced cell death (~ 20% vs 90 % at 1mM As), (Fig. 5B). These data suggest that Fet3 mediates, directly or indirectly As toxicity.

Considering that the expression of *FET3-FTR1* is inversely proportional to cell resistance to As, their inhibition by As could be interpreted as a defense response mechanism which consequently induces the deprivation of Fe by the loss of import as high affinity pathway. To address whether Fe deficiency, which in turn leads to Aft1 activation, occurs as a consequence of impaired high affinity Fe uptake, we performed a time course study of *CTH2* and *FET3* expression during 1hr of exposure to As (Fig. 5C). While *FET3* mRNA expression starts decreasing right after the first 10 minutes of exposure, becoming almost undetectable over the time, *CTH2* mRNA levels are inversely induced reaching their peak at 60 minutes. This suggests that upon As addition, Aft1 activation occurs as a secondary event triggered by defective high affinity Fe uptake. This result is also consistent with the sensitivity of the *aft1* mutant to As and its recovery by addition of Fe.

A


AsV 1mM
BPS 100uM
Physiologic
WT
fet3ftr1
xrn1

B


100
90
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As (mM)
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WT

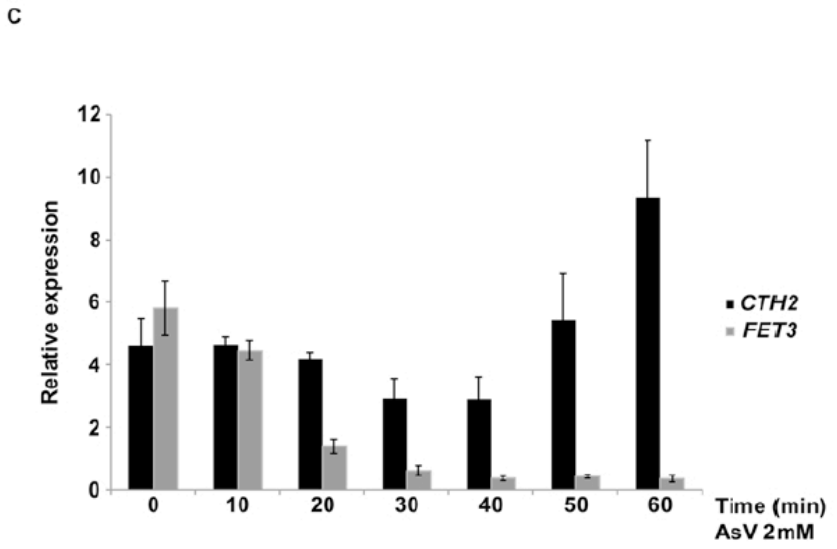

FIGURE 5: *fet3ftr1* mutant is tolerant to arsenate exposure. A. *fet3ftr1*, *xrn1* mutants and the respective wild-type cells were spotted on YPD or YPD plus AsV 1mM and BPS 100uM. B. Arsenic cell survival curve. Logarithmic-phase *fet3ftr1* mutant and wild-type cells were grown in YPD and incubated on YPD plates containing different concentrations of AsV at 30°C for 2 days to allow formation of colonies. Data are expressed as percentages of colonies formed compared with control cultures. C. Kinetic study of *CTH2* and *FET3* expression upon addition of AsV 2mM by RT-PCR. All assays were made in triplicates.

Arsenic trioxide causes Fe uptake destabilization in mammalian cells

Arsenic trioxide [12] is a small molecule arsenic compound used in the treatment of acute promyelocytic leukemia (PML). Although this compound was shown to cause damage to/or degradation of the PML protein/retinoic acid receptor-alpha (PML/RARa) fusion protein, thereby inducing apoptosis of acute PML cells [26], its mechanism of action is not well understood. To extend the results obtained in yeast, we checked whether ATO could also perturb Fe uptake in mammalian cells, using mouse embryo fibroblasts (MEFs). Ferritin is an Fe-storage protein that has also been implicated in Fe-transport pathways [27]. Ferritin synthesis is regulated post-transcriptionally by cytosolic Fe. In cells that are replete with Fe, the Iron Regulatory Protein (IRP) is released from the 5' Iron Response Element (IRE) of the ferritin mRNA, thereby allowing its translation, whereas in Fe deplete cells IRP binds the ferritin mRNA 5' UTR IRE preventing its translation [27]. As shown in Fig. 6 the expression of ferritin is reduced under low Fe conditions (DFX 5uM) and increased in the presence of Fe (FAC 100uM). Interestingly when ATO is added together with FAC the ferritin levels decrease significantly compared to Fe replete conditions (FAC). Most importantly, the ATO effect is specific for Fe since the Copper Chaperone for superoxide dismutase (Ccs1) levels, that change inversely with copper (Cu) concentration [12, 28], remain similar among the samples. This result is consistent with the data obtained from yeast demonstrating that As compounds disrupt either the Fe uptake or its intracellular regulation.

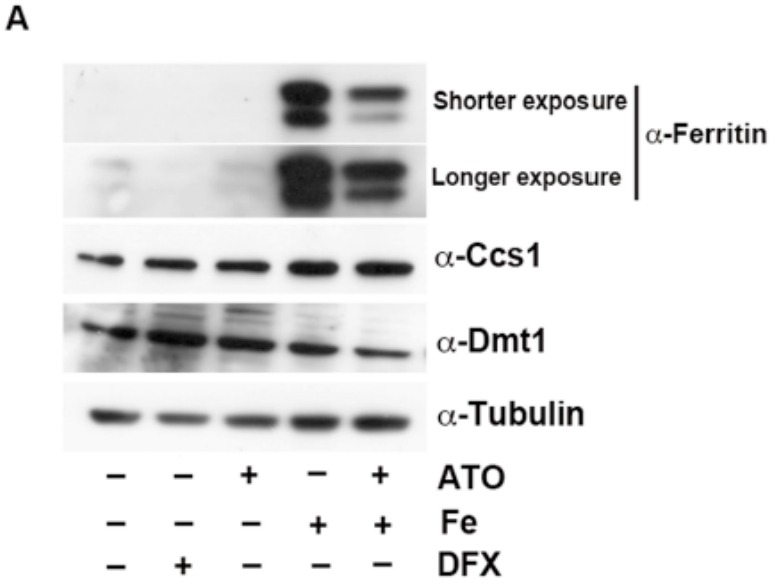

FIGURE 6: Arsenic trioxide destabilizes iron uptake in mammals. A. MEF cells were incubated with iron chelator 5uM DFX, 5uM ATO, and 100uM FAC, or 5uM ATO together with 100uM FAC. After 24hr media were collected and cells were lysed. Ferritin levels were detected by western blot using a mouse anti-Ferritin. Protein extracts were also analyzed for Ccs1 levels using rabbit anti-Ccs1 as well as Dmt1 using a rabbit anti-Nramp22D.

Discussion

Over the past decade extensive studies on As toxicity have suggested several modes of action such as production of chromosomal abnormalities, promotion of carcinogenesis and oxidative stress, among others [29, 30]. To date studies have not shown a link between As and Fe homeostasis.

Our DNA microarray data indicate that As exposure disturbs the expression of a battery of genes that encode for proteins involved in the Fe homeostasis by inducing a cellular response similar to that induced by Fe deficiency [16-18, 31, 32]. Upon As addition Aft1 translocates into the nucleus and activates the expression of the genes collectively known as the Fe regulon with the exception of *FET3* and *FTR1* genes related to the high affinity Fe uptake. Moreover upon As exposure the *aft1* mutant shows a defect in growth that is recovered by addition of Fe. These observations support the idea that when As is present in the environment cells undergo Fe deficiency, which is caused by *FET3-FTR1* degradation mediated by the Xrn1 pathway.

Similarly in presence of As Fet3 protein levels also decrease after 4 hours of exposure. Most importantly Fet3 upon As exposure is unable to localize in the plasma membrane getting restrained in the ER. This observation together with the decrease of the mRNA levels seem to indicate that As induced-Fe deficiency is caused by impairment of the high-affinity Fe uptake system is compromised. Indeed WT cells exposed to As for 4 hours are unable to consume as much Fe as in the absence of As. The Fe assimilation is then achieved through the non-reductive pathway, mediated by the ARN family genes, as WT cells exposed to As together with siderophores show higher levels of intracellular Fe content.

Interestingly *fet3ftr1* mutant is resistant to As while *xrn1* mutant

expressing high levels of *FET3* is sensitive, which indicates that *FET3-FTR1* expression is deleterious in the presence of As. These results suggest that *FET3* has a pivotal role in As toxicity. An intriguing possibility is that Fet3/Ftr1 complex might also have affinity to transport As inside the cells and *FET3* mRNA degradation by Xrn1 is a negative feedback regulation specific for As stress adaptation. The impairment of the high affinity Fe uptake by As is followed by the activation of *CTH2* expression, thereby allowing an mRNA decay-mediated global metabolic reprogramming in order to facilitate the utilization of limiting Fe pools.

Taking into consideration that As is a chemotherapeutic drug used to treat APL, we investigated whether this compound could also interfere with the Fe homeostasis in mammals. In mammals the response to Fe deficiency is post-trascriptionally controlled by the Fe-regulatory proteins IRP1 and IRP2 [27, 33, 34]. Consistent with our data from yeast, when arsenic trioxide is added together with Ferric Ammonium Citrate the ferritin levels decrease significantly compared to Fe replete conditions, confirming that As compounds impair the cellular Fe uptake. Finally in our study we have found novel effects of As in Fe metabolism in both yeast and mammalian cells. These observations might shed new light on the mechanism of action of arsenic, which may be relevant to better understand its toxicity and possibly its effect when used as chemotherapy in cancer patients.

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Footnotes

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Chapter 2

Introduction

Hsf1, the Molecular Thermometer

HSF1, OUR OLDEST FOUNTAIN OF YOUTH!

In 1962, Ferruccio Ritossa at the Genetics Institute in Pavia, Italy, made a discovery that gave birth to the heat shock research field. After someone had accidentally turned up the temperature in an incubator holding fruit flies he noticed that their chromosomes had puffed up at discrete locations a puffing pattern in the polytene chromosomes of Drosophila larvae. This puffy appearance was a sign that genes were being activated to give rise to their encoded proteins, the heat shock proteins. These proteins were detected in mammals only 15 years later. Since then the heat shock proteins have been recognized as part of the central role in life protecting the cells from several types of stress. Newly recognized roles in cancer and immunity as well as in ageing make them potential therapeutic allies.

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

For successful adaptation and survival to extreme proteotoxic insults such as heat, oxidative stress, heavy metals, toxins and bacterial infections, all living organisms must maintain a cellular quality control. One way they do this is through the activation of the heat shock response (HSR) via the heat shock transcription factor 1 (Hsf1), leading to increased expression of heat shock proteins (HSPs) which are involved in maintaining protein homeostasis/proteostasis, a hallmark of stressed cells. The heat shock response is a highly conserved mechanism in all organisms from yeast to human, which suggests that this response is essential for survival in a stressful environment. Upon stress, Hsf1 binds to an heat shock element (HSE) composed of at least three contiguous inverted repeats, nTTCnnGAAnnTTCn [1] (Figure 1) found on its target genes. The promoters of HSF target genes can also contain more than one HSE, thereby allowing the simultaneous binding of multiple HSFs.

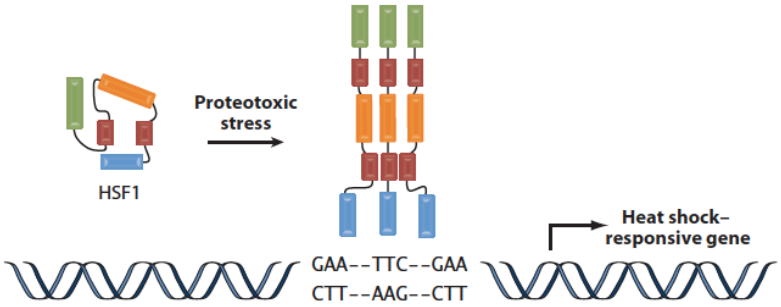

Figure 1: HSF1 is activated by proteotoxic stress, trimerizes and binds to HSEs, consisting in inverted repeats of the nGAAn pentamer in the promoters of hsps and other target genes. Taken from [2].

In yeast, nematodes, and fruit flies Hsf is encoded by a single gene whereas vertebrates have evolved a family of four HSF members, HSF1 that is the stress-activated prototype of this transcription family, and three more members, HSF2 that has mainly been linked to development and differentiation events, HSF4 and the avian-specific HSF3. HSFs possess unique and overlapping functions (Figure 2), exhibit tissue-specific patterns of expression and have multiple interacting protein partners [3].

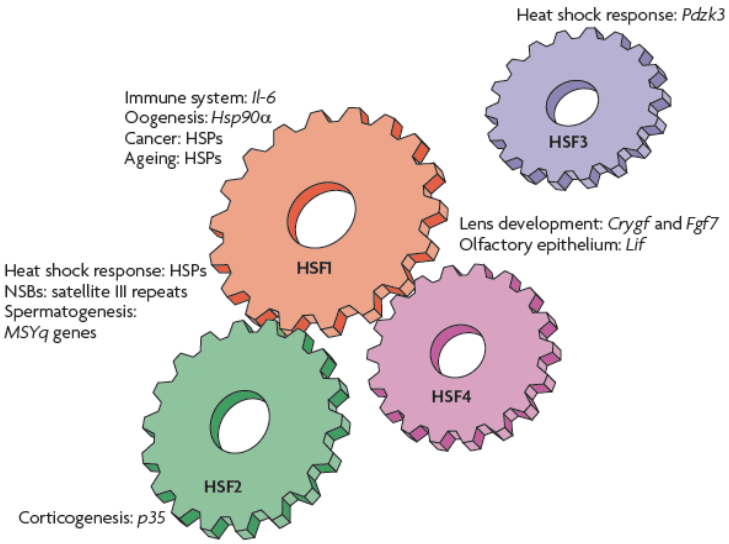

Figure 2: Overview of the mammalian HSF machinery. HSF1 is recognized as the principal stress-responsive regulator of the HSR, and HSF2 modulates HSF1-mediated expression of HSP through heterocomplex formation. Both HSF1 and HSF2 have regulatory functions in developmental processes, such as oogenesis, spermatogenesis and corticogenesis. HSF4 is involved in the development of different sensory organs in cooperation with HSF1. HSF3 is the most recently identified HSF

and participates in the HSR. Currently, HSF3 is not known to crosstalk with any member of the HSF family. Taken from [2].

In plants the Hsf family has so far been fully described only in few model species such as *Arabidopsis* and rice. *A. thaliana*, that serves as the prototype for the Hsf family, has a set of 21 Hsf. Recent analyses of Hsfs in other species indicated that both size and composition of the Hsf family is subject to evolutionary change. It is thought that plants evolved such an extended family of HSFs so as to withstand the constant and often severe environmental aggressions as those faced by immotile organisms. The wide multiplicity of plant Hsfs is in sharp contrast to the situation in most other organisms [4, 5].

Mammalian HSF1 is the master regulator of chaperone expression in vertebrates, as mice and cell models lacking hsf1 gene are unable to elevate Hsp levels in response to thermal insult displaying reduced survival after challenge with the bacterial toxin lipopolysaccharide [6]. Hsf1 is the best characterized member of the HSF family, it is constitutively expressed in most tissues and cell types [7], and is kept as an inactive monomer through post-translational modifications (PTMs) that include protein acetylation, sumoylation and phosphorylation (Figure 3) and through the activity of the chaperone proteins HSP90, HSP70, and HSP40 and other co-chaperones (Figure 4).

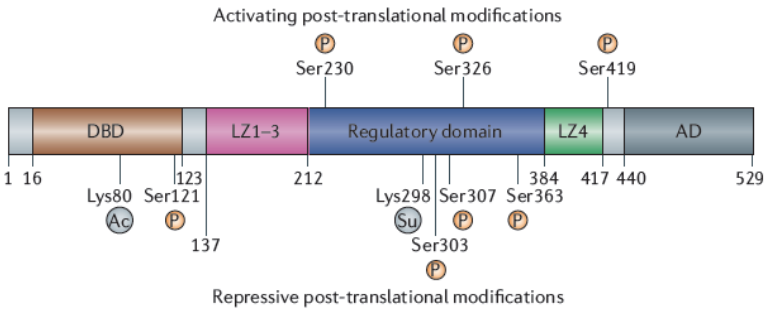

Figure 3: Overview of HSF1 post-translational modifications (PTMs). Sites for acetylation (Ac), phosphorylation (P) and sumoylation (Su) are indicated. Taken from [8].

HSF, like many other transcription factors, are composed of functional domains. All HSF contain two conserved domains, the amino-terminal DNA binding domain (DBD) and an adjacent oligomerization domain. The DBD is the only functional domain of HSFs being considered as the signature domain for HSFs target-gene recognition. DBD consists on a loop that allows protein–protein interface between adjacent subunits of the HSF trimer that enhances high-affinity binding to DNA [9].

HSF1 trimerization is regulated by the hydrophobic heptad repeats (LZ1-3) (Figure 3) that form a coiled coil, which is characteristic for many Leu zippers [10]. The suppression of HSF1 spontaneous trimerization is mediated by the additional hydrophobic heptad repeat region, LZ4 (Figure 3). The LZ4 domain is thought to fold back and interact with the

LZ1-3 domain to keep HSF1 in an inactive state as introduction of mutations in this domain allows constitutive HSF1 trimerization and DNA-binding activity [11, 12]. *Saccharomyces cerevisiae* HSF1 and mammalian HSF4 lack the LZ4 domain, which might explain its constitutive trimerization and DNA binding activity at normal growth temperatures [13, 14]. In response to stress HSF1 homotrimerizes, accumulates into the nucleus, binds DNA, becomes hyper-phosphorylated and activates the expression of stress response genes (Figure 4) [8, 15]. The conversion of the inactive monomeric HSF1 to high-affinity DNA-binding trimers is the initial step in the multistep activation process and is a common feature of all eukaryotic HSFs [16]. Hsp90 has been shown to have a role in maintaining HSF1 in an inert state, as its immunodepletion or inhibition by geldamycin let to activation of HSF1 DNA binding domain. Hsp70 effect on trimer formation is minor since it rather suppresses the transcriptional activity of HSF1 by binding to its activation domain. Elevated levels of both HSP90 and HSP70 negatively regulate HSF1 and prevent trimer formation during heat shock. Activated HSF1 trimers also interact with HSP70 and the co-chaperone HSP40, but instead of suppressing the DNA-binding activity of HSF1, this interaction inhibits its transactivation capacity [17]. Regardless of how HSF1 is converted from a monomer to a trimer the DNA-bound HSF1 is insufficient for transcriptional induction of hsp genes, suggesting that other mechanisms operate to facilitate gain of HSF1 trans-activating competence. A prominent feature of HSF1 is that its conversion into a transcriptionally active trimer occurs with extensive hyper-phosphorylation of serine residues [2], most of which reside within the Regulatory Domain (RD) (Figure 3) [18].

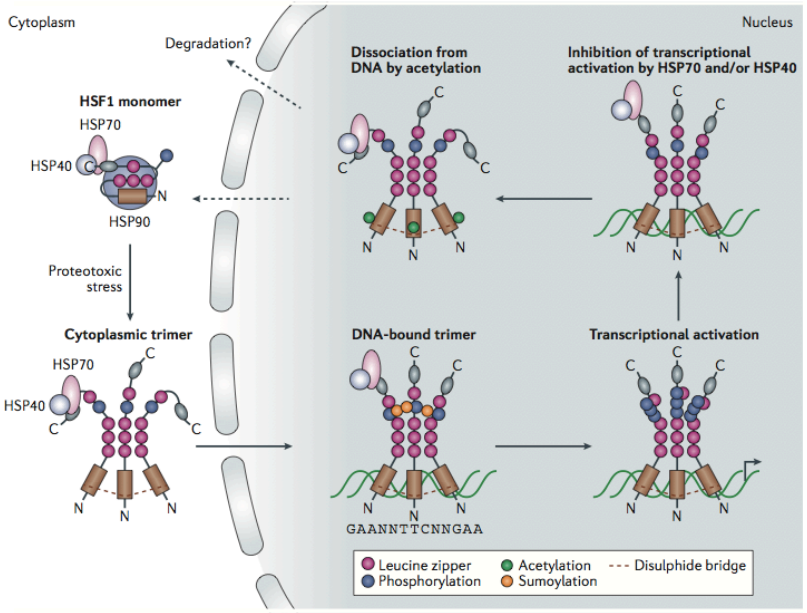

Figure 4: HSF1 multistep activation and attenuation process. In the resting state, HSF1 is a monomer in both the cytoplasm and nucleus. Monomeric HSF1 is already a phosphoprotein under non-stress conditions and it interacts with HSP90. Upon stress, HSF1 dissociates from the HSP90 complex, allowing HSF1 to trimerize and bind to the HSEs in HSP genes. HSF1 undergoes phosphorylation and sumoylation and acquires transcriptional activity. Attenuation involves two regulatory steps: negative feedback from HSPs, which represses the transactivation of DNA-bound HSF1, and inhibition of DNA binding by the acetylation of Lys80 in the DBD of HSF1. Taken from [8].

Results from mass spectrometry (MS) analyses combined with phosphopeptide mapping experiments indicate that at least 12 Ser residues are phosphorylated [19] (Table 1). As a monomer HSF1 is phosphorylated on at least Ser230, Ser303, Ser307 and Ser363, and its DNA-binding and transcriptional activities are repressed. In contrast to the repressive role of phosphoserines 303, 307 and 363, the Ser230 was

identified as a phosphorylation site that promotes the transcriptional activity of HSF1 (Figure 3) [20]. Equally important is that the basal phosphorylation state of Ser230 is enhanced upon heat shock, showing a stoichiometric shift towards phosphoserine 230 in a subset of HSF1 molecules [21]. *Hong, Y. et al.* [22] has also shown that HSF1 undergoes stress-induced sumoylation on Lys298, which lies close to the residue Ser303 that needs to be phosphorylated before a small ubiquitin-related modifier can be conjugated. Recently *Westerheide et al* [23] showed that HSF1 is also subject to stress-inducible acetylation. By mass spectrometry it were identified at least 9 lysines in HSF1 that were acetylated in response to stress (Table 1) of which Lys80 appears to control the HSF1 binding to DNA. This stress-inducible acetylation event seems to be regulated by the balance of acetylation by p300–CBP (CREB-binding protein) and deacetylation by the NAD^+-dependent sirtuin, SIRT1. Increased expression and activity of SIRT1 enhance and prolong the DNA-binding activity of HSF1 at the human HSP70.1 promoter, whereas downregulation of SIRT1 enhances the acetylation of HSF1 and the attenuation of DNA-binding without affecting the formation of HSF1 trimers [23].

Table 1: Heat shock factor 1 (HSF1) post-translational modifications (PTMs) events [15].

Modification	Enzyme	Effect
Ac-K80	p300/SIRT1	Inhibitory
Ac-K116	p300/SIRT1	?
Ac-K118	p300/SIRT1	?

Modification	Enzyme	Effect
Ph-S121	MAPKAPK2	Inhibitory (promotes Hsp90 binding)
Ac-K126	p300/SIRT1	?
Ph-T142	CK2	Inhibitory
Ac-K148	p300/SIRT1	?
Ac-K157	p300/SIRT1	?
Ac-K208	p300/SIRT1	?
Ph-S216	Plk1	Promotes mitotic progression
Ac-K224	p300/SIRT1	?
Ph-S230	CamKII	Stimulatory
Ph-S292	?	?
Su-K298	Ubc9	Inhibitory
Ac-K298	p300/SIRT1	?
Ph-S303	MAPKGSK3β	Inhibitory (promotes K298 sumoylation)
Ph-S307	MAPK	?
Ph-S314	?	?
Ph-S319	?	?
Ph-S320	?	?
Ph-T323	?	?
Ph-S326	?	Stimulatory (promotes Daxx binding)
Ph-S344	?	?

Modification	Enzyme	Effect
Ph-S363	PKC, JNK	Inhibitory
Ph-T367	?	?
Ph-S368	?	?
Ph-T369	?	?
Ph-S419	PLK1	Stimulatory
Ph-S444	?	?
O-glycosylation	?	?

Finally, in the light of current knowledge, the attenuation phase of the HSF1 cycle is regulated by a dual mechanism: a dependency on the levels of HSPs that feedback directly by weak interactions with HSF1, and a parallel step that involves the SIRT1-dependent control of the DNA-binding activity of HSF1. SIRT1 is the mammalian ortholog of the yeast Sir2, has been implicated in caloric restriction and ageing. Ageing and cellular senescence reduce the organism's ability to respond to stress and maintain homeostasis [24]. Whereas HSF1 protein concentration increases with age, the amount of SIRT1 protein decreases. The age-dependent loss of SIRT1 and impaired HSF1 activity correlated with an impairment of the heat shock response and proteostasis in senescent cells, suggests that SIRT1 and HSF1 function together to protect cells from various stresses, promote survival, and extend life span. Age-dependent onset applies to both degenerative conditions that have a clear genetic determinant, such as Huntington's disease, and to pathologies in which sporadic forms are predominant, such as Parkinson's, amyotrophic lateral

sclerosis (ALS), and Alzheimer's diseases. These neurodegenerative diseases often occur later in life when heat shock genes seem to be poorly induced [25].

Nevertheless tumor cells typically express higher levels of heat shock proteins leading to the suggestion that the aberrant expression of chaperones is associated with the tumorigenic state [26]. Also tumor cells might be dependent on elevated levels of Hsps, perhaps as a generalized mechanism to suppress cumulative mutations that would otherwise result in the expression of deleterious proteins. Given the unique role of HSF1 in stress biology and proteostasis, this has been considered as a potential target for therapeutics and many academic researchers and industrial laboratories have actively been searching for small molecule regulators of HSF1 [2].

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Abstract

Heat shock transcription factor 1 (HSF1) plays an important role in the cellular response to proteotoxic stresses. Under normal growth conditions HSF1 is repressed as an inactive monomer in part through post-translation modifications that include protein acetylation, sumoylation and phosphorylation. Upon exposure to stress HSF1 homotrimerizes, accumulates in nucleus, binds DNA, becomes hyper-phosphorylated and activates the expression of stress response genes. While HSF1 and the mechanisms that regulate its activity have been studied for over two decades, our understanding of HSF1 regulation remains incomplete. As previous studies have shown that HSF1 and the heat shock response promoter element (HSE) are generally structurally conserved from yeast to metazoans, we have made use of the genetically tractable budding yeast as a facile assay system to further understand the mechanisms that regulate human HSF1 through phosphorylation of serine 303. We show that when human HSF1 is expressed in yeast its phosphorylation at S303 is promoted by the MAP-kinase Slt2 independent of a priming event at S307 previously believed to be a prerequisite. Furthermore, we show that phosphorylation at S303 in yeast and mammalian cells occurs independent of GSK3, the kinase primarily thought to be responsible for S303 phosphorylation. Lastly, while previous studies have suggested that S303 phosphorylation represses HSF1-dependent transactivation, we now show that S303 phosphorylation also represses HSF1 multimerization in both yeast and mammalian cells. Taken together, these studies suggest that yeast cells will be a powerful experimental tool for deciphering aspects of human HSF1 regulation by post-translational modifications.

Introduction

All organisms are exposed to proteotoxic stresses that result in the accumulation of misfolded proteins. In response to these stresses cells have evolved adaptive responses to protect and stabilize cellular proteins until more favorable conditions for cell proliferation are encountered [1]. The heat shock transcription factor, HSF, is a homotrimeric transcription factor that activates gene expression in response to a variety of stresses including heat and oxidative stress, as well as inflammation and infection [2]. Recent evidence has shown that the *S. cerevisiae* HSF directly activates the expression of genes whose protein products are involved in protein folding and degradation, ion transport, signal transduction, energy generation, carbohydrate metabolism, vesicular transport, cytoskeleton formation and other cellular functions [3].

While mammalian cells express four distinct HSF proteins encoded by separate genes, HSF1 is the primary factor responsible for stress responsive gene transcription [2]. In the absence of stress, mammalian HSF1 is repressed through mechanisms that are not well understood. HSF1 is thought be maintained in an inactive monomeric state through intramolecular interactions between a hydrophobic coiled-coil domain in the carboxyl-terminus of the protein and three amino-terminal coiled-coils required for homotrimerization and transcriptional activation [4-6]. HSF1 is also thought to be bound and repressed by the protein chaperones Hsp90 and Hsp70, though it is not clear how these chaperones repress HSF1 activity [7-10]. Studies suggest that during the initial phase of the stress response, the inactive HSF1 monomer dissociates from Hsp90, homotrimerizes, is transported to the nucleus and binds to heat shock elements (HSE) found in the promoters of HSF target genes [10, 11]. The DNA-bound homotrimer, remains relatively transcriptionally inert [12], potentially due to the

continued interaction with Hsp70 and the HSF1-transactivation domain [9]. Stress-dependent hyperphosphorylation of HSF1 by potentially multiple protein kinases has been proposed to, in part, promote HSF1 dependent transactivation [13-15].

The activity of HSF1 is also thought to be negatively regulated through a number of post-translational modifications including phosphorylation, sumoylation and acetylation [16-19]. Mass spectrometry analyses have shown HSF1 to be phosphorylated on at least 12 serine residues [13] and phosphorylation of S121, S303, S307 and S363 have been correlated with a repression in HSF1 activity [18, 20, 21]. The most comprehensively studied of these phosphorylation events are the phosphorylation of S303 and S307. However, much of what is known about S303 and S307 phosphorylation stems from *in vitro* phosphorylation experiments and *in vivo* studies using either lexA or Gal4-HSF1 fusion proteins lacking the native HSF1 DNA binding domain. As such, many of the earlier studies exploring S303 and S307-dependet regulation of HSF1 activity have resulted in conflicting results. For example, previous *in vitro* phosphorylation experiments suggested that S307 was phosphorylated by ERK which, in turn, acted as an essential priming step for GSK3-dependent phosphorylation of S303 [22]. However, subsequent *in vitro* studies suggested that S303 could also be phosphorylated by a variety of mitogen activated protein kinases (MAPK) including the stress responsive MAPK p38 [17, 18]. In addition, subsequent *in vivo* data suggested S303 phosphorylation could occur independently of S307 phosphorylation [16].

While the specific mechanism by which S303 and S307 phosphorylation repress HSF1 activity remains unclear, evidence has suggested that S303 and S307 phosphorylation represses the transactivation potential of HSF1 [18, 22, 23]. S303 and S307 are constitutively phosphorylated in the absence of stress and S303 phosphorylation levels increase after exposure to stress, suggesting that this

phosphorylation event might also contribute to HSF1 inactivation during the recovery phase [16, 17]. Interestingly, phosphorylation of S303, but not S307, promotes sumoylation of K298 [16] which, like S303 phosphorylation, also increases in response to stress exposure and represses HSF1-dependent transactivation [24]. However, it remains unclear if the repressive effects of S303 phosphorylation on HSF1 activity are exclusively mediated through K298 sumoylation or occur through additional mechanisms.

While HSF1 and the cognate HSEs are quite well conserved from yeast to humans, our previous results demonstrated that human HSF1 expressed in *S. cerevisiae* is unable to complement for the loss of the essential yeast HSF protein [25]. Further analysis showed that human HSF1 expressed in yeast was unable to form a homotrimer and consequently unable to activate HSE-dependent gene expression to support cell viability. Human HSF1 homotrimerized, became active and complemented for the loss of yeast HSF when three derepressing mutations, collectively known as LZ4m, were introduced into the repressive carboxyl-terminal coiled-coil domain [6, 25]. Further studies in yeast identified an amino-terminal linker-domain as well as a loop in the DNA binding domain as repressive elements that contributed to HSF1 repression in both yeast and mammalian cells [26, 27]. We have also used to the yeast assay system to screen for and indentify novel pharmacological activators of human HSF1 [28]. Together, these results suggest that human HSF1 expressed in yeast is maintained in a constitutively repressed state through mechanisms similar to those of mammalian cells and that the yeast system can serve as a simplified assay system to decipher the complex mechanisms regulating human HSF1 activity.

Here we report the use of the yeast assay system to further understand the mechanisms that regulate human HSF1 through phosphorylation of serine 303. Our results suggest that S303 phosphorylation blocks human HSF1

homotrimerization thereby preventing human HSF1 activation and complementation of the loss of yeast HSF. Furthermore, we demonstrate that S303 phosphorylation also blocks HSF1 homotrimerization in mammalian cells. We show that phosphorylation of HSF1 S303 in yeast occurs via the action of the MAPK Slt2 and not via the action of GSK3 and we extend these findings to show that S303 phosphorylation also occurs independent of GSK3 in mammalian cells.

Materials and Methods

Yeast Strains, Plasmids

S. cerevisiae strains used in this study are listed in Table I. Yeast expression plasmids pRS424-GPD-HSF1 and pRS424-GPD-HSF1LZ4m were described previously [25]. Point mutations were introduced into the HSF1 coding sequence using the QuickChange Site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Mammalian expression plasmids were generated by subcloning the HSF1 open reading frame from yeast vectors into the mammalian vector pcDNA3.1.

Table 1. Yeast strains used in this study.

Strain	**Genotype**
PS145	*MAT*a *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1 hsf1*Δ::*LEU2* Ycp50gal-*yHSF*
YPH499	*MAT*a *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
LNY1	*MAT*a *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1 hsf1*Δ::*LEU2* Ycp50gal-*yHSF rim11Δ::HIS3*
LNY2	*MAT*a *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1 hsf1*Δ::*LEU2* Ycp50gal-*yHSF slt2Δ::HIS3*
LNY3	*MAT*a *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rim11Δ::TRP1 mck1Δ::HIS3 mrk1Δ::URA3 ygk3Δ::kanMX*

Cell culture maintenance, transfection and siRNA

Mammalian cell lines used in the study were hsf1$^{-/-}$ MEF cells [29] and HeLa cells (ATCC, CCL-2). The MEF cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 100 U/ml penicillin/streptomycin and 55 µM 2-mercaptoethanol. HeLa cells were maintained in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. MEF cells were transfected with HSF1 expressing plasmids using a Nucleofector (Lonza) and Nucleofector solution MEF2. siRNA was purchased from Dharmacon and 2 nmoles of each siRNA were transfected into HeLa cells using Dharmafect 1. Knock-down of proteins was assayed 72 h after siRNA transfection by immunoblot analysis.

Complementation assays

Growth curve experiments were carried out in 96-well plates as described previously [28]. For spot assays yeast cells were grown overnight in galactose-containing medium to allow for expression of GAL1-yHSF and reseeded the following day at $O.D._{600}$ = 0.2 and spotted on either galactose or dextrose supplemented growth media.

Immunoblot and Crosslinking Analysis

Protein extracts were generated from yeast cultures using glass bead lysis in cell lysis buffer (25 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitors (Roche) and Halt phosphate inhibitor cocktail (Thermo Scientific Pierce). Proteins extracts were generated from mammalian cell culture using cell lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentrations were quantified using the BCA assay and 80-100µg of total protein was resolved by SDS-PAGE and transferred to

a nitrocellulose membrane. HSF1 oligomerization was assessed using the amine-specific cross-linker ethylene glycol bis-succinimidyl succinate (EGS) [30]. Crosslinking analysis were carried out as described previously [28]. Antibodies used in this study were anti-phospho-S303 (pS303) (ab47369, Abcam), anti-HSF1 [28], anti-Pgk1, anti-FLAG (M2, Sigma), anti-Hsp70 (C92, Stressmarq), anti-β-catenin (6B3, Cell Signaling), anti-GAPDH (6C5, Ambion) and anti-GSK3α/β (D75D3, Cell Signaling). Quantification of immunoblot data was done using Photoshop.

Results

Phosphorylation of S303 contributes to repression of human HSF1 in yeast.

When human HSF1 is expressed in yeast it is unable to homotrimerize, promote gene expression and complement for the loss of the essential yeast HSF protein [25]. Because our previous work suggested that when HSF1 is expressed in yeast it exists in a constitutively repressed monomeric state, we sought to use the yeast assay system to better understand the complex mechanisms regulating HSF1 activity in mammalian cells. An important component of HSF1 repression occurs through the phosphorylation of serine 303 and serine 307 [17, 18]. Because S303 and S307 are constitutively phosphorylated in mammalian cells and alanine substitution of S303 or S307 promotes constitutive activation of HSF1 in mammalian cells in reporter assays [17, 18] we tested whether S303 and/or S307 contribute to HSF1 repression in yeast. Wild-type HSF1 or the individual S303A, S307A or S303/307A double mutants were expressed in yeast strain PS145 which lacks a chromosomal copy of the essential yeast HSF gene and constitutively expresses yeast HSF episomally from a galactose inducible and dextrose repressible promoter [31]. When PS145 is grown in the presence of dextrose as the sole carbon source, yeast HSF expression is extinguished and growth becomes

solely dependent on HSF1 which is episomally expressed [28]. While wild-type human HSF1 was unable to complement for the loss of yeast HSF, expression of the S303A, S307A or S303/307A HSF1 mutants allowed for human HSF1-dependent yeast growth (Figure 1A, B). Interestingly, the S303/307A double HSF1 mutant did not display enhanced activity over the S303A mutant (Figure 1B) suggesting that phosphorylation of both S303 and S307 modulate HSF1 repression through similar mechanisms.

To ascertain whether HSF1 is being phosphorylated in yeast, we employed a commercially available antibody specific for phospho-S303 (pS303). Because this antibody has not previously been characterized in the literature, we tested its specificity in human HeLa cells where S303 is known to be constitutively phosphorylated [17]. As shown in Figure 1C, using this antibody we detected that endogenous HSF1 was constitutively phosphorylated in HeLa cells in the absence of stress. We also observed an increase in S303 phosphorylation in response to a heat shock, which correlated with previous reports [16]. Importantly, the pS303-specific antibody did not detect HSF1 when HeLa extracts were treated with lambda protein phosphatase prior to immunoblot analysis (Figure 1C), nor does it detect HSF1 when S303 is mutated to alanine (Figure 1D). Together, these data suggest that the antibody is specific for HSF1 that is phosphorylated on S303. The detection of HSF1 using a polyclonal anti-HSF1 antibody demonstrates that there are no significant differences in the steady state levels of HSF1 either treated or untreated with lambda phosphatase (Figure 1C).

Consistent with a contribution to HSF1 repression (Figure 1A, B) S303 is robustly phosphorylated when HSF1 is expressed in yeast (Figure 1D, E). Interestingly, phosphorylation of S303 was also observed when the S307A mutant was expressed in yeast though it was reduced by approximately 50% when compared

to wild-type HSF1 (Figure 1D, E). While this observation supports a previous report indicating that S303 phosphorylation could occur independently of S307 phosphorylation in mammalian cells [16], these data also suggests that under certain circumstances S303 phosphorylation may be enhanced by S307 phosphorylation. In addition, although a correlation between S303 and S307 phosphorylation and HSF1 protein stability has not been previously reported, we repeatedly observed two to three-fold higher steady state levels of HSF1 when the S303A, S307A and S303/307A mutants were expressed in yeast (Figure 1D, F). While an antibody specific for phospho-S307 is commercially available, we have been unable to detect S307 phosphorylation of human HSF1. As such we focused our investigation on S303-phosphorylation dependent repression of human HSF1. Human HSF1 S303 phosphorylation is known to promote sumoylation of lysine 298, which also contributes to the repression of HSF1 activity [16]. Therefore, to further investigate the idea that human HSF1 is being actively repressed in yeast, we explored the possibility that K298, like S303, contributes to HSF1 repression in yeast. However, unlike the S303A HSF1 mutant, the K298R mutant did not promote HSF1-dependent growth (Figure 1E), suggesting that at least in yeast, K298 does not significantly contribute to HSF1 repression. We also did not observe a reduction in human HSF1-dependent yeast growth for the S303A/K298R double mutant, indicating that K298 is also not required for HSF1 activity in yeast (Figure 1E).

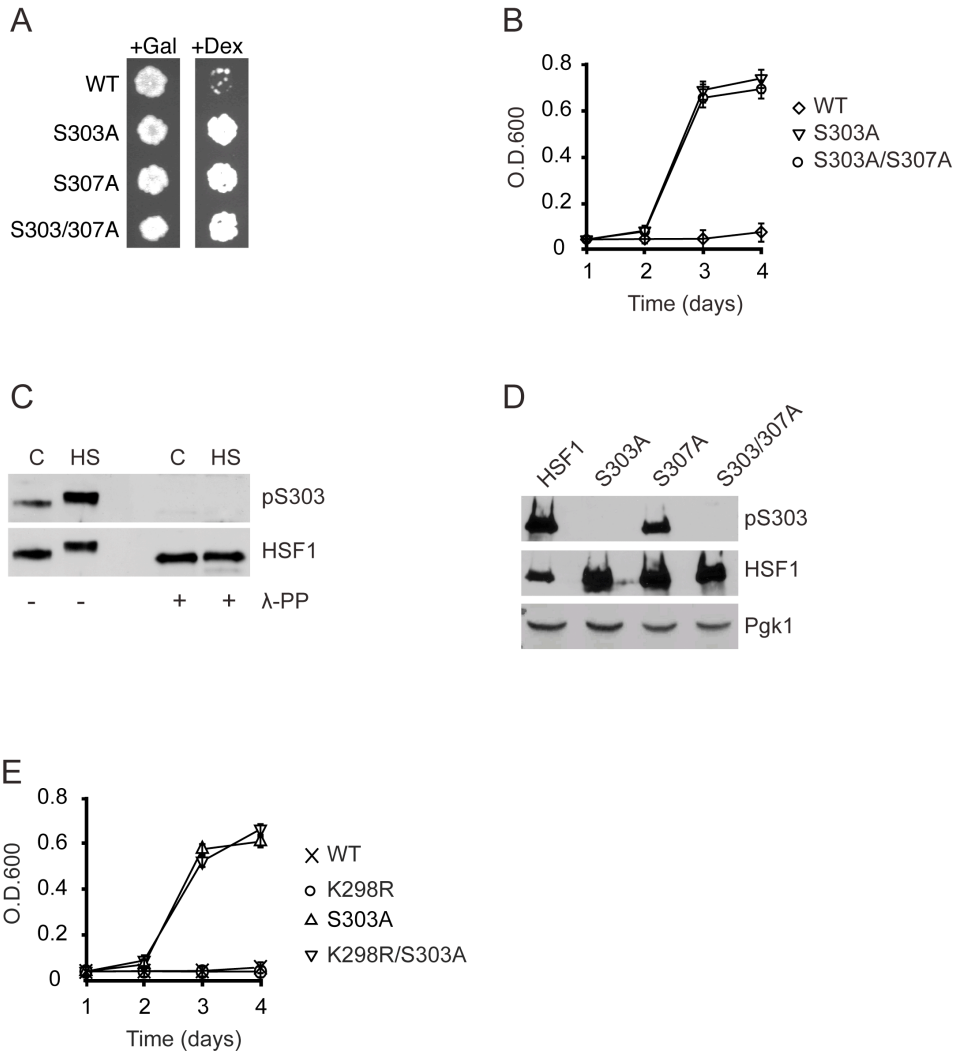

Figure 1. S303 phosphorylation represses HSF1 activity in yeast. (**A**) PS145 yeast strains expressing wild-type HSF1 [18] or the S303A, S307A or S303/307A mutants were plated on either galactose or dextrose supplemented medium. (**B**) PS145 expressing either wild-type HSF1 or the S303A or S303/307A mutants were grown in dextrose containing medium for 4 d. Growth was monitored by

measuring $O.D._{600}$. (**C**) HeLa cells were grown at 37˚C (C) or heat shocked for 2 h at 42˚C (HS). Total protein extracts were treated with lambda protein phosphatase and analyzed for phospho-S303 (pS303) and total HSF1 levels by immunoblotting. (**D**) PS145 was transformed with a plasmid expressing wild-type HSF1 [18] or mutant alleles of HSF1 and grown on galactose containing medium. Total protein extracts were analyzed for pS303, HSF1 and Pgk1 by immunoblotting. (**E**) Levels of HSF1 phosphorylated at S303 were quantified and are shown as a percent of total HSF1, from panel D. (**F**) Protein levels of HSF1 were normalized to Pgk1, from panel D. (**G**) PS145 expressing either wild-type HSF1 or mutant HSF1 alleles were assayed for HSF1-dependent growth as in **B**.

S303 represses trimer formation of HSF1 in yeast and mammalian cells

Previous reports have suggested that phosphorylation of S303 represses the ability of HSF1 to transactivate gene expression [18, 22, 23]. Here we show that the HSF1 S303A mutant functionally complements for the lost of yeast HSF (Figure 1A, B). Based on our previous work this indicates that S303 phosphorylation might also regulate the ability of human HSF1 to homotrimerize [25]. To test this hypothesis we carried out EGS cross-linking experiments in conjunction with immunoblot analysis to ascertain if S303 phosphorylation regulates the homotrimerization of human HSF1 in yeast. When the S303A HSF1 mutant was expressed in yeast we detected approximately 2-fold higher levels of trimerized HSF1 at the intermediate EGS concentration than when wild-type HSF1 was expressed in yeast (Figure 2A). However, trimerization of the S303A HSF1 mutant was lower than trimerization of the LZ4 HSF1 mutant, previously demonstrated to be constitutively trimerized in yeast and mammalian cells and

able to complement for the loss of yeast HSF [6, 25]. We observed similar results for the S307A and S303/307A HSF1 mutants (data not shown) further supporting the notion that S303 and S307 phosphorylation repress HSF1 activity through similar mechanisms. We next evaluated whether HSF1 S303 phosphorylation could also function to repress homotrimer formation in mammalian cells. To test this hypothesis we expressed wild-type HSF1 or the S303A, S307A or S303/307A mutants in hsf1$^{-/-}$ mouse embryonic fibroblasts (MEF) [29] and assayed for HSF1 trimerization in the absence of thermal stress by EGS crosslinking and immunoblotting. While wild type HSF1 could be detected as a multimer in these extracts, we observed approximately 2-fold higher levels of the HSF1 trimer for the HSF1 S303A mutant (Figure 2B) as well as the S307A and S303/307A mutants (data not shown).

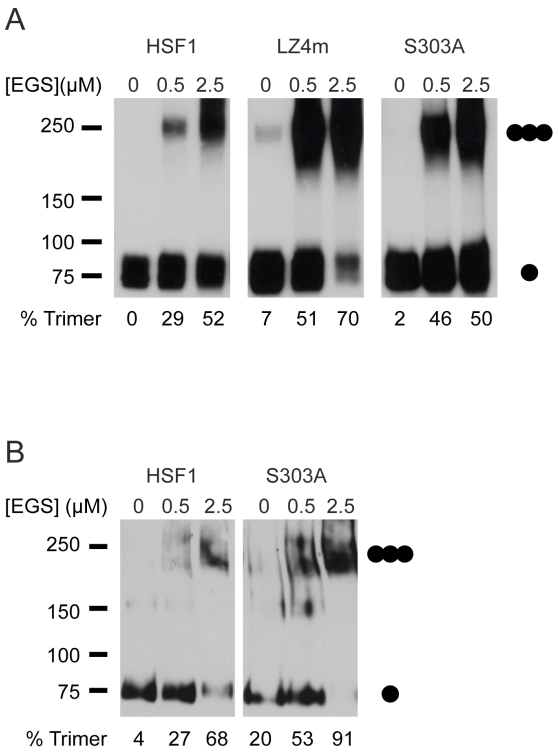

Figure 2. S303 represses trimer formation of HSF1 in yeast and mammalian cells. (**A**) PS145 was transformed with wild-type HSF1, the LZ4m mutant or the S303A mutant and grown on galactose containing medium. Total protein extracts were evaluated for HSF1 multimerization by EGS crosslinking, SDS-PAGE, and immunoblotting using an HSF1 specific antibody. The positions of molecular weight markers are indicated on the left, and circles indicating the expected migration of HSF1 monomers and trimers are on the right. Levels of HSF1 trimer as percent of total HSF1 are shown below. (**B**) $hsf1^{-/-}$ MEFs were transfected with a plasmid expressing wild-type HSF1 or the S303A mutant and analyzed for HSF1 multimerization by EGS cross-linking as in **A**.

S303 phosphorylation and coiled-coil interactions synergize in HSF1 repression.

In addition to post-translational modifications, HSF1 activity is also thought to be repressed through intramolecular interactions between carboxyl- and amino-terminal coiled-coil domains and mutations in these domains render HSF1 constitutively trimerized, nuclear localized and bound to DNA in mammalian cells [6]. Because our results suggest that S303 phosphorylation might also regulate homotrimer formation, we tested the combined affects of both the S303A as well as the LZ4m mutations on human HSF1 activity in yeast. A human HSF1 mutant containing both the S303A and LZ4m mutations was created and its ability to promote human HSF1-dependent yeast growth was compared to the individual HSF1 mutants as well as wild-type HSF1 in quantitative cell growth assays. The individual S303A and LZ4m HSF1 mutants promoted human HSF1-dependent yeast growth to a similar extent, though neither the LZ4m nor the S303 mutant were fully derepressed, as the S303A/LZ4m double mutant displayed enhanced human HSF1-dependent yeast growth (Figure 3A). While we currently do not know if the S303A/LZ4m double HSF1 mutant has an increased propencity to trimerize, previous studies have shown that the LZ4m mutant, when expressed in yeast is not maximally trimerized and trimerization can be further enhanced via the addition of pharmacological HSF1 activators [28]. While we observed higher steady state protein levels for both the S303A and LZ4m mutants in comparison to wild-type HSF1 when expressed in yeast, no further increases in protein levels were observed for the double mutant (Figure 3B, C). These results suggest that while both HSF1 S303 phosphorylation and coiled-coil interactions regulate human HSF1 multimerization in yeast, they do so via distinct mechanisms. We also did not observe changes in HSF1 S303 phosphorylation when the LZ4m

mutant was expressed in yeast, consistent with the notion that HSF1 trimerization does not affect HSF1 S303 phosphorylation.

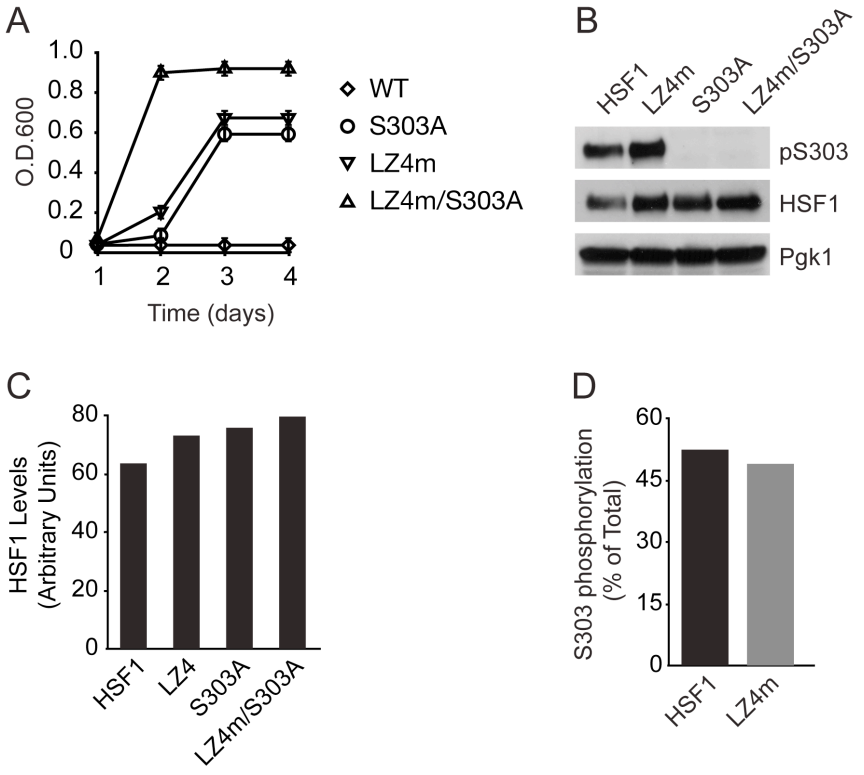

Figure 3. Phosphorylation of S303 and coiled-coil domains synergize in the repression of HSF1 in yeast. (**A**) PS145 expressing either wild-type HSF1 or mutant alleles of HSF1 were grown in dextrose supplemented medium for 4 d. Growth was monitored by measuring $O.D._{600}$. (**B**) PS145 was transformed with a plasmid expressing wild-type HSF1 [18] or mutant alleles of HSF1 and grown on galactose containing medium. Total protein extracts were analyzed for pS303, total HSF1 and Pgk1 by immunoblotting. **(C)** Protein levels of HSF1 were

normalized to Pgk1, from panel B. **(D)** Levels of HSF1 phosphorylated at S303 were quantified and are shown as a percent of total HSF1, from panel **B**.

Gsk3 regulates human HSF1 activity in yeast independent of S303 phosphorylation.

Previous reports using *in vitro* phosphorylation experiments have suggested that HSF1 is phosphorylated at S303 by glycogen synthase kinase 3 (GSK3) [20, 22, 32]. However, it remains unclear if GSK3 phosphorylates and represses HSF1 via S303 phosphorylation *in vivo*. To test if GSK3 contributes to HSF1 repression, we assayed human HSF1-dependent yeast growth in a strain also lacking the yeast GSK3 homolog Rim11. Supporting the notion that yeast GSK3 can repress human HSF1 activity in yeast we observed human HSF1-dependent yeast growth as well as HSF1 multimerization in the *rim11*Δ strain (Figure 4A, B). However, HSF1-dependent yeast growth in the *rim11*Δ strain was less robust than growth of a wild-type strain expressing the S303A HSF1 mutant (Figure 4A). Furthermore, when we expressed the S303A HSF1 mutant in the *rim11*Δ strain we observed HSF1-dependent growth at a rate similar to the growth of the S303A mutant in wild-type cells. This suggested the possibility that HSF1 might not be fully derepressed in the *rim11*Δ strain. Consistent with this idea, we did not detect a reduction in S303 phosphorylation in the *rim11*Δ strain (Figure 4C). *S. cerevisiae* encodes four separate yet partially functionally redundant GSK3 homologues [33], suggesting the possibility that S303 remains phosphorylated in the *rim11*Δ strain due to phosphorylation through other GSK3 proteins. To test this hypothesis we assayed the phosphorylation state of HSF1 at S303 in a yeast strain lacking all four isoforms of yeast GSK3. As shown in Figure 4D, no reduction in S303 phosphorylation was observed in the 4x*gsk3*Δ strain suggesting that while

yeast GSK3 does contribute to HSF1 repression, it does so independently of S303 phosphorylation.

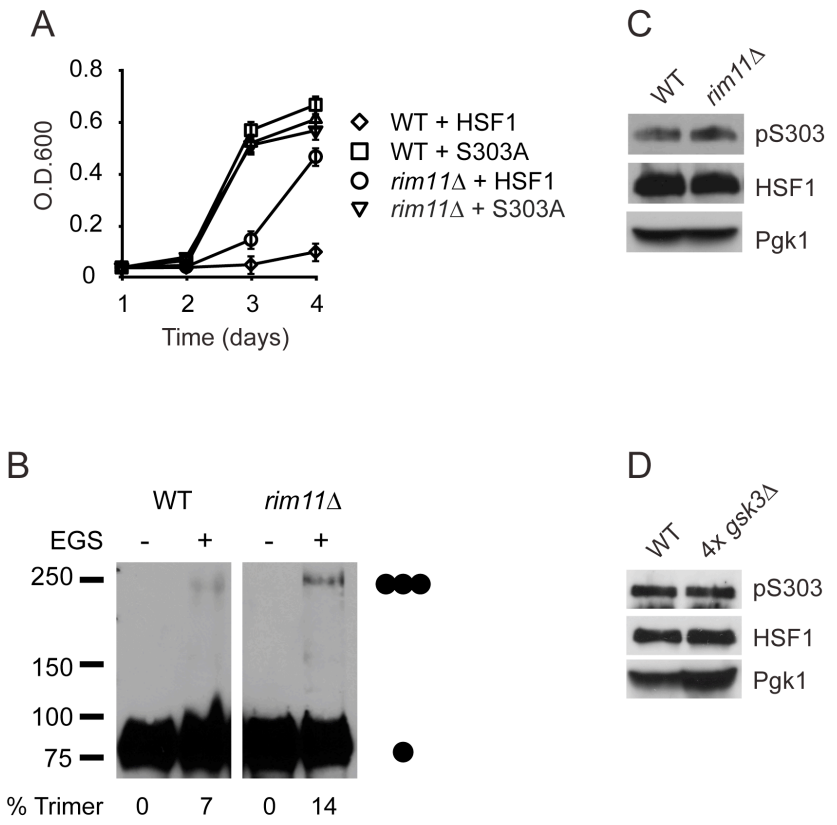

Figure 4. GSK3 represses HSF1 activity in yeast independent of S303. (**A**) PS145 [18] expressing wild-type HSF1 or the S303A HSF1 mutant and LNY1 (*rim11*Δ) expressing wild-type HSF1 were grown in dextrose supplemented medium for 4 d. Growth was monitored by measuring $O.D._{600}$. (**B**) PS145 [18] and LNY1 (*rim11*Δ) expressing wild-type HSF1 were grown on galactose containing medium and were

evaluated for HSF1 multimerization by EGS crosslinking, SDS-PAGE, and immunoblotting using an HSF1 specific antibody. The positions of molecular weight markers are indicated on the left, and circles indicating the expected migration of HSF1 monomers and trimers are on the right. Levels of HSF1 trimer as percent of total HSF1 are shown below. (**C**) PS145 [18] and LNY1 (*rim11*Δ) were transformed with a plasmid expressing wild-type HSF1 and were grown on galactose containing medium. Total protein extracts were analyzed for pS303, total HSF1 and Pgk1 by immunoblotting. (**D**) YPH499 [18] and LNY3 (4x*gsk3*Δ) were transformed with a plasmid expressing wild-type HSF1 and were grown in dextrose containing medium. Total protein extracts were analyzed for pS303, total HSF1 and Pgk1 by immunoblotting.

Results shown here for the S303A HSF1 mutant and previously published for the LZ4m HSF1 mutant suggest that mechanisms that regulate HSF1 in mammalian cells are at least partially conserved with regulation of human HSF1 expressed in yeast cells. Therefore, we carried out experiments to ascertain if GSK3 might also repress HSF1 independently of S303 phosphorylation in mammalian cells. To explore this possibility HeLa cells were treated with the GSK3 inhibitor SB-216763 [34] and assayed for HSF1 S303 phosphorylation as ascertained by immunoblotting with the anti-pS303 antibody. While SB-216763 treatment strongly inhibited GSK3 activity as shown by increased β-catenin levels [35], no reduction in S303 phosphorylation was observed (Figure 5A). However, similar to the results obtained from our yeast experiments, SB-216763 did promote activation of HSF1 under normal growth conditions, as determined by immunoblot analysis of Hsp70 expression (Figure 5A). This result is consistent with a previous report showing increased Hsp70 expression in response to lithium

treatment, which also inhibits GSK3 function [36]. siRNA mediated knock-down of the two GSK3 isoforms in mammals, GSK3α and GSK3β, either singly or in combination, further confirmed that, while β-catenin expression was elevated, HSF1 S303 was not appreciably phosphorylated by GSK3 in unstressed mammalian cells (Figure 5B). Together, data from experiments in both yeast and mammalian cells support a model in which GSK3 inhibits HSF1 activity through a mechanism that is independent of S303 phosphorylation.

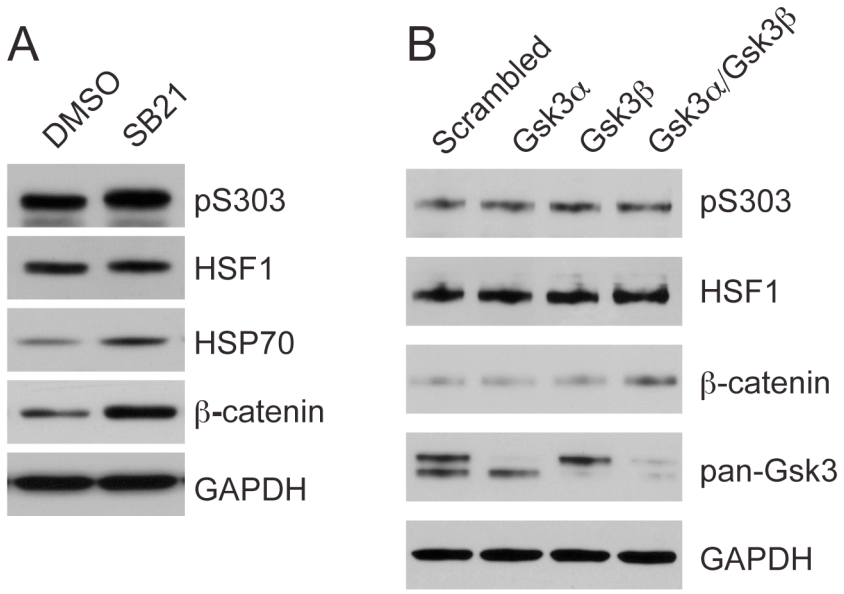

Figure 5. GSK3 represses HSF1 activity in HeLa cells independent of S303 phosphorylation. (**A**) HeLa cells were treated with DMSO solvent or the GSK3 inhibitor SB-216763 (25 μM) for 15 h. Total protein was analyzed for pS303, HSF1, and β-catenin by immunoblotting. GAPDH serves as a loading control. (**B**) HeLa

cells were treated with siRNA specific for GSK3α and GSK3β either individually or together or a scrambled siRNA for 72h. Total protein was analyzed for pS303, total HSF1, β-catenin, GSK3α/β and GAPDH by immunoblotting.

Slt2 represses human HSF1 activity via S303 phosphorylation in yeast.

To begin to identify which protein kinase(s) in yeast phosphorylate human HSF1 at S303 to promote HSF1 repression, we assayed S303 phosphorylation in several previously generated protein kinase deletion strains obtained from the yeast gene deletion collection [37]. One strain in which we detected severely reduced levels of human HSF1 S303 phosphorylation was a strain deleted for the *SLT2* gene, encoding a stress-responsive MAPK [38, 39], consistent with S303 lying within a consensus site for MAPK-dependent phosphorylation (Figure 6A) [40]. This suggests that Slt2 either directly or indirectly promotes the phosphorylation of human HSF1 expressed in yeast. This hypothesis was further supported by the observation that an *slt2*Δ strain allowed wild type human HSF1-dependent yeast growth at a rate similar to the HSF1 S303A mutant, while no growth was observed in the *SLT2* wild-type strain (Figure 6B). Homotrimerization of wild-type human HSF1 was observed in the *slt2*Δ strain at levels similar to the S303A and LZ4m HSF1 mutants, further supporting the notion that the Slt2 MAPK represses human HSF1 multimerization in yeast (Figure 6C). In mammalian cells the most closely related homolog of Slt2 is the MAPK ERK5 [41]. However, using siRNA-mediated knock-down of ERK5 we were unable detect an effect of ERK5 on HSF1 S303 phosphorylation in mammalian cells (data not shown). This may suggest that in mammalian cells S303 can be phosphorylated by multiple MAPKs. This hypothesis is supported by previous data showing that ERK1/2 as well as the stress-responsive MAPK p38 could phosphorylate HSF1 at S303 *in vitro* [18]. In

addition, our data showing reduced, but not eliminated phosphorylation of S303 in the *slt2*Δ strain (Figure 6A) also support a model where S303 may be phosphorylated by multiple MAPKs.

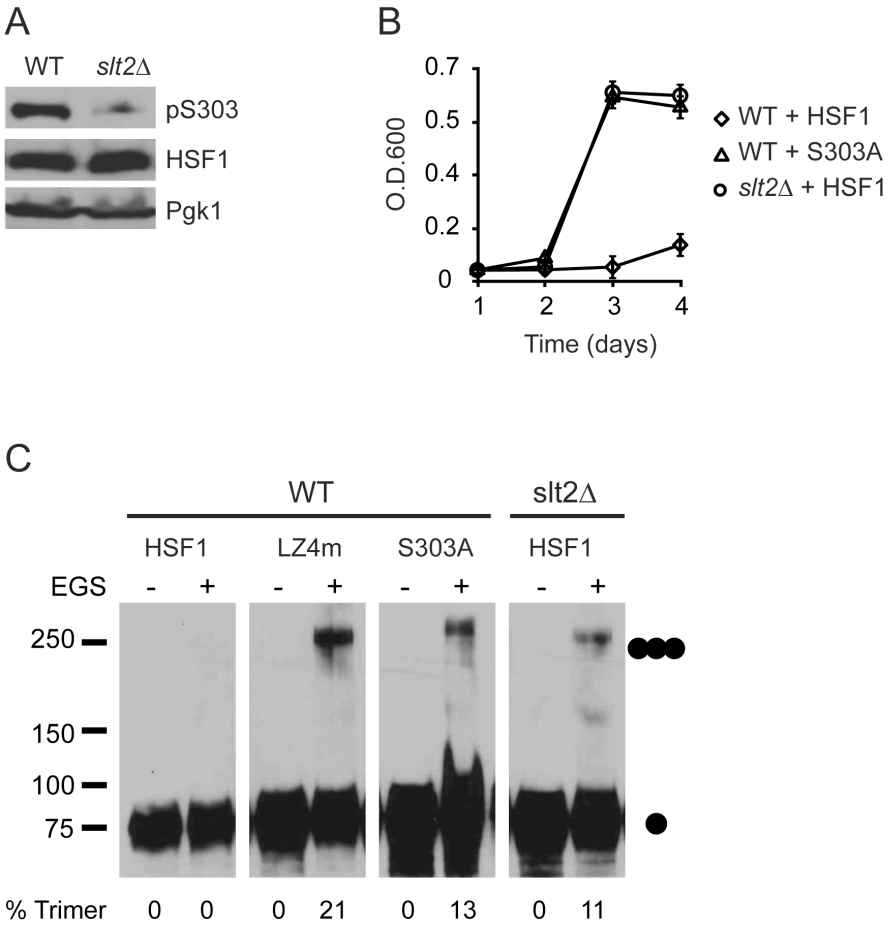

Figure 6. S303 phosphorylation of HSF1 in yeast is modulated by Slt2. (**A**) PS145 and LNY2 (*slt2*Δ) were transformed with a plasmid expressing wild-type HSF1 and were grown on galactose containing medium. Total protein extracts were

analyzed for pS303, total HSF1 and Pgk1 by immunoblotting. (**B**) PS145 [18] expressing wild-type HSF1 or the S303A mutant or LNY2 (*slt2*Δ) expressing wild-type HSF1 were grown in dextrose supplemented medium for 4 d. Growth was monitored by measuring $O.D._{600}$. (**C**) PS145 [18] expressing wild-type HSF1, the LZ4m mutant or the S303A mutant and LNY2 (*slt2*Δ) expressing HSF1 were evaluated for HSF1 multimerization by EGS cross-linking, SDS-PAGE, and immunoblotting. The positions of molecular weight markers are indicated on the left and circles indicating the expected migration of HSF1 monomers and trimers are on the right. Levels of HSF1 trimer as percent of total HSF1 are shown below.

Expression of S303A and S307A mutants in hsf1 $^{-/-}$ cells results in constitutive activation of Hsp70 expression.

Previous studies have assayed the function of S303 and S307 phosphorylation in HSF1 regulation via *in vitro* phosphorylation experiments [22], *in vivo* using lexA/Gal4-HSF1 fusion proteins lacking the native HSF1 DNA binding domain [17, 18] or via overexpression of a S303A HSF1 mutants in mammalian cells expressing endogenous wild-type HSF1 [16]. We tested the consequences of loss of S303 and S307 phosphorylation on HSF1 activity in the context of the entire protein using hsf1$^{-/-}$ MEFs, which lack endogenous HSF1. When we expressed S303A, S307A or S303/307A HSF1 mutants in hsf1$^{-/-}$ MEFs we observed a modest elevation of Hsp70 expression under normal growth conditions (Figure 7A, B) consistent with the hypothesis that S303 phosphorylation modulates both homotrimerization as well as transactivation by HSF1. However, HSF1 was not fully activated through the S303A and S307A mutations, as expression of Hsp70 was further enhanced when the transfected cells were exposed to low levels of

the proteasome inhibitor MG132 (Figure 7A, B). This is consistent with our data generated in yeast demonstrating that while the S303A mutation did activate human HSF1-dependent yeast growth, this was further enhanced when the S303A HSF1 mutant was combined with the LZ4m mutation (Figure 3A). Interestingly, in hsf1$^{-/-}$ cells we observed a faster electrophoretic mobility on SDS-PAGE gels for the HSF1 S303A and S303/307A mutant proteins that was not observed for wild-type HSF1 or the S307A mutant (Figure 7A), nor did we observe this change in mobility in the yeast system (Figure 1D). While the nature of this electrophoretic mobility shift is unknown, the HSF1 S303A and S303/S307A mutant alleles also exhibited lower steady state levels when exposed to MG132, suggesting that these proteins, despite having increased activity, might be less stable (Figure 7A, C). Because S303 phosphorylation has been proposed to promote HSF1 sumoylation in mammalian cells [16] it is possible that lack of sumoylation results in the altered electrophoretic mobility. Despite the fact that equal amounts of plasmid DNA were transfected for each mutant, we observed elevated steady state protein levels for the HSF1 S307A mutant (Figure 7A, C). While we have not definitively demonstrated that the S307A mutant protein has increased stability in comparison to wild-type HSF1, this finding correlates with the increased protein levels we observed for the HSF1 mutants expressed in yeast (Figure 1D, F) and will require further investigation. Interestingly, when we expressed the HSF1 S307A mutant in hsf1$^{-/-}$ cells we did not observe a reduction in S303 phosphorylation (Figure 7A, D) as was observed in yeast cells (Figure 1D, E) suggesting that priming requirements for S303 phosphorylation may change in different expression systems.

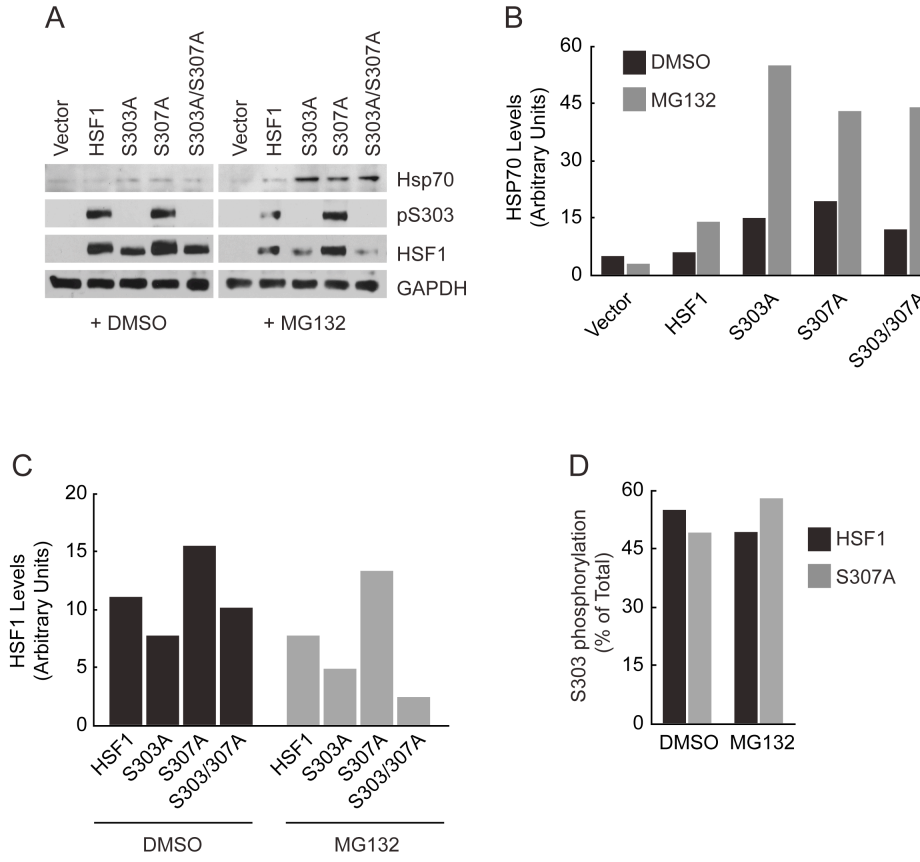

Figure 7. (**A**) hsf1$^{-/-}$ MEFs were transfected with an empty vector or plasmids expressing wild-type HSF1 or the S303A, S307A or the S303/307A mutants. The transfected cells were treated with DMSO solvent or MG132 (10 µM) for 5 h. Total protein extracts were analyzed for Hsp70, pS303 and HSF1 by immunoblotting. GAPDH serves as a loading control. (**B**) Protein levels of Hsp70 were normalized to GAPDH, from panel A. (**C**) Protein levels of HSF1 were normalized to GAPDH, from panel A. (**D**) Levels of HSF1 phosphorylated at S303 were quantified and are shown as a percent of total HSF1, from panel **A**.

Discussion

Mammalian HSF1 activity is regulated via complex regulatory mechanisms that include post-translation modifications as well as inter- and intra-molecular protein-protein interactions [2]. While our understanding of these regulatory mechanisms remains incomplete, earlier work has suggested that many of these mechanisms may be conserved in yeast [25-28]. This is evident, in part, by repression of the human HSF1 protein when it is expressed in *S. cerevisiae* via coiled-coil domain and HSF1 loop interactions. In this report we show that evaluation of the mechanisms that regulate HSF1 activity in yeast via post-translational modifications can lead to important insights into the mechanisms that regulate HSF1 in mammalian cells.

Previous experiments using HSF1 fusions with the constitutively bound Gal4 or lexA DNA-binding domains demonstrated that phosphorylation of S303 contributed to the repression of HSF1 transactivation [17, 18]. In this report we show that alanine substitution of S303, in the context of full length HSF1, also results in increased levels of trimerized HSF1 both in un-stressed yeast and in mammalian cells. This suggests that aside from repressing transactivation, S303 phosphorylation can also repress earlier points in the HSF1 activation pathway. Interestingly, we also show that repression of HSF1 activity through S303 phosphorylation may occur independent of K298 sumoylation in yeast, as arginine substitution of K298 does not promote HSF1 activation in yeast. It should be noted that not all of the mechanisms that regulate human HSF1 in mammalian cells are conserved in yeast. While human HSF1 is repressed in both yeast and mammalian cells through an amino-terminal coiled-coil as well as a carboxyl-terminal linker domain, the ability of wild type human HSF1 to respond to

proteotoxic compounds or thermal stress, for example, appears to be strikingly absent in yeast [25, 27, 28]. Nevertheless, the ability of S303 phosphorylation to promote repression of human HSF1 in yeast independent of K298 sumoylation suggests that our understanding of the mechanisms by which S303 phosphorylation represses HSF1 activity remains incomplete. S303 and S307 are located in the regulatory domain of HSF1, a proposed binding site for the protein chaperone Hsp90 [42]. As such, it is tempting to speculate that phosphorylation of these residues might affect binding to Hsp90.

An understanding of how phosphorylation regulates HSF1 activity and what protein kinases phosphorylate HSF1 remains largely incomplete [20, 22]. Early reports showed that *in vitro*, HSF1 S307 phosphorylation acted as an essential priming event for S303 phosphorylation [22]. However, a subsequent report showed this priming event was not required *in vivo* and that HSF1 S303 phosphorylation occurred independent of S307 phosphorylation in K562 cells [16]. The work presented here using the yeast model system furthers our understanding of these regulatory mechanisms and may begin to clarify the conflicting mechanisms underlying S303 phosphorylation. Specifically, our data suggest that while phosphorylation of S303 can occur independently of S307 phosphorylation in both yeast and mammalian cells, S303 phosphorylation may be enhanced by S307 phosphorylation in the non-native yeast system. While a mechanistic basis for this difference in the requirements for S303 phosphorylation remains unknown when HSF1 is expressed in yeast, structural differences could change the priming requirements for S303 phosphorylation. Such changes in HSF1 might occur due to different protein interactions and as such it is not surprising that in *in vitro* experiments, using only recombinant HSF1 protein, phosphorylation of S303 is fully dependent on S307 phosphorylation.

However, further studies will be required to fully test these hypotheses.

Here, we demonstrate that in both yeast and mammalian cells phosphorylation of HSF1 S303 appears to occur independently of GSK3, previously thought to be the primary kinase responsible for S303 phosphorylation [20, 22]. Rather, as suggested by loss of function analysis, we propose that the MAPK Slt2 is one candidate that phosphorylates HSF1 at S303 in yeast though residual phosphorylation of HSF1 at S303 in an *slt2*Δ strain suggests that other MAPKs may also contribute to S303 phosphorylation. Differences in HSF1 structure between the *in vivo* and *in vitro* systems may also explain why different kinases can target S303 for phosphorylation under different conditions. We speculate that under some cellular conditions, for example physiological stress or different cell types, HSF1 structure may be altered, thereby shifting the S303-kinase specificity from a MAPK to GSK3. This might, in part, contribute to the complexity in identifying all of the mammalian kinases that phosphorylate S303. While GSK3 does not appear to phosphorylate HSF1 at S303 *in vivo*, data presented here nevertheless support a role for GSK3 as a repressor of HSF1 activity. It should be noted that several other serine residues in the HSF1 coding sequence, including S307, are located within putative GSK3 consensus sites [40].

The importance in understanding HSF1 regulation is underscored by recent findings showing that pharmacological activation of HSF1 can increase protein chaperone expression and ameliorate cytotoxicity in models of protein folding disease [28, 43-46]. As such, it is important to further our understanding of the mechanisms that repress HSF1 activity as potential points of therapeutic intervention in disease. For example, our data has shown that the loss of S303-dependent HSF1 repression can lead to the accumulation of protein chaperones and as such could be efficacious in the treatment of protein folding diseases. In

support of this possibility Rimoldi *et al* showed that over-expression of the HSF1 S303G mutant in HeLa cells reduced aggregation and inclusion formation of an aggregation prone Ataxin1-31Q mutant protein [47] In addition, Fujimoto *et al* showed that overexpression of a constitutively active HSF1 mutant lacking the regulatory domain, which includes S303 and S307, suppressed the aggregation and cytotoxicity of a mutant Huntingtin protein in both cell culture and mice [48]. Furthermore, Carmichael *et al* suggested that GSK3-inhibitors might prove useful in the treatment of polyQ-expansion diseases [49].

Acknowledgements

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Chapter 3

Discussion and Future Perspectives

Discussion

Arsenic is one of the most widely studied elements in the field of metal intoxication after lead (Pb). As is a metalloid found in water, soil, and air from natural and anthropogenic sources and exists in inorganic and organic forms [1]. The major inorganic forms of As include the trivalent arsenite (AsIII) and the pentavalent arsenate (AsV). AsIII is known to be more toxic than the AsV [2]. Humans can be exposed to As via air and food, but the major exposure route to As is through drinking water, especially in India, Bangladesh, China, and some Central and South American countries [3]. For instance the As concentrations in drinking water in Argentina are 200 ppb, Mexico 400 ppb, Taiwan 50–1980 ppb and in the Indo-Bangladesh region 800 ppb, values well above the WHO guidelines' maximum permissible value, 10 ppb. Chronic arsenicosis due to drinking arsenic-contaminated water is reported to affect more than 200 million people worldwide [4]. Several studies have reported that arsenic intoxication can cause internal cancers [5], blackfoot disease [6], vascular diseases [7] and diabetes [8].

Although As is considered a human carcinogen it is also a therapeutic agent and its cellular metabolism has been extensively studied to further understand its mechanisms of action. It has been suggested that As toxicity has several modes of action such as production of chromosomal abnormalities, promotion of carcinogenesis and oxidative stress, among others [9, 10]. To date studies have not shown a link between As and Fe homeostasis. Our data in chapter 1 suggests for the first time that As stress deregulate the expression of genes involved in cellular iron homeostasis. It activates Aft1 iron-responsive transcription factor and

promotes the uses of the non-reductive pathway mediated by the ARN family permeases, to uptake iron. Most Importantly it causes *FET3* mRNA down-regulation by the 5'-3' Xrn1 and causes Fet3 mislocalization in the ER being degraded after 4hrs of exposure. In conclusion As stress induces a response similar to that induced by iron deficiency. Moreover, we also obtained preliminary data suggesting that As disruption of iron homeostasis also occurs in mammalian cells. This connection between arsenic and iron might shed new light on the mechanism of action of arsenic.

Over the past two decades, the role of HSF1 has been solidly established in several biochemical and genetic experiments. Yet, we are far from fully understanding the biology of HSF1. HSF1 is the major stress-activated transcription factor enhancing the expression of *hsp* genes that encode molecular chaperones that facilitate protein folding and suppress protein aggregation, this response plays a major role in maintaining protein homeostasis and is called as the heat shock response (HSR) [11]. The requirement of an essential response to acute and chronic proteotoxic damage imposes aspects as detection, signaling, and response to misfolding. This is achieved by hierarchical regulation of HSF1. In the chapter 2 of this work we emphasize the biochemistry of HSF1 during its complex multistep mechanism of regulation, in particular its posttranslational signature. Several kinases have been linked to the phosphorylation of HSF1. The specific kinases that phosphorylate at Ser303 and Ser307, serine residues involved in the HSF1 repression step, remain unknown. Early *in vitro* experiments suggested that Ser303 was phosphorylated via glycogen synthase kinase 3 (GSK3) and that Ser307

was phosphorylated via extracellular signal-regulated kinase 1 and 2 (ERK1/2) [12]. However, our data suggest that although GSK3 does repress HSF1 activity, such happens independently of the phosphorylation at Ser303. Moreover our results show that Ser303 may be phosphorylated by a mitogen-activated protein kinase (MAPK). In conclusion, the identification of the kinase(s) that phosphorylate these residues *in vivo* could be important for developing agents to target neurodegenerative diseases via HSF1 activation.

Future Perspectives

The results obtained with this graduation work helped us to elucidate the mechanisms regarding two different stress responses, the stress response to arsenic exposure and the stress response to misfolding proteins. However throughout this dissertation these stress mechanisms were handle using different approaches a very recent study as just shown that HSF1 can be activated by arsenite (AsIII) [13]. The authors show that arsenic trioxide (ATO) promotes HSF1 phosphorylation at S326 and induces the expression of the Hsp27 and Hsp70, which in turn may protect cells from ATO cytotoxicity. On the other side the inhibition of HSF1 function not only reduced ATO induction of HSP27 and 70, but enhanced ATO cytotoxicity by increasing apoptosis. Moreover the treatment of cells with ATO and PI103, an inhibitor of the members of the phosphatidylinositol 3-kinase (PI3K) family, suppressed not only ATO-induced Hsp70 expression but also the phosphorylation at S326.

These findings represent a very promising discovery toward a new field of research establishing a potential connection between HSF1 and arsenic toxicity with great impact for human diseases.

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Appendix

Table 1: Human disease genes that are found in Yeast.

Human Disease Gene	Yeast Gene	Brief disease description
N-Acetylglucosamine phosphatidylinositol biosynthetic protein	SPT14	Haemolytic blood disorder (venous thrombosis)
Adenine phosphoribosyltransferase	APT1/APT2	Urolithiasis
Adenosine deaminase	Yn1141w	Immunodeficiency
Adenylate kinase	URA6/ADK1	Haemolytic anaemia
S-Adenosylmethionine synthetase	SAM1/SAM2	Hypermethioninaemia; mental and motor retardation
Adenylosuccinate lyase	Y1r359w	Purine nucleotide biosynthesis defect; autism features
Aldehyde dehydrogenase	ALD2	Delayed oxidation of acetaldehyde; acute alcohol intoxication
δ-Aminolevulinate dehydratase	HEM2	Hepatic porphyria
Ankyrine defect (spherocytose)	Yil112w	Spherocytic anaemia
Arginosuccinate lyase	ARG4	Neonatal infantile chronic hyperammonaemia
Arginosuccinate synthetase	ARG1	Argininaemia; severe psychomotor retardation
Bartter's syndrome (NKCC2 cotransporter)	Ybr235w	Hypokalaemic alkalosis with hypercalciura
Carbamoylphosphate synthase	URA2/CPA2	Hyperammonaemia
Carboxypeptidase C	PRC1	Galactosialidosis
Carnitine palmitoyltransferase	YAT1	Lipid metabolism defect; cardiomyopathy
Catalase	CTA1	Acatalasia
Coproporphyrinogen oxidase	HEM6	Coproporphyria; psychiatric symptoms
Cyclin DI (CCND1)	CGSG	Oncogene; parathyroid adenomas

Human Disease Gene	Yeast Gene	Brief disease description
Cystathionine-*β*-synthase	Ygr155w	Homocystinuria
Dihydrolipoyl dehydrogenase	LPD1	Lactic acidosis; `maple syrup' urine disease
Ferrochelatase	HEM15	Protoporphyria, erythropoietic
Fumarate hydratase	FUM1	Fumaric aciduria; encephalopathy
Glucose-6-phosphate dehydrogenase	ZWF1	Haemolytic anaemia
α-Glucosidase	Ybr229c	Cardiomyopathy; muscular hypotonia
Glucokinase	HXK1/HXK2	Hyperglycaemia; diabetes
γ-Glutamyltranspeptidase	Y1r299w	Glutathionuria
Glutathione peroxidase	HYR1	Haemolytic anaemia
Glycine decarboxylase	Ymrl89w	Non-ketotic hyperglycinaemia; lethargy; severe mental retardation
Guanine nucleotide binding protein	GPA1	Defect in adenylcyclase regulation; osteodystrophy
Gulonolo-*γ*-lactone oxidase (pseudogene)	Ydr305c	Ascorbic acid biosynthesis defect
Holocarboxylase synthetase	ACC2	Biotin-responsive carboxylase deficiency; ataxia
α-Ketoglutarate dehydrogenase (El)	KGD1	Lactic acidosis; neurodisorders
LIM kinase	Yol113w	Williams syndrome; brain development
Lipoamide acyltransferase (E2)	KGD2/PDA2	Lactic acidosis; `maple syrup' urine disease
Methylene tetrahydrofolate reductase	Ypl023c	Homocystinuria; psychotic symptoms
Mevalonate kinase	ERG12	Mevalonicaciduria; variety of symptoms
Monoamine oxidase	FMS1	Mental retardation and keratocunjunctivis
Nucleoside diphosphate kinase	Ykl067w	Tumour metastatic process
Nucleotide pyrophosphatase	Ycr026c	Insulin resistance

Human Disease Gene	Yeast Gene	Brief disease description
Ornithine-δ-aminotransferase	CAR2	Hyperornithinemia; atrophy of choroid and retina
Ornithine transcarbamylase	ARG3	Hyperammonaemia in males
Phosphatidylinositol glycan class A	SPT14	Haemolytic blood disorder (venous thrombosis)
Phosphoglycerate mutase	GPM1	Myopathy
Phospholipid-cholesterolacyltransferase	Ynr008w	Cholesterol esterification defects; cornea lipid deposits
Porphobilinogen deaminase	HEM3	Acute intermittent porphyria
Propionyl-COA-carboxylase	PYC2	Hyperglycinemia; intolerance to proteins
Protoporphyrinogen oxidase	Yer0l4w	Variegate porphyria; light-sensitive dermatis
Purine phosphorylase	Ylr209c	Immunodeficiency; neurodisorders
Pyruvate carboxylase	PYC1	Lactic acidosis; death
Pyruvate dehydrogenase	PDA1	Lactic acidosis; ataxia
Pyruvate kinase	PYK1	Non-spherocytic anaemia
SA gene	ACS1	Hypertension-associated gene
Serine pyruvate aminotransferase	Yfl030w	Hyperoxaluria; urolithiase; nephrocalcinosis
SLC4 (anion exchanger)	Ynl275w	Hereditary spherocytosis
Sterol-26-hydroxylase	ERG11	Cerebral cholesterinosis
Succinate dehydrogenase	SDH1	Flavoprotein subunit defect; Leigh syndrome
Succinic semialdehyde dehydrogenase	Ybr00Gw	Mental retardation and ataxia
Sucrose isomaltase	Ybr229c	Sucrose intolerance
Sulfonylurea receptor	YCF1/YHD5	ABC transporter; unregulated insulin secretion
TAP1/TAP2 transporters	MDL2	ABC transporters; immunodeficiency
α-Tocopherol transferase	SEC14	Vitamin E deficiency; ataxia

Human Disease Gene	Yeast Gene	Brief disease description
Triosephosphate isomerase	TPI1	Chronic haemolytic anaemia and neuromuscular disorders
Tyrosine transaminase	Yjl060w	Tyrosinemia
Uroporphyrinogen decarboxylase	HEM12	Porphyria, cutanea tarda
Uroporphyrinogen synthase	HEM4	Porphyria, congenital erythropoietic
X. pigmentosum (XP-D, 1990)	RAD3/RAD25	DNA helicase; TFIIH complex subunit; photosensitivity; cancer
X. pigmentosum (XP-G, 1990)	RAD2	Structure specific endonuclease; photosensitivity; cancer
X. pigmentosum (XP-A, 1992)	RAD14	Zinc finger damaged DNA binding protein; photosensitivity; cancer
X. pigmentosum (XP-C, 1992)	RAD4	125 kDa ssDNA binding protein; photosensitivity; cancer
Cockayne syndrome (CS-B, 1992)	RAD26	Progressive neurological dysfunction; photosensitivity
Cockayne syndrome (CS-A, 1995)	Ydr030c	WD-repeat protein; same phenotype as above
Achondroplasia (FGFR3)	IPL1	Membrane Ser/Thr protein kinase
Adrenoleukodystrophy (ADL)	PAL1	ABC transporter; neurodegenerative disease
Amyotrophic lateral sclerosis (SOD1)	SOD1	Superoxide dismutase
Ataxia telangiectasia (ATM)	TEL1/MEC1	Phosphatidylinositol kinase-related protein
Barth syndrome (G4.5.)	Ypr140w	Unknown function; cardioskeletal myopathy
Bloom syndrome (BLM)	SGS1/YABC	RecQ DNA helicase-related protein; growth defect; predisposition to all types of cancer
Chediak-Higashi syndrome (CHS)	Ycr032w	`Beige' protein; decreased pigmentation; immunodeficiency
Choroideremia (CHM)	GDI1k	Component A of RAB geranylgeranyltransferase
Cystic fibrosis (CFTR)	YCF1	ABC transporter; impaired clearance in a variety of organs

Human Disease Gene	Yeast Gene	Brief disease description
Deafness, DFN-1 (DDP)	Yjr135a	Unknown function; neurodegenerative syndrome
Diastrophic dysplasia (DTD)	SUL1	Sulfate transporter; undersulfation of proteoglycans
Fanconi syndrome (CLCN5)	GEF1	Kidney chloride channel; nephrolithiasis
Fragile histidine triad protein (FHIT)	Ydr305c	Dideadenosine tetraphosphate hydrolase; cancer
Friedreich ataxia (FRD)	Ydl120w	Neurodegenerative disease
Glycerol kinase (GK)	GUT1	Hyperglycerolaemia; poor growth; mental retardation
HNPCC (MSH2)	MSH2	Mismatch-repair; hereditary nonpolyposis colon cancer
HNPCC (MLH1)	MLH1	Mismatch repair; hereditary nonpolyposis colon cancer
Lissencephaly (LIS1)	MET30	Subunit of platelet-activating factor acetylhydrolase
Lowe syndrome (OCRL)	Yil002c	Inositol polyphosphate 5 phosphatase-related protein; cataracts and glaucoma
Menkes disease (MNK)	CCC2	Copper-transporting ATPase; neurodegenerative disease and death
Migraine (CACNL1-A4)	Ygr2l7w	Calcium channel; familial hemiplegic migraine and episodic ataxia
Multiple endocrine neoplasia	PHO85	Related to transmembrane receptors with a cytoplasmic tyrosine kinase domain
Myotonic dystrophy (DM)	Ynl161w	Ser/Thr protein kinase; neurodegenerative disease
Myotubular myopathy (MTM1)	Yjr110	Probable tyrosine phosphatase; muscle-specific disease
NBCC syndrome (PTC)	Ypl006w	Homologue of Drosophila patched; nevoid basal cell carcinoma syndrome
Neurofibromatosis (NF1)	IRA2	GTPase-activating protein

Human Disease Gene	Yeast Gene	Brief disease description
Retinitis pigmentosa (RPGR)	SRM1	RCC1-related protein; progressive retinal degeneration
Thomsen disease (CLCN1)	GEF1	Muscle chloride channel; myotonic disorders
Werner syndrome (WRN)	SGS1	Premature ageing and strong predisposition to cancer
Wilms tumour (WT1)	FZF1	Zinc finger protein; nephroblastoma
Wilson disease (WND)	CCC2	Copper transporting ATPase; toxic accumulation of copper in liver and brain
Wiskott-Aldrich syndrome (WASP)	LAS17	Eflector for CDC42H GTPase; immunodeficiency

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Supplemental Material – Chapter 1

Table S2: Oligonucleotides used in the Real-Time PCR Analyses

ARN1	**5'-**GGGTGTGGGCACTACTTTGT-3' 5'-GAACCGACAGCAGAACCAAT-3'
ARN2	5'-AGGTATGCTGCTGGAGCTGT-3' **5'-**GAGGGCCATGAAGGTATCAA-3'
FTR1	**5'-**GATTCAACCTTGCCAGTGGT-3' **5'-**ATTGTCCAGTTCTGGGTTGC-3'
FET3	5'-ACGGTGTGAATTACGCCTTC-3' 5'-TTGGAAAGCGTGACCATGTA
CTH2	**5'-**CAAAAGCCCAAAAATGATGC-3' **5'-**TTCGCACAGCTCTGTCTTGT-3'
ACT1	5'-CTATTGGTAACGAAAGATTCAG-3' 5'-CCTTACGGACATCGACATCA-3'

Table S3
Iron homeostasis genes whose mRNA levels were down- or up-regulated in a WT strain upon As treatment.

ORF	Gene	Function	Fold change
Iron Regulon			
YPL202C	AFT2	Iron-regulated transcriptional activator	3.3
YKR052C	MRS4	Mitochondrial iron transporter of the mitochondrial carrier family (MCF	2.49
YER174C	GRX4	Hydroperoxide and superoxide-radical responsive glutathione-dependent oxidoreductase	-2.76
YMR308C	PSE1	Importin for specific proteins, including Pdr1, Yap1, Ste12, and	-2.24
YDR534C	FIT1	Cell wall mannoprotein involved in siderophore-iron uptake	-2.55
YOR382W	FIT2	Cell wall mannoprotein involved in siderophore-iron uptake	1.2
YOR383C	FIT3	Cell wall mannoprotein involved in siderophore-iron uptake	1.73
YHL040C	ARN1	Ferrirubin, ferrirhodin, and related Fe-siderophores transporter	2.17
YHL047C	ARN2/TAF1	Triacetylfusarinine C transporter	2.07
YOL158C	ARN4/ENB1	Enterobactin transporter	1.63
YMR058W	FET3	Multicopper ferroxidase involved in high-affinity Fe uptake	-2.14
YER145C	FTR1	High-affinity Fe permease that forms a complex with Fet3	-1.55
YLR214W	FRE1	Ferric and cupric reductase	1.36
YOR381W	FRE3	Ferric reductase that reduces siderophore bound Fe	1.43
YNR060W	FRE4	Ferric reductase, reduces a specific subset of siderophore-bound iron	1.22
YLL051C	FRE6	Putative ferric reductase	3.29
YLR047C	FRE8	Protein with sequence similarity to iron/copper reductases	2.76
YMR319C	FET4	Low-affinity Fe transport protein	-2.94
YBR207W	FTH1	Vacuolar Ftr1 homologue	1.7
YMR177W	MMT1	Putative metal transporter involved in mitochondrial iron accumulation	2.57
YPL224C	MMT2	Putative metal transporter involved in mitochondrial iron accumulation	2.15
YDR151C	CTH1	Member of the CCCH zinc finger family,has a role in mRNA degradation	3.72
YLR136C	CTH2/TIS11	mRNA-binding protein expressed during iron starvation	5.69
YLR205C	HMX1	Heme-binding peroxidase	1.87
YOR176W	HEM15	Ferrochelatase, catalyzes insertion of Fe(II) into protoporphyrin	-1.2
YLR256W	HAP1	Zinc finger transcription factor involved in response to levels of heme and oxygen	-2.09
Iron-sulfur Proteins			
Fe-S Cluster Synthesis			
YKL040C	NFU1	NifU-like protein	4.28
YDR091C	RLI1	Essential Fe/S protein required for ribosome biogenesis and translation initiation	-5.32

YPL135W	ISU1	Member of Fe-S cluster bisynthesis machinery	2.17
YOR226C	ISU2	Member of Fe-S cluster bisynthesis machinery	3.23
YPR067W	ISA2	Member of Fe-S cluster bisynthesis machinery	2.97
YCL017C	NFS1	Cysteine desulfurase involved in iron-sulfur cluster (Fe/S) biogenesis	2.47
YPL059W	GRX5	Mitochondrial matrix protein involved in the synthesis/assembly of Fe/S centers	2.23
YMR301C	ATM1	(ABC) transporter, exports mitochondrially synthesized precursors of iron-sulfur (Fe/S) clusters to the cytosol	2.98
YER048W	ISD11	Protein required for mitochondrial iron-sulfur cluster biosynthesis	2.18
YGL018C	JAC1	Specialized J-protein that functions with Hsp70 in Fe-S cluster biogenesis in mitochondria	2.02
YNL240C	NAR1	Component of the cytosolic iron-sulfur (FeS) protein assembly	3.01
YPR048W	TAH18	Component of an early step in the cytosolic Fe-S protein assembly (CIA) machinery	2.54
Fe-S Cluster-containing proteins			
YGL009C	LEU1	Isopropylmalate isomerase, catalyzes the second step in the leucine biosynthesis pathway	-2.96
YOR196C	LIP5	Protein involved in biosynthesis of the coenzyme lipoic acid	1.78
YPL252C	YAH1	Fe-S cluster protein homologous to human adrenodoxin	-1.26
YGR286C	BIO2	Biotin synthase	-1.97
YIL003W	CFD1	Highly conserved, iron-sulfur cluster binding protein localized in the cytoplasm	4.46
YLR304C	ACO1	Mitochondrial aconitase, Fe-S cluster protein	-3.86
YJL200C	ACO2	Mitochondrial aconitase, Fe-S cluster protein	-1.61
Mitochondrial respiraion/electron transport chain			
Cytochrome c oxidase			
YNL111C	CYB5	Cytochrome b5	-5.53
YML054C	CYB2	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase)	3.04
YOR065W	CYT1	Cytochrome c1	-1.82
YJR048W	CYC1	Iso-1-cytochrome c	-1.1
YAL039C	CYC3	Cytochrome c heme lyase (holocytochrome c synthase)	1.07
YEL039C	CYC7	Cytochrome c isoform 2	1.69
YKL087C	CYT2	Cytochrome c1 heme lyase	1.57
YHR051W	COX6	Subunit VI of cytochrome c oxidase	-1.04
YKR066C	CCP1	Mitochondrial cytochrome-c peroxidase	2.08
Sterol and fatty acid synthesis and metabolism			
YHR042W	NCP1	NADP-cytochrome P450 reductase involved in ergosterol biosynthesis	-2.08
YLR056W	ERG3	C-5 sterol desaturase	-9.03
YMR015C	ERG5	C-22 sterol desaturase	-3.49
YHR007C	ERG11	Lanosterol C-14 demethylase	-1.58
YGR060W	ERG25	C-4 methyl sterol oxidase	-5.31
YGL055W	OLE1	Fatty acid desaturase	-5.06
YMR272C	FAH1/SCS7	Hydroxylation of C-26 fatty acid in ceramide	-3.85
Other Functions			
YJR070C	LIA1	Deoxyhypusine hydroxylase	-4.85
YPL207W	TYW1	Protein required for the synthesis of wybutosine	-4.56
YGR123C	PPT1	Protein serine/threonine phosphatase with similarity to human phosphatase PP5	-3.33

YJR016C	ILV3	Dihydroxyacid dehydratase	-2.78
YGL256W	ADH4	Alcohol dehydrogenase isoenzyme type IV	-2.69
YPL086C	ELP3	Subunit of Elongator complex	-2.45
YDL171C	GLT1	NAD(+)-dependent glutamate synthase (GOGAT)	-1.79
YJR025C	BNA1	Required for biosynthesis of nicotinic acid	2.1
YJR078W	BNA2	Required for biosynthesis of nicotinic acid	1.31
YER051W	JHD1	JmjC domain family histone demethylase specific for H3-K36	1.63
YGR234W	YHB1	Nitric oxide oxidoreductase, flavohemoglobin involved in nitric oxide detoxification	1.63
YML016C	PPZ1	Serine/threonine protein phosphatase Z	1.76
YML057W	CMP2	Calcineurin A, regulates Crz1p (a stress-response transcription	1.9
YDR436W	PPZ2	Serine/threonine protein phosphatase Z	2
YDR402C	DIT2	Microsomal enzyme involved in the production of N,N-bisformyl dityrosine	2.08
YNR032W	PPG1	Putative serine/threonine protein phosphatase	2.79
YOL043C	NTG2	DNA N-glycosylase and apurinic/apyrimidinic (AP) lyase	2.83
YBR018C	GAL7	Galactose-1-phosphate uridyl transferase	4.13
YJR137C	ECM17	Sulfite reductase beta subunit, involved in amino acid biosynthesis	7.93
YDR256C	CTA1	Catalase A, breaks down hydrogen peroxide in the peroxisomal matrix	9.16
YLL057C	JLP1	Fe(II)-dependent sulfonate/alpha-ketoglutarate dioxygenase	23.9

The average fold induction of triplicate samples is represented. An increase of 1.2-fold and a p-value <0.005were used.

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Deciphering Human Heat Shock Transcription Factor 1 Regulation via Post-Translational Modification in Yeast

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Abstract

Heat shock transcription factor 1 (HSF1) plays an important role in the cellular response to proteotoxic stresses. Under normal growth conditions HSF1 is repressed as an inactive monomer in part through post-translation modifications that include protein acetylation, sumoylation and phosphorylation. Upon exposure to stress HSF1 homotrimerizes, accumulates in nucleus, binds DNA, becomes hyper-phosphorylated and activates the expression of stress response genes. While HSF1 and the mechanisms that regulate its activity have been studied for over two decades, our understanding of HSF1 regulation remains incomplete. As previous studies have shown that HSF1 and the heat shock response promoter element (HSE) are generally structurally conserved from yeast to metazoans, we have made use of the genetically tractable budding yeast as a facile assay system to further understand the mechanisms that regulate human HSF1 through phosphorylation of serine 303. We show that when human HSF1 is expressed in yeast its phosphorylation at S303 is promoted by the MAP-kinase Slt2 independent of a priming event at S307 previously believed to be a prerequisite. Furthermore, we show that phosphorylation at S303 in yeast and mammalian cells occurs independent of GSK3, the kinase primarily thought to be responsible for S303 phosphorylation. Lastly, while previous studies have suggested that S303 phosphorylation represses HSF1-dependent transactivation, we now show that S303 phosphorylation also represses HSF1 multimerization in both yeast and mammalian cells. Taken together, these studies suggest that yeast cells will be a powerful experimental tool for deciphering aspects of human HSF1 regulation by post-translational modifications.

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Introduction

All organisms are exposed to proteotoxic stresses that result in the accumulation of misfolded proteins. In response to these stresses cells have evolved adaptive responses to protect and stabilize cellular proteins until more favorable conditions for cell proliferation are encountered [1]. The heat shock transcription factor, HSF, is a homotrimeric transcription factor that activates gene expression in response to a variety of stresses including heat and oxidative stress, as well as inflammation and infection [2]. Recent evidence has shown that the *S. cerevisiae* HSF directly activates the expression of genes whose protein products are involved in protein folding and degradation, ion transport, signal transduction, energy generation, carbohydrate metabolism, vesicular transport, cytoskeleton formation and other cellular functions [3].

While mammalian cells express four distinct HSF proteins encoded by separate genes, HSF1 is the primary factor responsible for stress responsive gene transcription [2]. In the absence of stress, mammalian HSF1 is repressed through mechanisms that are not well understood. HSF1 is thought be maintained in an inactive monomeric state through intramolecular interactions between a hydrophobic coiled-coil domain in the carboxyl-terminus of the protein and three amino-terminal coiled-coils required for homotrimerization and transcriptional activation [4,5,6]. HSF1 is also thought to be bound and repressed by the protein chaperones Hsp90 and Hsp70, though it is not clear how these chaperones repress HSF1 activity [7,8,9,10]. Studies suggest that during the initial phase of the stress response, the inactive HSF1 monomer dissociates from Hsp90, homotrimerizes, is transported to the nucleus and binds to heat shock elements (HSE) found in the promoters of HSF target genes [10,11]. The DNA-bound homotrimer, remains relatively transcriptionally inert [12], potentially due to the continued interaction with Hsp70 and the HSF1-transactivation domain [9]. Stress-dependent hyperphosphorylation of HSF1 by potentially multiple protein kinases has been proposed to, in part, promote HSF1 dependent transactivation [13,14,15].

The activity of HSF1 is also thought to be negatively regulated through a number of post-translational modifications including

phosphorylation, sumoylation and acetylation [16,17,18,19]. Mass spectrometry analyses have shown HSF1 to be phosphorylated on at least 12 serine residues [13] and phosphorylation of S121, S303, S307 and S363 have been correlated with a repression in HSF1 activity [18,20,21]. The most comprehensively studied of these phosphorylation events are the phosphorylation of S303 and S307. However, much of what is known about S303 and S307 phosphorylation stems from *in vitro* phosphorylation experiments and *in vivo* studies using either lexA or Gal4-HSF1 fusion proteins lacking the native HSF1 DNA binding domain. As such, many of the earlier studies exploring S303 and S307-dependet regulation of HSF1 activity have resulted in conflicting results. For example, previous phosphorylation experiments suggested that S307 was phosphorylated by ERK which, in turn, acted as an essential priming step for GSK3-dependent phosphorylation of S303 [22]. However, subsequent *in vitro* studies suggested that S303 could also be phosphorylated by a variety of mitogen activated protein kinases (MAPK) including the stress responsive MAPK p38 [17,18]. In addition, subsequent *in vivo* data suggested S303 phosphorylation could occur independently of S307 phosphorylation [16].

While the specific mechanism by which S303 and S307 phosphorylation repress HSF1 activity remains unclear, evidence has suggested that S303 and S307 phosphorylation represses the transactivation potential of HSF1 [18,22,23]. S303 and S307 are constitutively phosphorylated in the absence of stress and S303 phosphorylation levels increase after exposure to stress, suggesting that this phosphorylation event might also contribute to HSF1 inactivation during the recovery phase [16,17]. Interestingly, phosphorylation of S303, but not S307, promotes sumoylation of K298 [16] which, like S303 phosphorylation, also increases in response to stress exposure and represses HSF1-dependent transactivation [24]. However, it remains unclear if the repressive effects of S303 phosphorylation on HSF1 activity are exclusively mediated through K298 sumoylation or occur through additional mechanisms.

While HSF1 and the cognate HSEs are quite well conserved from yeast to humans, our previous results demonstrated that human HSF1 expressed in *S. cerevisiae* is unable to complement for the loss of the essential yeast HSF protein [25]. Further analysis showed that human HSF1 expressed in yeast was unable to form a homotrimer and consequently unable to activate HSE-dependent gene expression to support cell viability. Human HSF1 homotrimerized, became active and complemented for the loss of yeast HSF when three derepressing mutations, collectively known as LZ4m, were introduced into the repressive carboxyl-terminal coiled-coil domain [6,25]. Further studies in yeast identified an amino-terminal linker-domain as well as a loop in the DNA binding domain as repressive elements that contributed to HSF1 repression in both yeast and mammalian cells [26,27]. We have also used to the yeast assay system to screen for and indentify novel pharmacological activators of human HSF1 [28]. Together, these results suggest that human HSF1 expressed in yeast is maintained in a constitutively repressed state through mechanisms similar to those of mammalian cells and that the yeast system can serve as a simplified assay system to decipher the complex mechanisms regulating human HSF1 activity.

Here we report the use of the yeast assay system to further understand the mechanisms that regulate human HSF1 through phosphorylation of serine 303. Our results suggest that S303 phosphorylation blocks human HSF1 homotrimerization thereby preventing human HSF1 activation and complementation of the loss of yeast HSF. Furthermore, we demonstrate that S303 phosphorylation also blocks HSF1 homotrimerization in mammalian cells. We show that phosphorylation of HSF1 S303 in yeast occurs via the action of the MAPK Slt2 and not via the action of GSK3 and we extend these findings to show that S303 phosphorylation also occurs independent of GSK3 in mammalian cells.

Results

Phosphorylation of S303 contributes to repression of human HSF1 in yeast

When human HSF1 is expressed in yeast it is unable to homotrimerize, promote gene expression and complement for the loss of the essential yeast HSF protein [25]. Because our previous work suggested that when HSF1 is expressed in yeast it exists in a constitutively repressed monomeric state, we sought to use the yeast assay system to better understand the complex mechanisms regulating HSF1 activity in mammalian cells. An important component of HSF1 repression occurs through the phosphorylation of serine 303 and serine 307 [17,18]. Because S303 and S307 are constitutively phosphorylated in mammalian cells and alanine substitution of S303 or S307 promotes constitutive activation of HSF1 in mammalian cells in reporter assays [17,18] we tested whether S303 and/or S307 contribute to HSF1 repression in yeast. Wild-type HSF1 or the individual S303A, S307A or S303/307A double mutants were expressed in yeast strain PS145 which lacks a chromosomal copy of the essential yeast HSF gene and constitutively expresses yeast HSF episomally from a galactose inducible and dextrose repressible promoter [29]. When PS145 is grown in the presence of dextrose as the sole carbon source, yeast HSF expression is extinguished and growth becomes solely dependent on HSF1 which is episomally expressed [28]. While wild-type human HSF1 was unable to complement for the loss of yeast HSF, expression of the S303A, S307A or S303/307A HSF1 mutants allowed for human HSF1-dependent yeast growth (Figure 1A, B). Interestingly, the S303/307A double HSF1 mutant did not display enhanced activity over the S303A mutant (Figure 1B) suggesting that phosphorylation of both S303 and S307 modulate HSF1 repression through similar mechanisms.

To ascertain whether HSF1 is being phosphorylated in yeast, we employed a commercially available antibody specific for phospho-S303 (pS303). Because this antibody has not previously been characterized in the literature, we tested its specificity in human HeLa cells where S303 is known to be constitutively phosphorylated [17]. As shown in Figure 1C, using this antibody we detected that endogenous HSF1 was constitutively phosphorylated in HeLa cells in the absence of stress. We also observed an increase in S303 phosphorylation in response to a heat shock, which correlated with previous reports [16]. Importantly, the pS303-specific antibody did not detect HSF1 when HeLa extracts were treated with lambda protein phosphatase prior to immunoblot analysis (Figure 1C), nor does it detect HSF1 when S303 is mutated to alanine (Figure 1D). Together, these data suggest that the antibody is specific for HSF1 that is phosphorylated on S303. The detection of HSF1 using a polyclonal anti-HSF1 antibody demonstrates that there are no significant differences in the steady state levels of HSF1 either treated or untreated with lambda phosphatase (Figure 1C).

Consistent with a contribution to HSF1 repression (Figure 1A, B) S303 is robustly phosphorylated when HSF1 is expressed in yeast (Figure 1D, E). Interestingly, phosphorylation of S303 was also observed when the S307A mutant was expressed in yeast though it was reduced by approximately 50% when compared to wild-type HSF1 (Figure 1D, E). While this observation supports a previous report indicating that S303 phosphorylation could occur

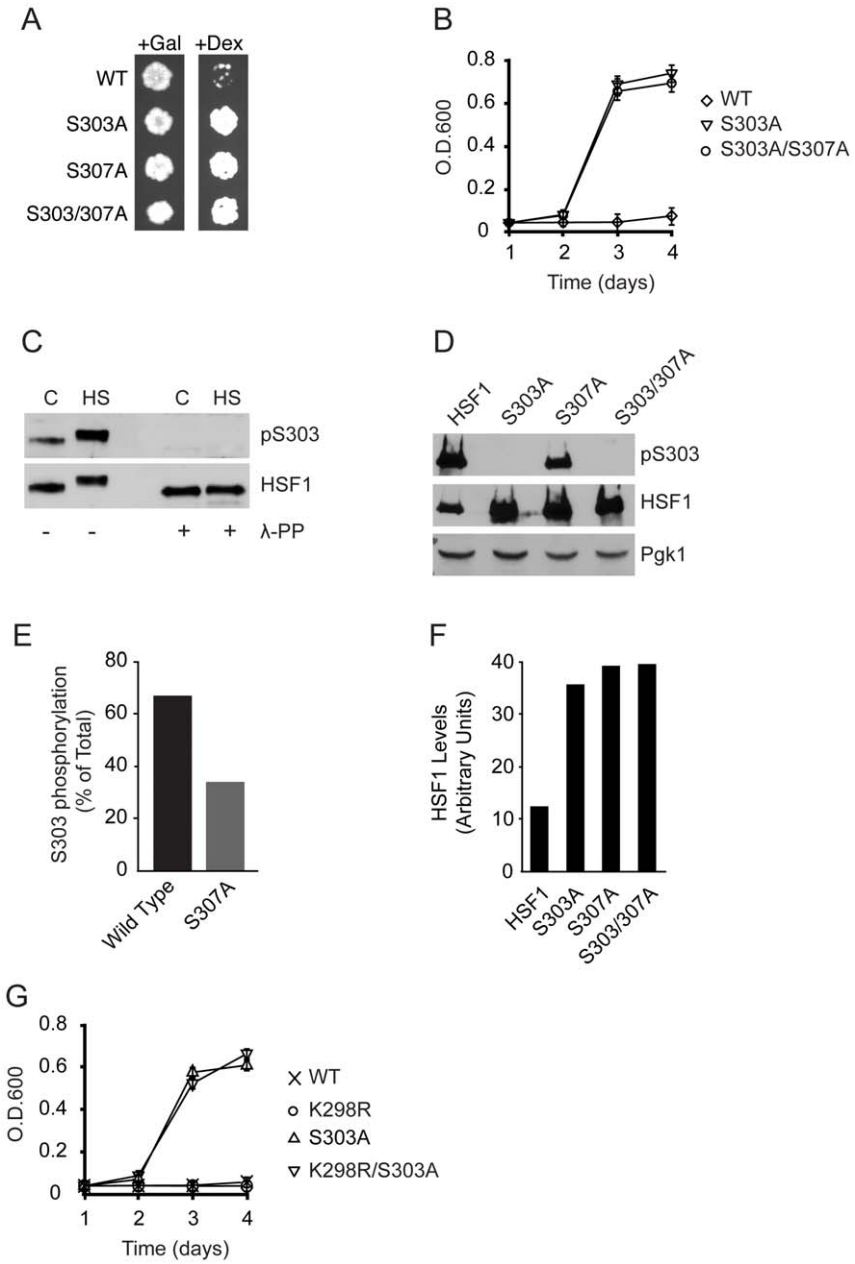

Figure 1. S303 phosphorylation represses HSF1 activity in yeast. (**A**) PS145 yeast strains expressing wild-type HSF1 (WT) or the S303A, S307A or S303/307A mutants were plated on either galactose or dextrose supplemented medium. (**B**) PS145 expressing either wild-type HSF1 or the S303A or S303/307A mutants were grown in dextrose containing medium for 4 d. Growth was monitored by measuring $O.D._{600}$. (**C**) HeLa cells were grown at 37°C (C) or heat shocked for 2 h at 42°C (HS). Total protein extracts were treated with lambda protein phosphatase and analyzed for phospho-S303 (pS303) and total HSF1 levels by immunoblotting. (**D**) PS145 was transformed with a plasmid expressing wild-type HSF1 (WT) or mutant alleles of HSF1 and grown on galactose containing medium. Total protein extracts were analyzed for pS303, HSF1 and Pgk1 by immunoblotting. (**E**) Levels of HSF1 phosphorylated at S303 were quantified and are shown as a percent of total HSF1, from panel D. (**F**) Protein levels of HSF1 were normalized to Pgk1, from panel D. (**G**) PS145 expressing either wild-type HSF1 or mutant HSF1 alleles were assayed for HSF1-dependent growth as in **B**.
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independently of S307 phosphorylation in mammalian cells [16], these data also suggests that under certain circumstances S303 phosphorylation may be enhanced by S307 phosphorylation. In addition, although a correlation between S303 and S307 phosphorylation and HSF1 protein stability has not been previously reported, we repeatedly observed two to three-fold higher steady state levels of HSF1 when the S303A, S307A and S303/307A mutants were expressed in yeast (Figure 1D, F). While an antibody specific for phospho-S307 is commercially available, we have been unable to detect S307 phosphorylation of human HSF1. As such we focused our investigation on S303-phosphorylation dependent repression of human HSF1.

Human HSF1 S303 phosphorylation is known to promote sumoylation of lysine 298, which also contributes to the repression of HSF1 activity [16]. Therefore, to further investigate the idea that human HSF1 is being actively repressed in yeast, we explored the possibility that K298, like S303, contributes to HSF1 repression in yeast. However, unlike the S303A HSF1 mutant,

the K298R mutant did not promote HSF1-dependent growth (Figure 1E), suggesting that at least in yeast, K298 does not significantly contribute to HSF1 repression. We also did not observe a reduction in human HSF1-dependent yeast growth for the S303A/K298R double mutant, indicating that K298 is also not required for HSF1 activity in yeast (Figure 1E).

S303 represses trimer formation of HSF1 in yeast and mammalian cells

Previous reports have suggested that phosphorylation of S303 represses the ability of HSF1 to transactivate gene expression [18,22,23]. Here we show that the HSF1 S303A mutant functionally complements for the lost of yeast HSF (Figure 1A, B). Based on our previous work this indicates that S303 phosphorylation might also regulate the ability of human HSF1 to homotrimerize [25]. To test this hypothesis we carried out EGS cross-linking experiments in conjunction with immunoblot analysis to ascertain if S303 phosphorylation regulates the homotrimerization of human HSF1 in yeast. When the S303A HSF1 mutant was expressed in yeast we detected approximately 2-fold higher levels of trimerized HSF1 at the intermediate EGS concentration than when wild-type HSF1 was expressed in yeast (Figure 2A). However, trimerization of the S303A HSF1 mutant was lower than trimerization of the LZ4 HSF1 mutant, previously demonstrated to be constitutively trimerized in yeast and mammalian cells and able to complement for the loss of yeast HSF [6,25]. We observed similar results for the S307A and S303/307A HSF1 mutants (data not shown) further supporting the notion that S303 and S307 phosphorylation repress HSF1 activity through similar mechanisms. We next evaluated whether HSF1 S303 phosphorylation could also function to repress homotrimer formation in mammalian cells. To test this hypothesis we expressed wild-type HSF1 or the S303A, S307A or S303/307A mutants in hsf1$^{-/-}$ mouse embryonic fibroblasts (MEF) [30] and assayed for HSF1 trimerization in the absence of thermal stress by EGS crosslinking and immunoblotting. While wild type HSF1 could be detected as a multimer in these extracts, we observed approximately 2-fold higher levels of the HSF1 trimer for the HSF1 S303A mutant (Figure 2B) as well as the S307A and S303/307A mutants (data not shown).

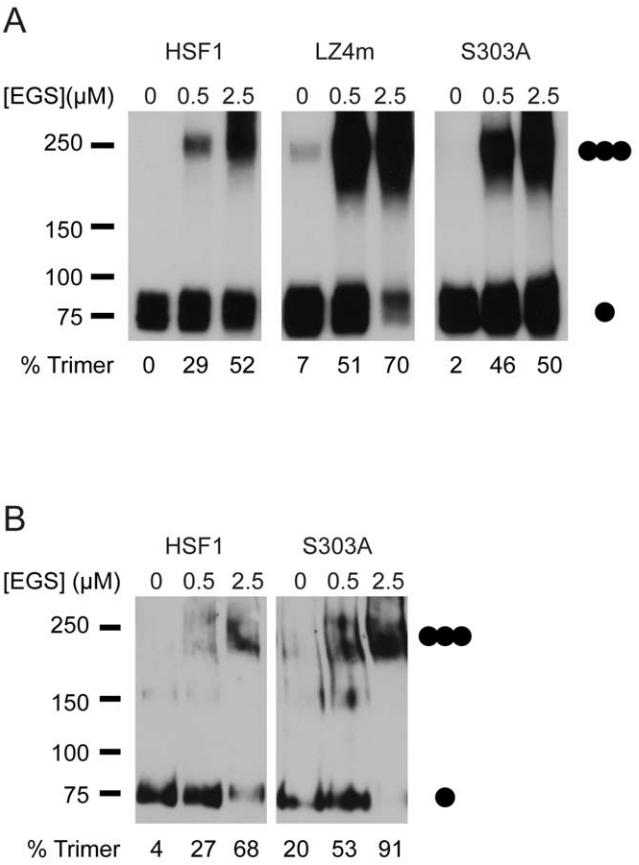

Figure 2. S303 represses trimer formation of HSF1 in yeast and mammalian cells. (**A**) PS145 was transformed with wild-type HSF1, the LZ4m mutant or the S303A mutant and grown on galactose containing medium. Total protein extracts were evaluated for HSF1 multimerization by EGS crosslinking, SDS-PAGE, and immunoblotting using an HSF1 specific antibody. The positions of molecular weight markers are indicated on the left, and circles indicating the expected migration of HSF1 monomers and trimers are on the right. Levels of HSF1 trimer as percent of total HSF1 are shown below. (**B**) hsf1$^{-/-}$ MEFs were transfected with a plasmid expressing wild-type HSF1 or the S303A mutant and analyzed for HSF1 multimerization by EGS cross-linking as in A.
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S303 phosphorylation and coiled-coil interactions synergize in HSF1 repression

In addition to post-translational modifications, HSF1 activity is also thought to be repressed through intramolecular interactions between carboxyl- and amino-terminal coiled-coil domains and mutations in these domains render HSF1 constitutively trimerized, nuclear localized and bound to DNA in mammalian cells [6]. Because our results suggest that S303 phosphorylation might also regulate homotrimer formation, we tested the combined affects of both the S303A as well as the LZ4m mutations on human HSF1 activity in yeast. A human HSF1 mutant containing both the S303A and LZ4m mutations was created and its ability to promote human HSF1-dependent yeast growth was compared to the individual HSF1 mutants as well as wild-type HSF1 in quantitative cell growth assays. The individual S303A and LZ4m HSF1 mutants promoted human HSF1-dependent yeast growth to a similar extent, though neither the LZ4m nor the S303 mutant were fully derepressed, as the S303A/LZ4m double mutant displayed enhanced human HSF1-dependent yeast growth (Figure 3A). While we currently do not know if the S303A/LZ4m double HSF1 mutant has an increased propencity to trimerize, previous studies have shown that the LZ4m mutant, when expressed in yeast is not maximally trimerized and trimerization can be further enhanced via the addition of pharmacological HSF1 activators [28]. While we observed higher steady state protein levels for both the S303A and LZ4m mutants in comparison to wild-type HSF1 when expressed in yeast, no further increases in protein levels were observed for the double mutant (Figure 3B, C). These results suggest that while both HSF1 S303 phosphorylation and coiled-coil interactions regulate human HSF1 multimerization in yeast, they do so via distinct mechanisms. We also did not observe changes in HSF1 S303 phosphorylation when the LZ4m mutant was expressed in yeast, consistent with the notion that HSF1 trimerization does not affect HSF1 S303 phosphorylation.

Gsk3 regulates human HSF1 activity in yeast independent of S303 phosphorylation

Previous reports using *in vitro* phosphorylation experiments have suggested that HSF1 is phosphorylated at S303 by glycogen synthase kinase 3 (GSK3) [20,22,31]. However, it remains unclear

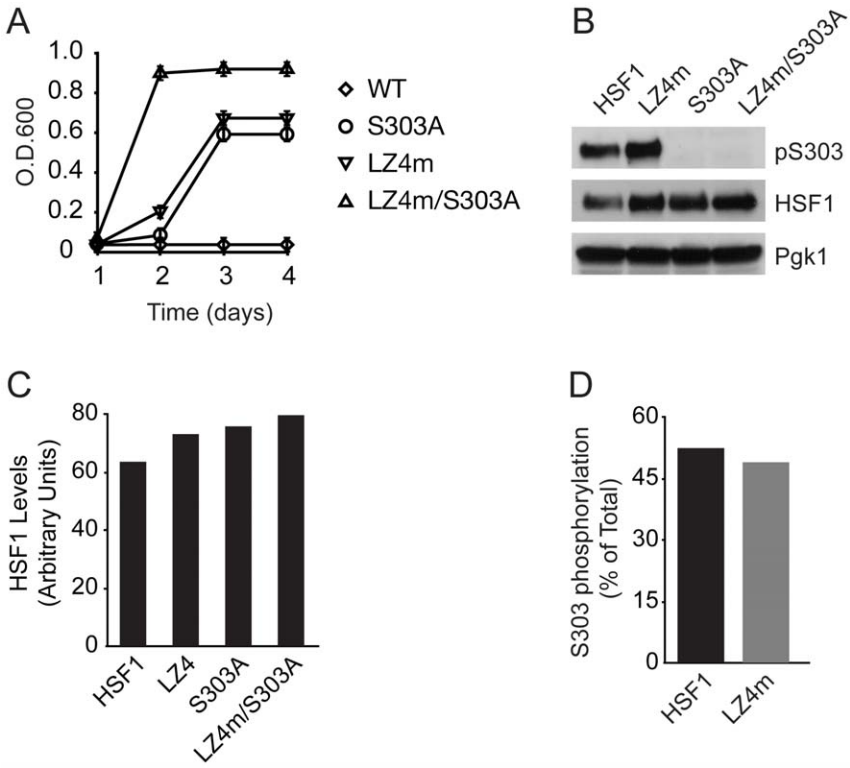

Figure 3. Phosphorylation of S303 and coiled-coil domains synergize in the repression of HSF1 in yeast. (**A**) PS145 expressing either wild-type HSF1 or mutant alleles of HSF1 were grown in dextrose supplemented medium for 4 d. Growth was monitored by measuring $O.D._{600}$. (**B**) PS145 was transformed with a plasmid expressing wild-type HSF1 (WT) or mutant alleles of HSF1 and grown on galactose containing medium. Total protein extracts were analyzed for pS303, total HSF1 and Pgk1 by immunoblotting. (**C**) Protein levels of HSF1 were normalized to Pgk1, from panel B. (**D**) Levels of HSF1 phosphorylated at S303 were quantified and are shown as a percent of total HSF1, from panel B.
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if GSK3 phosphorylates and represses HSF1 via S303 phosphorylation *in vivo*. To test if GSK3 contributes to HSF1 repression, we assayed human HSF1-dependent yeast growth in a strain also lacking the yeast GSK3 homolog Rim11. Supporting the notion that yeast GSK3 can repress human HSF1 activity in yeast we observed human HSF1-dependent yeast growth as well as HSF1 multimerization in the *rim11*Δ strain (Figure 4A, B). However, HSF1-dependent yeast growth in the *rim11*Δ strain was less robust than growth of a wild-type strain expressing the S303A HSF1 mutant (Figure 4A). Furthermore, when we expressed the S303A HSF1 mutant in the *rim11*Δ strain we observed HSF1-dependent growth at a rate similar to the growth of the S303A mutant in wild-type cells. This suggested the possibility that HSF1 might not be fully derepressed in the *rim11*Δ strain. Consistent with this idea, we did not detect a reduction in S303 phosphorylation in the *rim11*Δ strain (Figure 4C). *S. cerevisiae* encodes four separate yet partially functionally redundant GSK3 homologues [32], suggesting the possibility that S303 remains phosphorylated in the *rim11*Δ strain due to phosphorylation through other GSK3 proteins. To test this hypothesis we assayed the phosphorylation state of HSF1 at S303 in a yeast strain lacking all four isoforms of yeast GSK3. As shown in Figure 4D, no reduction in S303 phosphorylation was observed in the 4x*gsk3*Δ strain suggesting that while yeast GSK3 does contribute to HSF1 repression, it does so independently of S303 phosphorylation.

Results shown here for the S303A HSF1 mutant and previously published for the LZ4m HSF1 mutant suggest that mechanisms that regulate HSF1 in mammalian cells are at least partially conserved with regulation of human HSF1 expressed in yeast cells. Therefore, we carried out experiments to ascertain if GSK3 might also repress HSF1 independently of S303 phosphorylation in mammalian cells. To explore this possibility HeLa cells were treated with the GSK3 inhibitor SB-216763 [33] and assayed for HSF1 S303 phosphorylation as ascertained by immunoblotting with the anti-pS303 antibody. While SB-216763 treatment strongly inhibited GSK3 activity as shown by increased β-catenin levels [34], no reduction in S303 phosphorylation was observed (Figure 5A). However, similar to the results obtained from our yeast experiments, SB-216763 did promote activation of HSF1 under normal growth conditions, as determined by immunoblot analysis of Hsp70 expression (Figure 5A). This result is consistent with a previous report showing increased Hsp70 expression in response to lithium treatment, which also inhibits GSK3 function [35]. siRNA mediated knock-down of the two GSK3 isoforms in mammals, GSK3α and GSK3β, either singly or in combination, further confirmed that, while β-catenin expression was elevated, HSF1 S303 was not appreciably phosphorylated by GSK3 in unstressed mammalian cells (Figure 5B). Together, data from experiments in both yeast and mammalian cells support a model in which GSK3 inhibits HSF1 activity through a mechanism that is independent of S303 phosphorylation.

Slt2 represses human HSF1 activity via S303 phosphorylation in yeast

To begin to identify which protein kinase(s) in yeast phosphorylate human HSF1 at S303 to promote HSF1 repression, we assayed S303 phosphorylation in several previously generated protein kinase deletion strains obtained from the yeast gene

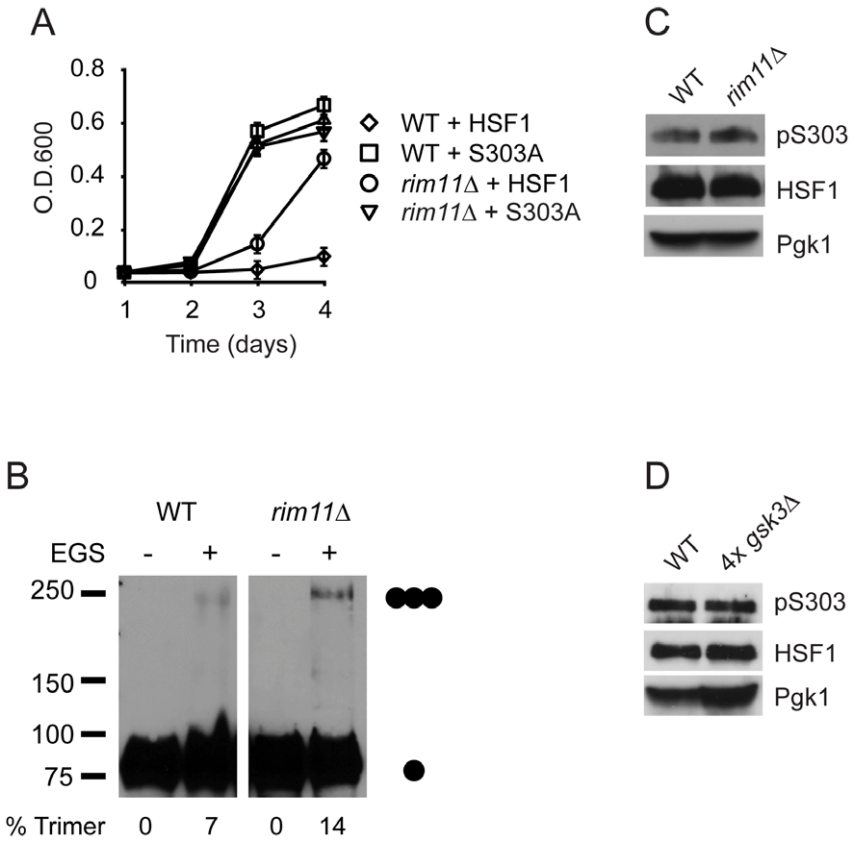

Figure 4. GSK3 represses HSF1 activity in yeast independent of S303. (**A**) PS145 (WT) expressing wild-type HSF1 or the S303A HSF1 mutant and LNY1 (*rim11*Δ) expressing wild-type HSF1 were grown in dextrose supplemented medium for 4 d. Growth was monitored by measuring $O.D._{600}$. (**B**) PS145 (WT) and LNY1 (*rim11*Δ) expressing wild-type HSF1 were grown on galactose containing medium and were evaluated for HSF1 multimerization by EGS crosslinking, SDS-PAGE, and immunoblotting using an HSF1 specific antibody. The positions of molecular weight markers are indicated on the left, and circles indicating the expected migration of HSF1 monomers and trimers are on the right. Levels of HSF1 trimer as percent of total HSF1 are shown below. (**C**) PS145 (WT) and LNY1 (*rim11*Δ) were transformed with a plasmid expressing wild-type HSF1 and were grown on galactose containing medium. Total protein extracts were analyzed for pS303, total HSF1 and Pgk1 by immunoblotting. (**D**) YPH499 (WT) and LNY3 (4x*gsk3*Δ) were transformed with a plasmid expressing wild-type HSF1 and were grown in dextrose containing medium. Total protein extracts were analyzed for pS303, total HSF1 and Pgk1 by immunoblotting.
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deletion collection [36]. One strain in which we detected severely reduced levels of human HSF1 S303 phosphorylation was a strain deleted for the *SLT2* gene, encoding a stress-responsive MAPK [37,38], consistent with S303 lying within a consensus site for MAPK-dependent phosphorylation (Figure 6A) [39]. This suggests that Slt2 either directly or indirectly promotes the phosphorylation of human HSF1 expressed in yeast. This hypothesis was further supported by the observation that an *slt2*Δ strain allowed wild type human HSF1-dependent yeast growth at a rate similar to the HSF1 S303A mutant, while no growth was observed in the *SLT2* wild-type strain (Figure 6B). Homotrimerization of wild-type human HSF1 was observed in the *slt2*Δ strain at levels similar to the S303A and LZ4m HSF1 mutants, further supporting the notion that the Slt2 MAPK represses human HSF1 multimerization in yeast (Figure 6C). In mammalian cells the most closely related homolog of Slt2 is the MAPK ERK5 [40]. However, using siRNA-mediated knock-down of ERK5 we were unable detect an effect of ERK5 on HSF1 S303 phosphorylation in mammalian cells (data not shown). This may suggest that in mammalian cells S303 can be phosphorylated by multiple MAPKs. This hypothesis is supported by previous data showing that ERK1/2 as well as the stress-responsive MAPK p38 could phosphorylate HSF1 at S303 *in vitro* [18]. In addition, our data showing reduced, but not eliminated phosphorylation of S303 in the *slt2*Δ strain (Figure 6A) also support a model where S303 may be phosphorylated by multiple MAPKs.

Expression of S303A and S307A mutants in hsf1$^{-/-}$ cells results in constitutive activation of Hsp70 expression

Previous studies have assayed the function of S303 and S307 phosphorylation in HSF1 regulation via *in vitro* phosphorylation experiments [22], *in vivo* using lexA/Gal4-HSF1 fusion proteins lacking the native HSF1 DNA binding domain [17,18] or via overexpression of a S303A HSF1 mutants in mammalian cells expressing endogenous wild-type HSF1 [16]. We tested the consequences of loss of S303 and S307 phosphorylation on HSF1 activity in the context of the entire protein using hsf1$^{-/-}$ MEFs which lack endogenous HSF1. When we expressed S303A, S307A or S303/307A HSF1 mutants in hsf1$^{-/-}$ MEFs we observed a modest elevation of Hsp70 expression under normal growth conditions (Figure 7A, B) consistent with the hypothesis that S303 phosphorylation modulates both homotrimerization as well as transactivation by HSF1. However, HSF1 was not fully activated through the S303A and S307A mutations, as expression of Hsp70 was further enhanced when the transfected cells were exposed to low levels of the proteasome inhibitor MG132

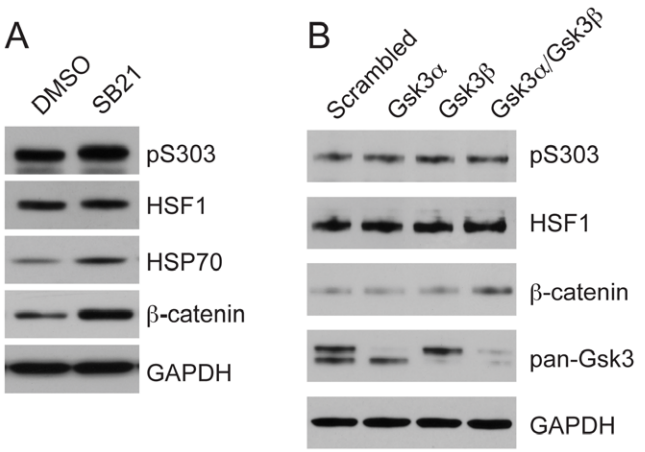

Figure 5. GSK3 represses HSF1 activity in HeLa cells independent of S303 phosphorylation. (**A**) HeLa cells were treated with DMSO solvent or the GSK3 inhibitor SB-216763 (25 μM) for 15 h. Total protein was analyzed for pS303, HSF1, and β-catenin by immunoblotting. GAPDH serves as a loading control. (**B**) HeLa cells were treated with siRNA specific for GSK3α and GSK3β either individually or together or a scrambled siRNA for 72 h. Total protein was analyzed for pS303, total HSF1, β-catenin, GSK3α/β and GAPDH by immunoblotting.
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(Figure 7A, B). This is consistent with our data generated in yeast demonstrating that while the S303A mutation did activate human HSF1-dependent yeast growth, this was further enhanced when the S303A HSF1 mutant was combined with the LZ4m mutation (Figure 3A). Interestingly, in $hsf1^{-/-}$ cells we observed a faster electrophoretic mobility on SDS-PAGE gels for the HSF1 S303A and S303/307A mutant proteins that was not observed for wild-type HSF1 or the S307A mutant (Figure 7A), nor did we observe this change in mobility in the yeast system (Figure 1D). While the nature of this electrophoretic mobility shift is unknown, the HSF1 S303A and S303/S307A mutant alleles also exhibited lower steady state levels when exposed to MG132, suggesting that these proteins, despite having increased activity, might be less stable (Figure 7A, C). Because S303 phosphorylation has been proposed to promote HSF1 sumoylation in mammalian cells [16] it is possible that lack of sumoylation results in the altered electrophoretic mobility. Despite the fact that equal amounts of plasmid DNA were transfected for each mutant, we observed elevated steady state protein levels for the HSF1 S307A mutant (Figure 7A, C). While we have not definitively demonstrated that the S307A mutant protein has increased stability in comparison to wild-type HSF1, this finding correlates with the increased protein levels we observed for the HSF1 mutants expressed in yeast (Figure 1D, F) and will require further investigation. Interestingly, when we expressed the HSF1 S307A mutant in $hsf1^{-/-}$ cells we did not observe a reduction in S303 phosphorylation (Figure 7A, D) as was observed in yeast cells (Figure 1D, E) suggesting that priming requirements for S303 phosphorylation may change in different expression systems.

Discussion

Mammalian HSF1 activity is regulated via complex regulatory mechanisms that include post-translation modifications as well as inter- and intra-molecular protein-protein interactions [2]. While our understanding of these regulatory mechanisms remains incomplete, earlier work has suggested that many of these mechanisms may be conserved in yeast [25,26,27,28]. This is evident, in part, by repression of the human HSF1 protein when it is expressed in *S. cerevisiae* via coiled-coil domain and HSF1 loop interactions. In this report we show that evaluation of the mechanisms that regulate HSF1 activity in yeast via post-translational modifications can lead to important insights into the mechanisms that regulate HSF1 in mammalian cells.

Previous experiments using HSF1 fusions with the constitutively bound Gal4 or lexA DNA-binding domains demonstrated that phosphorylation of S303 contributed to the repression of HSF1 transactivation [17,18]. In this report we show that alanine substitution of S303, in the context of full length HSF1, also results in increased levels of trimerized HSF1 both in un-stressed yeast and in mammalian cells. This suggests that aside from repressing transactivation, S303 phosphorylation can also repress earlier points in the HSF1 activation pathway. Interestingly, we also show that repression of HSF1 activity through S303 phosphorylation may occur independent of K298 sumoylation in yeast, as arginine substitution of K298 does not promote HSF1 activation in yeast. It should be noted that not all of the mechanisms that regulate human HSF1 in mammalian cells are conserved in yeast. While human HSF1 is repressed in both yeast and mammalian cells through an amino-terminal coiled-coil as well as a carboxyl-terminal linker domain, the ability of wild type human HSF1 to respond to proteotoxic compounds or thermal stress, for example, appears to be strikingly absent in yeast [25,27,28]. Nevertheless, the ability of S303 phosphorylation to promote repression of human HSF1 in yeast independent of K298 sumoylation suggests that our understanding of the mechanisms by which S303 phosphorylation represses HSF1 activity remains incomplete. S303 and S307 are located in the regulatory domain of HSF1, a proposed binding site for the protein chaperone Hsp90 [41]. As such, it is tempting to speculate that phosphorylation of these residues might affect binding to Hsp90.

An understanding of how phosphorylation regulates HSF1 activity and what protein kinases phosphorylate HSF1 remains largely incomplete [20,22]. Early reports showed that *in vitro*, HSF1 S307 phosphorylation acted as an essential priming event for S303 phosphorylation [22]. However, a subsequent report showed this priming event was not required *in vivo* and that HSF1 S303 phosphorylation occurred independent of S307 phosphorylation in K562 cells [16]. The work presented here using the yeast model system furthers our understanding of these regulatory mechanisms and may begin to clarify the conflicting mechanisms underlying S303 phosphorylation. Specifically, our data suggest that while phosphorylation of S303 can occur independently of S307 phosphorylation in both yeast and mammalian cells, S303 phosphorylation may be enhanced by S307 phosphorylation in the non-native yeast system. While a mechanistic basis for this difference in the requirements for S303 phosphorylation remains unknown when HSF1 is expressed in yeast, structural differences could change the priming requirements for S303 phosphorylation. Such changes in HSF1 might occur due to different protein interactions and as such it is not surprising that in *in vitro* experiments, using only recombinant HSF1 protein, phosphorylation of S303 is fully dependent on S307 phosphorylation. However, further studies will be required to fully test these hypotheses.

Here, we demonstrate that in both yeast and mammalian cells phosphorylation of HSF1 S303 appears to occur independently of GSK3, previously thought to be the primary kinase responsible for S303 phosphorylation [20,22]. Rather, as suggested by loss of function analysis, we propose that the MAPK Slt2 is one candidate that phosphorylates HSF1 at S303 in yeast though residual phosphorylation of HSF1 at S303 in an *slt2*Δ strain suggests that

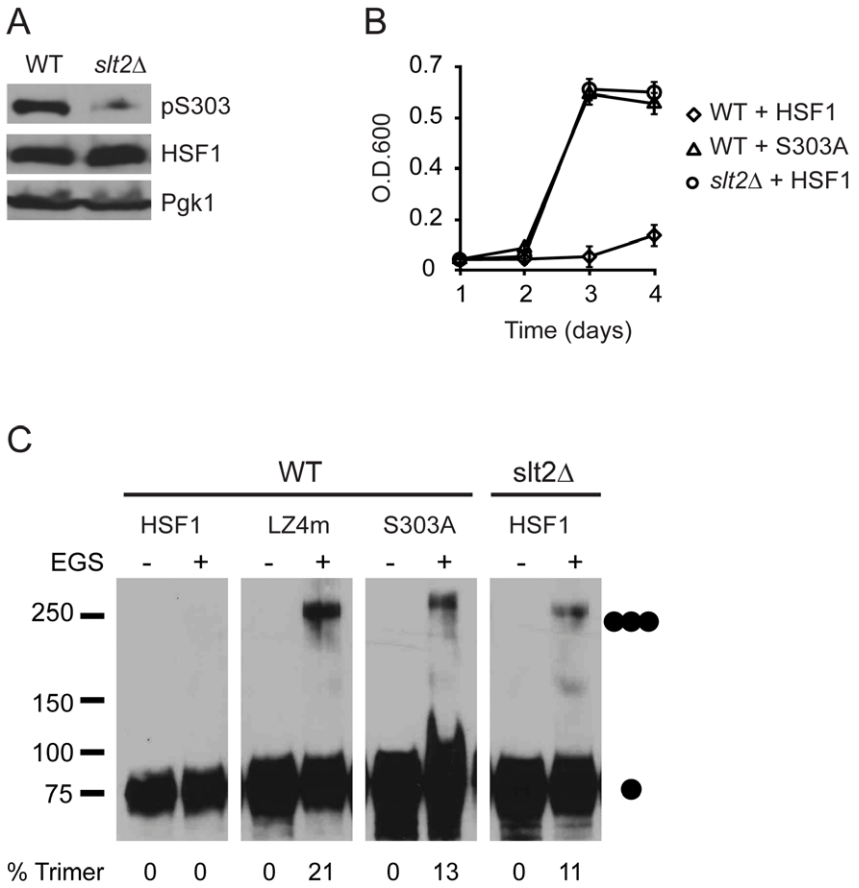

Figure 6. S303 phosphorylation of HSF1 in yeast is modulated by Slt2. (**A**) PS145 and LNY2 (*slt2*Δ) were transformed with a plasmid expressing wild-type HSF1 and were grown on galactose containing medium. Total protein extracts were analyzed for pS303, total HSF1 and Pgk1 by immunoblotting. (**B**) PS145 (WT) expressing wild-type HSF1 or the S303A mutant or LNY2 (*slt2*Δ) expressing wild-type HSF1 were grown in dextrose supplemented medium for 4 d. Growth was monitored by measuring $O.D._{600}$. (**C**) PS145 (WT) expressing wild-type HSF1, the LZ4m mutant or the S303A mutant and LNY2 (*slt2*Δ) expressing HSF1 were evaluated for HSF1 multimerization by EGS cross-linking, SDS-PAGE, and immunoblotting. The positions of molecular weight markers are indicated on the left and circles indicating the expected migration of HSF1 monomers and trimers are on the right. Levels of HSF1 trimer as percent of total HSF1 are shown below.
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other MAPKs may also contribute to S303 phosphorylation. Differences in HSF1 structure between the *in vivo* and *in vitro* systems may also explain why different kinases can target S303 for phosphorylation under different conditions. We speculate that under some cellular conditions, for example physiological stress or different cell types, HSF1 structure may be altered, thereby shifting the S303-kinase specificity from a MAPK to GSK3. This might, in part, contribute to the complexity in identifying all of the mammalian kinases that phosphorylate S303. While GSK3 does not appear to phosphorylate HSF1 at S303 *in vivo*, data presented here nevertheless support a role for GSK3 as a repressor of HSF1 activity. It should be noted that several other serine residues in the HSF1 coding sequence, including S307, are located within putative GSK3 consensus sites [39].

The importance in understanding HSF1 regulation is underscored by recent findings showing that pharmacological activation of HSF1 can increase protein chaperone expression and ameliorate cytotoxicity in models of protein folding disease [28,42,43,44,45]. As such, it is important to further our understanding of the mechanisms that repress HSF1 activity as potential points of therapeutic intervention in disease. For example, our data has shown that the loss of S303-dependent HSF1 repression can lead to the accumulation of protein chaperones and as such could be efficacious in the treatment of protein folding diseases. In support of this possibility Rimoldi *et al* showed that over-expression of the HSF1 S303G mutant in HeLa cells reduced aggregation and inclusion formation of an aggregation prone Ataxin1-31Q mutant protein [46] In addition, Fujimoto *et al* showed that overexpression of a constitutively active HSF1 mutant lacking the regulatory domain, which includes S303 and S307, suppressed the aggregation and cytotoxicity of a mutant Huntingtin protein in both cell culture and mice [47]. Furthermore, Carmichael *et al* suggested that GSK3-inhibitors might prove useful in the treatment of polyQ-expansion diseases [48].

Materials and Methods

Yeast Strains, Plasmids

S. cerevisiae strains used in this study are listed in Table 1. Yeast expression plasmids pRS424-GPD-HSF1 and pRS424-GPD-HSF1LZ4m were described previously [25]. Point mutations were introduced into the HSF1 coding sequence using the Quick-Change Site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. YEp351-*Slt2-FLAG* was kindly provided by Dr. David E. Levin [49]. Mammalian expression plasmids were

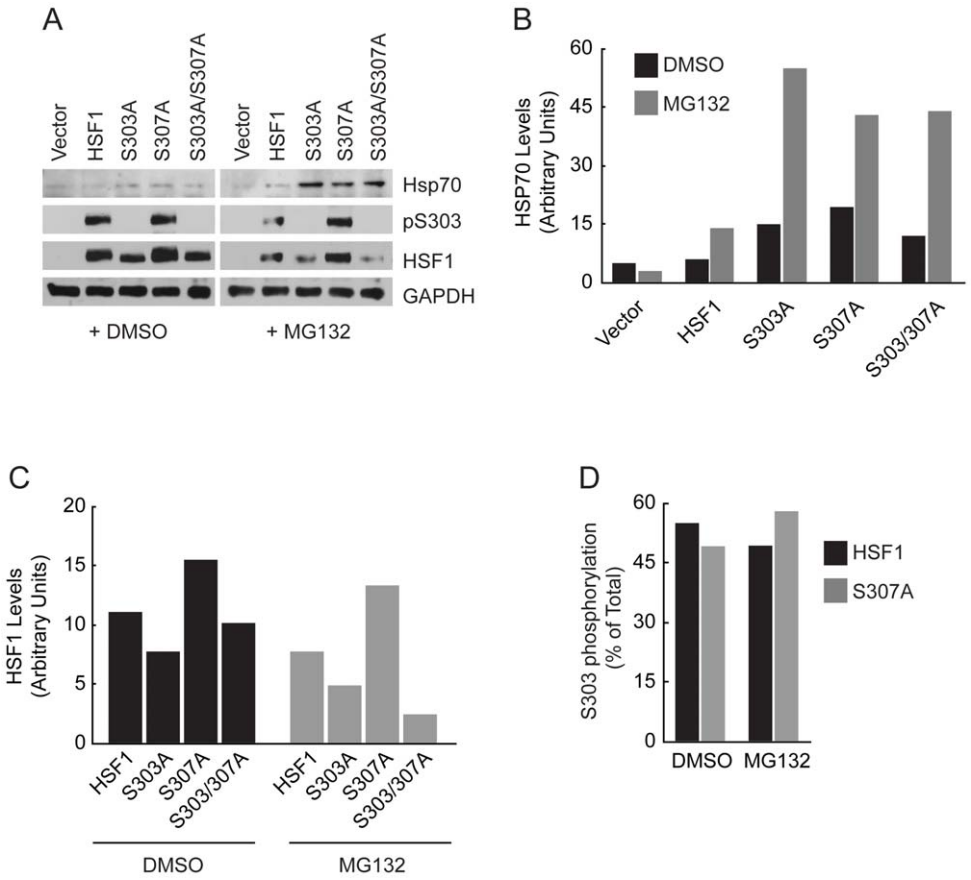

Figure 7. S303 and S307 repress HSF1 activity in hsf1$^{-/-}$ MEFs. (**A**) hsf1$^{-/-}$ MEFs were transfected with an empty vector or plasmids expressing wild-type HSF1 or the S303A, S307A or the S303/307A mutants. The transfected cells were treated with DMSO solvent or MG132 (10 μM) for 5 h. Total protein extracts were analyzed for Hsp70, pS303 and HSF1 by immunoblotting. GAPDH serves as a loading control. (**B**) Protein levels of Hsp70 were normalized to GAPDH, from panel A. (**C**) Protein levels of HSF1 were normalized to GAPDH, from panel A. (**D**) Levels of HSF1 phosphorylated at S303 were quantified and are shown as a percent of total HSF1, from panel A.
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generated by subcloning the HSF1 open reading frame from yeast vectors into the mammalian vector pcDNA3.1.

Cell culture maintenance, transfection and siRNA

Mammalian cell lines used in the study were hsf1$^{-/-}$ MEF cells [30] and HeLa cells (ATCC, CCL-2). The MEF cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 100 U/ml penicillin/streptomycin and 55 μM 2-mercaptoethanol. HeLa cells were maintained in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. MEF cells were transfected with HSF1 expressing plasmids using a Nucleofector (Lonza) and Nucleofector solution MEF2. siRNA was purchased from Dharmacon and 2 nmoles of each siRNA were transfected into HeLa cells using Dharmafect 1. Knock-down of proteins was assayed 72 h after siRNA transfection by immunoblot analysis.

Complementation assays

Growth curve experiments were carried out in 96-well plates as described previously [28]. For spot assays yeast cells were grown

Table 1. Yeast strains used in this study.

Strain	Genotype
PS145	*MAT*a *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1 hsf1Δ::LEU2* Ycp50gal-*yHSF*
YPH499	*MAT*a *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
LNY1	*MAT*a *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1 hsf1Δ::LEU2* Ycp50gal-*yHSF rim11Δ::HIS3*
LNY2	*MAT*a *ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3-1 hsf1Δ::LEU2* Ycp50gal-*yHSF slt2Δ::HIS3*
LNY3	*MAT*a *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rim11Δ::TRP1 mck1Δ::HIS3 mrk1Δ::URA3 ygk3Δ::kanMX*

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overnight in galactose-containing medium to allow for expression of GAL1-yHSF and reseeded the following day at $O.D._{600} = 0.2$ and spotted on either galactose or dextrose supplemented growth media.

Immunoblot and Crosslinking Analysis

Protein extracts were generated from yeast cultures using glass bead lysis in cell lysis buffer (25 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA) supplemented with protease inhibitors (Roche) and Halt phosphate inhibitor cocktail (Thermo Scientific Pierce). Proteins extracts were generated from mammalian cell culture using cell lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentrations were quantified using the BCA assay and 80–100 μg of total protein was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. HSF1 oligomerization was assessed using the amine-specific cross-linker ethylene glycol bis-succinimidyl succinate (EGS) (Pierce). Crosslinking analysis were carried out as described previously [28]. Antibodies used in this study were anti-phospho-S303(pS303) (ab47369, Abcam), anti-HSF1 [28], anti-Pgk1, anti-FLAG (M2, Sigma), anti-Hsp70 (C92, Stressmarq), anti-β-catenin (6B3, Cell Signaling), anti-GAPDH (6C5, Ambion) and anti-GSK3α/β (D75D3, Cell Signaling). Quantification of immunoblot data was done using Photoshop.

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Author Contributions

Conceived and designed the experiments: LB-N DWN PCCL DJT. Performed the experiments: LB-N DWN PCCL. Analyzed the data: LB-N DWN PCCL CR-P DT. Wrote the paper: DWN DJT.

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online data

Contribution of Yap1 towards *Saccharomyces cerevisiae* adaptation to arsenic-mediated oxidative stress

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In the budding yeast *Saccharomyces cerevisiae*, arsenic detoxification involves the activation of Yap8, a member of the Yap (yeast AP-1-like) family of transcription factors, which in turn regulates *ACR2* and *ACR3*, genes encoding an arsenate reductase and a plasma-membrane arsenite-efflux protein respectively. In addition, Yap1 is involved in the arsenic adaptation process through regulation of the expression of the vacuolar pump encoded by *YCF1* (yeast cadmium factor 1 gene) and also contributing to the regulation of *ACR* genes. Here we show that Yap1 is also involved in the removal of ROS (reactive oxygen species) generated by arsenic compounds. Data on lipid peroxidation and intracellular oxidation indicate that deletion of *YAP1* and *YAP8* triggers cellular oxidation mediated by inorganic arsenic. In spite of the increased amounts of As(III) absorbed by the *yap8* mutant, the enhanced transcriptional activation of the antioxidant genes such as *GSH1* (γ- glutamylcysteine synthetase gene), *SOD1* (superoxide dismutase 1 gene) and *TRX2* (thioredoxin 2 gene) may prevent protein oxidation. In contrast, the *yap1* mutant exhibits high contents of protein carbonyl groups and the GSSG/GSH ratio is severely disturbed on exposure to arsenic compounds in these cells. These results point to an additional level of Yap1 contribution to arsenic stress responses by preventing oxidative damage in cells exposed to these compounds. Transcriptional profiling revealed that genes of the functional categories related to sulphur and methionine metabolism and to the maintenance of cell redox homoeostasis are activated to mediate adaptation of the wild-type strain to 2 mM arsenate treatment.

Key words: arsenic stress, oxidative stress, transcriptional regulation, yeast AP-1-like transcription factor Yap1 gene (*YAP1*), yeast AP-1-like transcription factor Yap8 gene [*YAP8*; *ACR1* (arsenic compound resistance protein 1 gene), *ARR1* (arsenical-resistance protein 1 gene)].

INTRODUCTION

Arsenic (As) is a highly toxic metalloid widely distributed in Nature and mostly found in drinking water. The first step of inorganic As(V) removal from the cytoplasm consists of its two-electron reduction to As(III) using glutathione as the source of reducing potential [1]. Chronic exposure to this compound is generally associated with an increased risk of multiple cancers, vascular diseases, developmental anomalies and neurological disorders [2–4]. To counteract the deleterious effects caused by arsenic compounds, almost all living organisms have developed mechanisms to eliminate it. In the budding yeast *Saccharomyces cerevisiae*, resistance to arsenic is achieved through the activation of the transcriptional regulator Yap8 [yeast AP-1-like transcription factor Yap8, also called Acr1 (arsenic compound resistance protein 1)] [5], which in turn, induces the expression of an arsenate reductase and a plasma-membrane arsenite-efflux protein encoded by the genes *ACR2* and *ACR3* respectively [6-10]. In addition, the *YCF1* gene product, yeast cadmium factor 1, also facilitates the vacuolar extrusion of glutathione-conjugated arsenite molecules [11]. Although Yap8 is the main regulator of arsenic stress responses, Yap1 is also involved to a lesser extent through *YCF1* activation under these conditions and contributes to the full activation of enzymes encoded by the *ACR* genes [12]. Both regulators belong to the Yap family of bZIP (basic domain/leucine zipper) transcription factors, formed by eight members [10], which modulates the activation of specific genes in response to various stress (for a review, see [5]).

Arsenic toxicity and carcinogenicity in animals has been suggested to be probably due to the generation of an oxidative stress, thus provoking a deleterious effect by this metal [13]. Yeast mutants in genes related to several mitochondrial processes, which show sensitive phenotypes to arsenic compounds, were recently identified. A total of 20 specific-As(V)- sensitive mutants were found, from which 13 genes have orthologues in humans [14]. On the other hand, high-throughput arsenite-triggered changes in transcriptional profiling [15,16] indicate that cell antioxidant defences are up-regulated in yeast. Furthermore, other investigators have shown a dose-dependent increase in the levels of peroxidation of membrane lipids as a consequence of arsenite exposure [17]. Yap1, the best-characterized member of the Yap family and the major regulator in oxidative stress, is involved in arsenic stress responses. These facts together led to the hypothesis that arsenic induces oxidative stress in which Yap1 plays a major role. It is indeed known that arsenite [As(III)] can react with the thiol groups of proteins, inhibiting many biological pathways,

Abbreviations used: Arr1, arsenical-resistance protein 1; Acr1, arsenic compound resistance protein 1; DAPI, 4′,6-diamidino-2-phenylindole; DABCO, 1,4-diazabicyclo[2.2.2]octane; DNPH, 2,4-dinitrophenylhydrazine; DCF-DA, 2′,7′-dichlorofluorescein diacetate; FCT, Fundação para a Ciência e Tecnologia; GFP, green fluorescent protein; *GSH1*, γ-glutamylcysteine synthetase gene; LIFE, Laboratório de Investigação de Fatores de Estresse; MDA, malondialdehyde; ROS, reactive oxygen species; *SOD1*, superoxide dismutase 1 gene; TBARS, thiobarbituric acid-reactive substances; *TRX2*, thioredoxin 2 gene; UFRJ, Universidade Federal do Rio de Janeiro; Yap1 and Yap8, yeast AP-1-like transcription factors Yap1 and Yap8; *YCF1*, yeast cadmium factor 1 gene.

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whereas the pentavalent form [As(V)] of arsenic is a phosphate analogue interfering with phosphorylation reactions [1]. Although the toxic effect of both oxidation states, As(V) and As(III), appears to be very similar, the elimination of As(V) requires its reduction to As(III) using the redox potential of GSH and thus interfering with the GSH pool of the cell [1]. As recently pointed by other investigators [14], tolerance to either arsenate or arsenite also involves specific sets of mitochondrial genes.

We decided to evaluate, using biochemical and molecular approaches, the damage caused by As(V) and As(III). We show that oxidative stress is generated as an effect of arsenic exposure in strains defective in the arsenic-extrusion machinery and in the antioxidant defence system. By measuring the GSH/GSSG ratios, we provide evidence indicating that arsenic compounds trigger the disruption of the redox equilibrium (being the homoeostasis rapidly achieved through the enhancement of GSH generation). Transcriptional profiling of the wild-type strain under exposure to arsenate reveals the induction of many Yap1-dependent genes and genes involved in sulphur metabolism. Our results show that the antioxidant defences are up-regulated in the mutant *yap8*, which absorbs increased amounts of arsenite, in comparison with the parental strain. Since the status of protein carbonylation is not changed in the wild-type and *yap8* strains, we conclude that the activation of the antioxidant system under arsenic stress prevents the accumulation of oxidized proteins. Consistent with this notion, the *yap1* mutant displays high levels of protein oxidation.

EXPERIMENTAL

Strains, plasmids and growth conditions

The yeast strains used in the present study were: BY4741 *MAT a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0 (*EUROSCARF), BY4741 *Δyap1 MAT a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0; YML007w::kanMX4 (*EUROSCARF), BY*Δyap8 MAT a; his3Δ1; leuΔ0; met15Δ; lys2Δ0; ura3Δ0; YPR199c::kanMX4* [12], BY *Δyap1Δyap8 MAT a; his3Δ1; leuΔ0; met15Δ0; lys2Δ0; ura3Δ0; YPR199c::kanMX4; YML007w::HIS* [12] and FT4 *yap1 MAT a; ura3-52; trp1Δ63; his3-Δ200; leu2::PET56*; *yap1Δ* [18]. The complete coding region of *YAP8* gene was deleted by the microhomology PCR method [19] to create the strain FT4 *yap8 MAT a; ura3-52; trp1Δ63; his3-Δ200; leu2::PET56; yap8::KAN*. Deletion was confirmed by PCR analysis of genomic DNA using upstream and downstream primers. To overexpress *YAP8*, the corresponding chromosomal region was amplified by PCR using the primers 5′-CCATTGTAGGAGAGTAACCT-3′ and 5′-CATCGAATACTCCACATCGATC-3′. The product was first cloned using the Zero Blunt® TOPO® PCR cloning Kit (Invitrogen) and the XbaI/BamHI fragment was subcloned into the 2 μ vector YEplac195 [20]. The construct was sequenced using the ABI Prism DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 373A Automatic Sequencer (PerkinElmer). The plasmid overexpressing YAP1 was available in our laboratory [18]. The CEN plasmids expressing the myc-tagged YAP1 and GFP (green fluorescent protein)-tagged YAP1 and YAP8 versions were obtained from Dr M.B. Toledano's group [21], from Kuge and colleagues [22] or was available in our laboratory [12] respectively. Strains were grown in complete YPD [1 % yeast extract, 2 % (w/v) bactopeptone and 2 % (w/v) glucose] or selective media [SC (synthetic complete) or SD (minimal synthetic defined): 0.67 % ammonium sulfate/yeast nitrogen base without amino acids (Difco) and 2 % (w/v) glucose] supplemented with the appropriate selective amino acids. Early-exponential-phase cells [attenuance (D_{600}) 0.4–0.5] were stressed by the addition of 2 mM As(V) (Na_2HAsO) or As(III) ($NaAsO_2$) and samples were collected at the indicated time points. Phenotypic growth assays were carried out by spotting 5 μl of an early-exponential-phase sequentially diluted culture (approx. 2000–20 cells) in selective medium containing up to 2 mM Na_2HAsO or $NaAsO_2$. Growth was recorded after 2 days at 30 °C. The bacterial *Escherichia coli* strain XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZDM15 Tn10* (Tet^r)] (Stratagene) was used as the host for routine cloning purposes. Standard methods were used for genetic analysis, cloning and transformation [23].

Intracellular oxidation, lipid peroxidation and protein carbonylation

Measurements of intracellular oxidation and lipid peroxidation were performed with 50 mg (dry weight) of mid-exponential-phase cells grown under physiological conditions or exposed to arsenate or arsenite stress. Intracellular oxidative stress generated by arsenic compounds was monitored by measuring changes in fluorescence resulting from the oxidant-sensitive probe DCF-DA (2′,7′-dichlorofluorescein diacetate) [24]. A fresh ethanol stock solution of DCF-DA was added to the culture to a final concentration of 10 μM and cells were incubated for 15 min to allow uptake of the probe. Cells from each aliquot were cooled on ice, harvested by centrifugation at 2000 ***g*** for 5 min at 22 °C and washed twice with distilled water. The cell pellets were resuspended in 500 μl of water and lysed by vortex-mixing in the presence of 1.5 g of glass beads. The extracts obtained after centrifugation at 15 000 ***g*** for 5 min were diluted and fluorescence was measured using a Photo Technology International spectrofluorimeter set at an excitation wavelength of 504 nm and an emission wavelength of 524 nm with a slit width of 5 nm. The effect of deletions alone was measured under physiological conditions and the results under arsenic stress were expressed as the relation between the fluorescence of stressed and unstressed cells. Lipid peroxidation was determined by quantifying TBARS (thiobarbituric acid-reactive substances). After 24 h incubation with As compounds, cells were cooled on ice, harvested by centrifugation and washed twice with 20 mM Tris/HCl buffer, pH 7.4. The pellets were resuspended in 500 μl of the same buffer containing 10 % (w/v) trichloroacetic acid and 1.5 g of glass beads were added. The samples were lysed using six cycles of 20 s agitation on a vortex-mixer, followed by 20 s on ice. Lipid peroxidation of cell extracts was monitored spectrophotometricaly at 532 nm in an EDTA/thiobarbituric acid/NaOH solution as described in [25], which determines the accumulation of TBARS in cells. The control data for each mutant strain grown under physiological conditions was expressed as the amount of MDA (malondialdehyde)/mg of cell dry weight formed and the results under arsenic stress are expressed as the relation between the values of stressed and non-stressed cells. Several authors have recently used this technique [26,27]. All these experiments were carried out at least three times, with no fewer than three replicate measurements in each experiment and the results are presented are means ± S.D. To detect the presence of carbonyl groups introduced in proteins as a result of arsenic stress, an OxyBlot™ protein oxidation detection kit (Intergen) was used. The samples were analysed by immunoblotting and processed as described in [28], using rabbit anti-dinitrophenol antibody as the primary antibody. As a loading control, levels of the co-chaperone Sba1 were measured [29].

Glutathione determination

Measurements of thiols were performed by the spectrophotometric GR–DTNB [glutathione reductase–5,5-dithiobis-(2-nitrobenzoic acid)] recycling method originally described by Griffith

[30]. The wild-type and mutant strains grown to early exponential phase were induced with 2 mM As(V), and samples collected at the indicated time points were extracted in a solution consisting of 0.1 M HCl and 1 mM EDTA, pH 1.35 [28]. The kinetics of TNB [5-thio-2-nitrobenzoate] formation was monitored photometrically at 405 nm. GSSG concentrations were determined in the same extracts after a 30 min incubation of the supernatant with 2-vinylpyridine at room temperature (20–22 °C) to derivatize GSH [30]. The concentration was determined by reference to a standard prepared in HCl and was expressed as nmol of glutathione/mg cell dry weight. Measurements were carried out three times, with three replicates in each experiment and results are means ± S.D.

Determination of As (III) retention by atomic absorption

Analysis of the capacity of *S. cerevisiae* wild-type cells and *Δyap1* and *Δyap8* mutant versions to absorb and accumulate As(III) was determined using atomic-absorption spectrophotometry, as previously described [31]. Arsenite, to a final concentration of 2 mM, was added to the medium containing 1 mg (dry weight) of mid-exponential-phase cells, and the culture was incubated at 28 °C in a rotating bath for 4 h and 24 h. For measuring residual As(III) present in the medium, 5 ml aliquots were centrifuged at 2000 ***g*** for 5 min at 22 °C and the supernatant was collected and subjected to atomic-absorption spectrophotometry using a PerkinElmer 3100 atomic-absorption spectrometer. As(III) absorption was calculated by determining the difference in metalloid content between the control medium without cells and the test medium containing cells. Percentages of As(III) absorption were calculated by use of the following equation:

$$\text{Absorption (\%)} = [(\text{initial concentration} - \text{final concentration})/\text{initial concentration}] \times 100$$

The measurements were carried out three times, with three internal replicates and the results presented are means ± S.D.

Protein extraction and immunoblot analysis

Δyap1 mutant cells transformed with the plasmid encoding *c-myc–YAP1* [21] were grown to early exponential phase and induced or not with 2 mM As(V). Samples collected at the indicated time points were harvested by centrifugation at 4 °C and the protein extracts were prepared by the trichloracetic acid-lysis method and immunoblotted as described in [32]. To follow the kinetics of the recombinant *c*-Myc-Yap1 protein under arsenic stress, immunoblotting was performed with 50 μg of proteins that were probed with the 9E10 anti-*c*-Myc monoclonal antibody. Sba1 (p23), encoded by the *SBA1* gene [33], was used as a loading control [29,34]. Detection was performed using an ECL® (enhanced chemiluminescence) Western-blotting reagent kit (Amersham Pharmacia).

Fluorescence microscopy

FT4 *yap8* and FT4 *yap1* strains transformed with pRS encoding *cp-GFP-HA-YAP8* [12] or *cp-GFP-HA-YAP1* [22] respectively (where cp is centromeric plasmid and HA is haemagglutinin), were grown to early exponential phase and induced with either 2 mM As(V) or As(III) at the indicated time points. DAPI (4,6-diamidino-2-phenylindole) was added as a DNA marker at a final concentration of 5 μg/ml, 5 min before microscopy. After washing with PBS, cells were resuspended in a solution of 200 mM DABCO (1,4-diazadicyclo[2.2.2]octane) in 75 % (v/v) glycerol and 0.25 × PBS (Sigma–Aldrich). Both we and Delaunay et al. [21] have shown that DABCO does not affect the localization of the GFP fusions. 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.).

Northern Blot, real-time PCR and microarray analysis

RNA procedures were performed as described in [32]. RNA was isolated from cultures that were either untreated or exposed to 2mM Na_2HAsO or $NaAsO_2$ at the indicated time points. For Northern-blot analysis, approx. 40 μg of total RNA was separated in formaldehyde gels and transferred on to nylon membranes (Hybond XL;Amersham Pharmacia Biotech). Intragenic PCR fragments of *GSH1* and SNR17A/small nucleolar RNA U3 were used as probes. For real-time quantitative PCR the RNA samples were treated with DNase (TURBO DNAse-free; Ambion) according to the manufacturer's instructions. cDNAs were synthesized by reverse transcription from 0.5 μg of total RNA, using 50 pmol of $(dT)_{15}$, 1 mM dNTP and 5 units of Transcriptor Reverse transcriptase as described by the manufacturer (Roche). cDNA amplification was quantitatively analysed by incorporation of SYBR Green I (LightCycler FastStart DNA Master SYBR Green I; Roche) into double-stranded DNA, according to the manufacturer's instructions, on a Roche LightCycler II Instrument, using the *ACT1* (actin) gene as a loading control. The fold change was determined by the $2^{\Delta\Delta CT}$ method [35]. The primers used were as follows: *GSH1*: 5′-GCTGCTGGTAAAAGAGACAATG-3′ and 5′-ACTCACATCGTTAGCCTCACAA-3′; *TRX2*: 5′-GGTCACTCAATTAAAATCCGCTTC-3′ and 5′-CGACGACTCTGGTAACCTCCTTAC-3′; *SOD1*: 5′-AGCCAACCACTGTCTCTTACGA-3′ and 5′-ACACCATTTTCGTCCGTCTTTA-3′; and *ACT1*: 5′-CTA TTG GTA ACG AAA GAT TCA G-3′ and 5′-CCT TAC GGA CAT CGA CAT CA-3′. For transcript profiling, total RNA was purified using the RNAeasy kit (Qiagen), followed by the RNA clean-up procedure. A 10 μg portion of RNA was used to generate labelled cDNA, which was hybridized on the DNA arrays as described in [36]. We used arrays containing probes for most of the yeast open reading frame, obtained from the plate-forme transcriptome of the IFR36 (www.transcriptome.ens.fr). Slides were read using a Genepix 4000B scanner from Axon. The images were analysed with the Genepix pro 6.0 software. Data were normalized using global lowess followed by print tip group median from Goulphar software [37]. Complete microarray data are available as Supplementary Table S1 at http://www.BiochemJ.org/bj/414/bj4140301add.htm. The gene-ontology analyses were performed by submitting the whole set of microarray results to the t-profiler tool [38] using default parameters. Redundant or meaningless functional categories were hidden (Table 1). The results presented in Table 1 and Supplementary Tables S1 and S2 (at http://www.BiochemJ.org/bj/414/bj4140301add.htm) are from three independent experiments. Only genes measured at least twice were kept for further functional analyses.

Statistical analysis

The results reported in the present study are the averages for at least three independent experiments, with three replicates in each experiment, and are expressed as the mean ± S.D. Statistical differences among treatments were analysed by one-way ANOVA with Tukey's HSD (honest significant difference) multiple comparisons test ($\alpha = 0.05$) using STATISTICA for Windows (StatSoft Inc., Tulsa, OK, U.S.A.).

Table 1 Gene-ontology analyses of the arsenate transcriptional response

T-profiler [38] was used to conduct gene-ontology analyses from the global set of microarray data (see the Experimental values). The T-values and E-values are directly taken from the t-profiler output. The T-test is to assess whether the means of two groups are *statistically* different from each other. E-value is a parameter that describes the number of hits one can 'expect' to see by chance when searching a database of a particular size. It decreases exponentially as the score (S) of the match increases.Redundant or meaningless gene-ontology categories were hidden. Examples of genes belonging to each category are indicated, together with their fold induction.

Category	T-value	E-value	Genes
Heat-shock-protein-activity	10.46	$< 1.0 \times 10^{-15}$	*HSP42* (11.2), *SSA4* (11.0, *HSP26* (7.5). *SSE2* (5.9), *HSP12* (5.5), *HSP78* (5.1, *HSP30* (5.1), *SSA3* (3.5), *SSA1* (3.0), *HSP60* (2.2), *HSP104* (2.2). . . .
Sulfur metabolism	6.61	5.3×10^{-8}	*CYS3* (5.4), *MET16* (5.2), *MET10* (4.9), *MET22* (4.0), *HOM3* (4.0), *MET6* (3.7), *STR3* (3.7), *MET28* (3.5), *MET32* (3.3), *MET2* (2.8), *MET8* (2.7), *CYS4* (2.1). . . .
Response to stimulus	5.89	5.4×10^{-6}	*CUP1-2* (19.4), *CUP1-1* (18.8), *HSP42* (11.2), *SSA4* (11.0), *ARR2* (9.7), *HSP26* (7.5), *GRE2* (6.6), *HSP12* (5.5), *HSP78* (5.1), *HSP30* (5.1), *TRX2* (4.5), *MXR1* (4.4), *MET22* (4.0), *FLR1* (3.7), *SSA3* (3.5), *HSP82* (3.5), *TTR1* (3.4), *DDR2* (3.0), *POS5* (2.5), *STF2* (2.4), *ATR1* (2.3), *TSL1* (2.3), *UBC4* (2.2), *HSP104* (2.2), *SNG1* (2.1), *RDS1* (2.1), *GRE3* (2.0), *GPX2* (2.0). . . .
Oxidoreductase activity	5.67	2.0×10^{-5}	*OYE3* (14.2), *ARR2* (9.7), *AAD6* (8.3), *AAD16* (7.1), *AAD14* (6.8), *GRE2* (6.6), *SER33* (5.5), *MET16* (5.2), *MET10* (4.9), *TRX2* (4.5), *MXR1* (4.4), *OYE2* (4.0), *TTR1* (3.4), *PRX1* (3.1), *YPR1* (2.9), *TSA2* (2.8), *MET8* (2.7), *SER3* (2.5), *ADE3* (2.4), *ZWF1* (2.3), *FMO1* (2.2), *DLD3* (2.1), *ALD4* (2.1), *GRE3* (2.0), *GPX2* (2.0) . . .
Response to oxidative stress	4.70	3.6×10^{-3}	*HSP12* (5.5), *TRX2* (4.5), *MXR1* (4.4), *FLR1* (3.7), *TTR1* (3.4), *POS5* (2.5), *GPX2* (2.0), *GLR1* (1.9), *GRX1* (1.8), *TRX1* (1.7) . . .
Proteasome complex	4.31	2.2×10^{-2}	*UBC4* (2.2), *RAD6* (1.9), *RPN8* (1.9), *RPN4* (1.9), *SCL1* (1.8), *UBP6* (1.7), *PRE8* (1.7) . . .

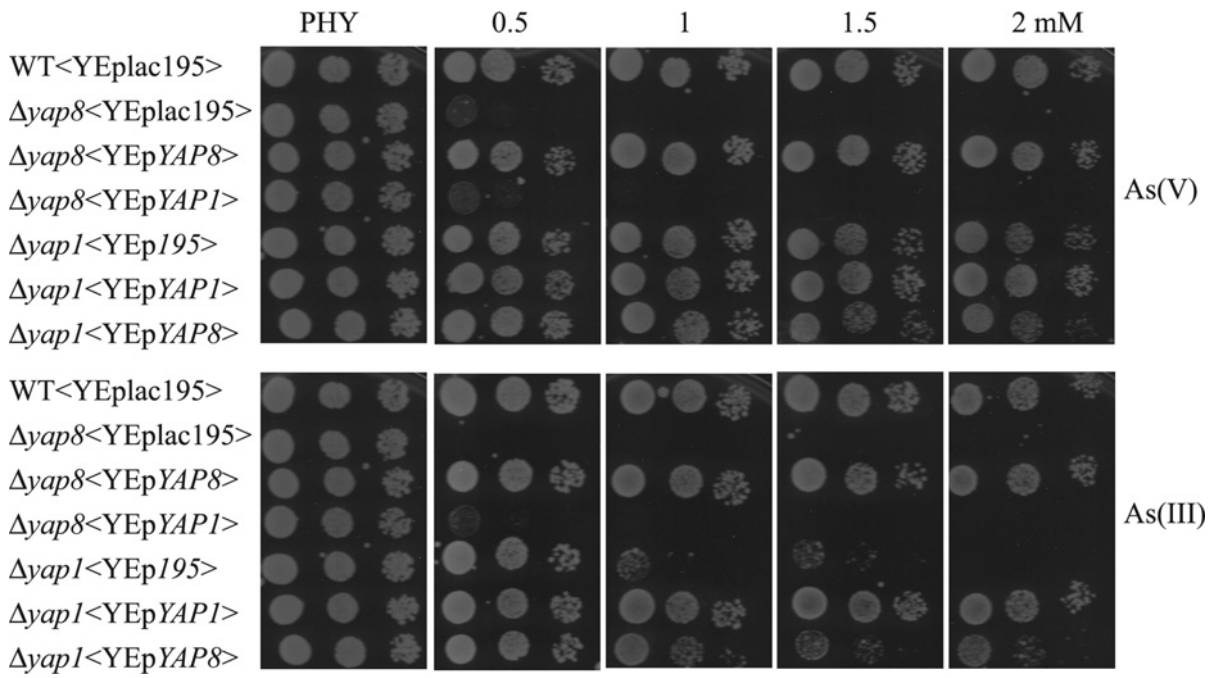


Figure 1 Arsenic-sensitivity phenotypes and respective recovery by overexpression of *YAP1* and *YAP8*

Wild-type (WT), Δ*yap1* and Δ*yap8* mutant strains were transformed with YEplac195 (empty vector), YEpYAP1 or YEpYAP8 and serially diluted cultures were spotted on selective medium supplemented with increasing concentrations of arsenate or arsenite (up to 2 mM). Plates were grown for 2 days at 30°C. A representative experiment is shown. Abbreviation: PHY, physiological conditions.

RESULTS

YAP8 overexpression does not alleviate *yap1* sensitivity under As(V)

It has been suggested that Yap1 and Yap8 play distinct and well-defined roles in the arsenic stress response by regulating distinct sets of genes [15,16]. In order to evaluate the physiological relevance of this specificity, we performed growth complementation assays (Figure 1). The *yap8* mutant reveals a severe growth-sensitive phenotype to both arsenate and arsenite, which is rescued by the overexpression of *YAP8*. The *yap1* mutant shows a mild growth-sensitive phenotype under arsenate conditions. In contrast, under arsenite treatment this phenotype is more accentuated. The overexpression of *YAP8* in the *yap1* mutant partially alleviates the growth-sensitivity of this strain under arsenite treatment, a finding consistent with the notion that Yap1 and Yap8 exert specific roles in arsenic stress responses. On the other hand, *YAP1* overexpression is not able to rescue the sensitive phenotype of the *yap8* mutant strain either under As(V) or As(III) conditions (Figure 1). Altogether, these results suggest some level of specificity of *YAP1* and *YAP8* in arsenic detoxification.

Induction of antioxidant defences by arsenate

In order to characterize global changes in cells subjected to arsenate treatment, we performed microarray analysis. We therefore compared the transcriptome of cells submitted to a 30 min exposure to 2 mM of arsenate with the one of cells mock-treated

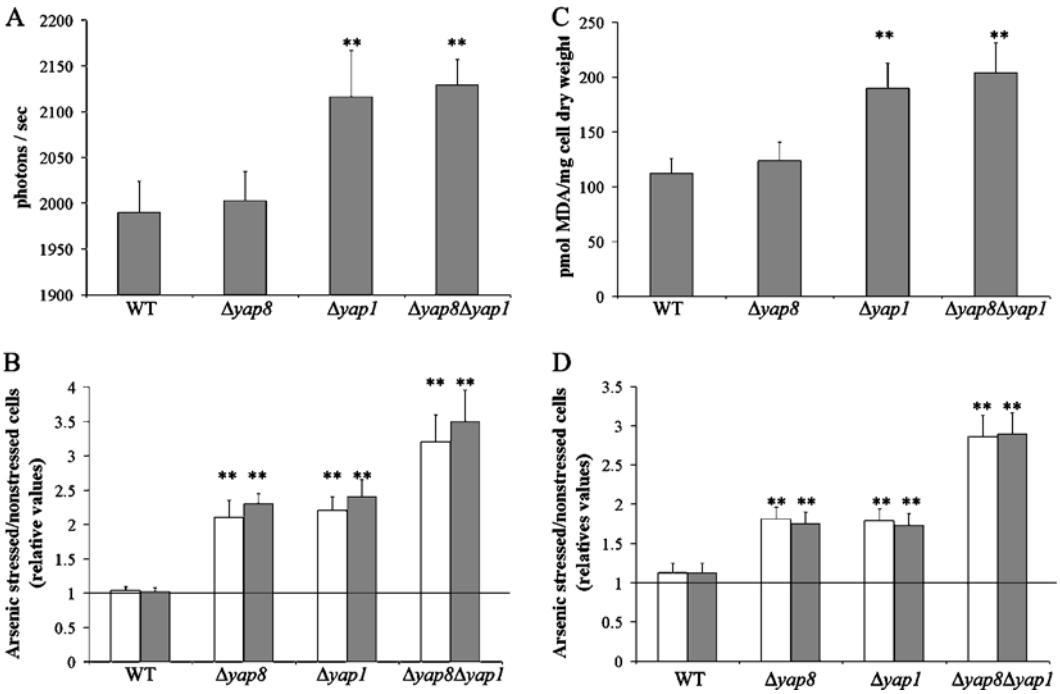


Figure 2 Increase in intracellular oxidation and lipid peroxidation caused by arsenic stress

(**A**) Changes in DCF-DA fluorescence caused by deletions were monitored under physiological conditions. The values are expressed as photons/s. (**B**) Control and mutant strains were stressed with 2 mM arsenate or arsenite for 24 h and the enhancement of the intracellular oxidation was expressed as ratio of the fluorescences of stressed and non-stressed cells. (**C**) The effect of deletions in the lipid-peroxidation content was measured as pmol of MDA/mg cell dry weight by the TBARS method. (**D**) The enhancement of lipid peroxidation was measured as the ratio of the levels observed for stressed and non-stressed cells. The horizontal line corresponding to a ratio of 1.00 in (**B**) and (**D**) indicates the relative value under non-stressed conditions. Values are means ± S.D. for three independent experiments. $^{**}P < 0.01$. WT, wild-type; white bars, arsenate; black bars, arsenite.

with water. We made a global gene-ontology search using the t-profiler software [38] to identify the cellular pathways that were affected by arsenate (see Table 1 and Supplementary Tables S1 and S2). This analysis indicated that arsenate up-regulated genes involved in protein folding, sulfur and methionine metabolism (mainly target genes of the transcription factor Met4p), redox homoeostasis (including most of the target genes of Yap1) and proteasome activity (including the proteasome gene transcriptional regulator *RPN4*). This pattern is indeed very characteristic of the oxidative stress response and is similar to what has been described in the case of cell exposure to arsenite [15,16] or cadmium [39]. Furthermore, the cellular pathway of response to stimulus, including the genes *ACR2* and *ACR3*, is also shown to be induced by arsenate treatment.

Arsenic treatment generates oxidative damage in the *yap1* mutant

Once having established that As(V) induces expression of genes involved in redox homoeostasis, we adopted several biochemical approaches to verify whether 2mM arsenate or arsenite treatment was associated with an oxidative environment in yeast cells. Intracellular oxidation was determined using the probe DCF-DA, which is sensitive to ROS (reactive oxygen species). Time-course experiments had been previously carried out in order to determine the maximum levels of intracellular oxidation, which occurred at 24 h of arsenic treatment (results not shown). The results in Figure 2(A) reveal that deletion of *YAP1*, but not of *YAP8*, interferes with the redox state of the cytoplasm under physiological conditions. The direct exposure of wild-type cells to either arsenate or arsenite does not lead to any detectable increase in the intracellular oxidation levels (Figure 2B), the level being identical in the absence of either *YAP8* or *YAP1* but increasing, however, when compared with the parental strain. This value is still more accentuated in the case of the double mutant. Furthermore, under physiological conditions, increased peroxidation levels of cellular lipids, as compared with the wild-type strain, were detected in the *yap1* mutant through the formation of MDA (Figure 2C). Exposure of wild-type cells to both arsenate and arsenite stress does not cause any significant increase in lipid peroxidation compared with the unstressed cells (Figure 2D), a finding that is in good agreement with the intracellular oxidation results. In single and double mutants these levels are increased about 1.6- and 2.6-fold respectively. Taken together, these results show that arsenic compounds generate oxidative stress under conditions where the arsenate extrusion system and/or the antioxidant machinery are deficient. Protein carbonyl content is an indicator of oxidative stress and is by far the most commonly used biomarker of protein oxidation [40]. To monitor the possible oxidative effects of arsenic treatment at the protein level, the changes in protein carbonyl status during exposure of yeast cells to both arsenate and arsenite were monitored up to 4 h. The resulting OxyBlots™ show that the amount of carbonylated proteins does not vary significantly in the wild-type strain and the mutant *yap8*, although in the latter the levels of oxidized proteins are slightly increased at all time points studied (Figure 3). A more severe effect was, however, observed in the *yap1* strain. When these cells are treated with arsenate, the protein carbonyl content is slightly enhanced compared with the untreated cells. By contrast, exposure to arsenite caused a strong increase in the protein oxidation levels. This effect is even more severe during the first hour of treatment. Furthermore, additional

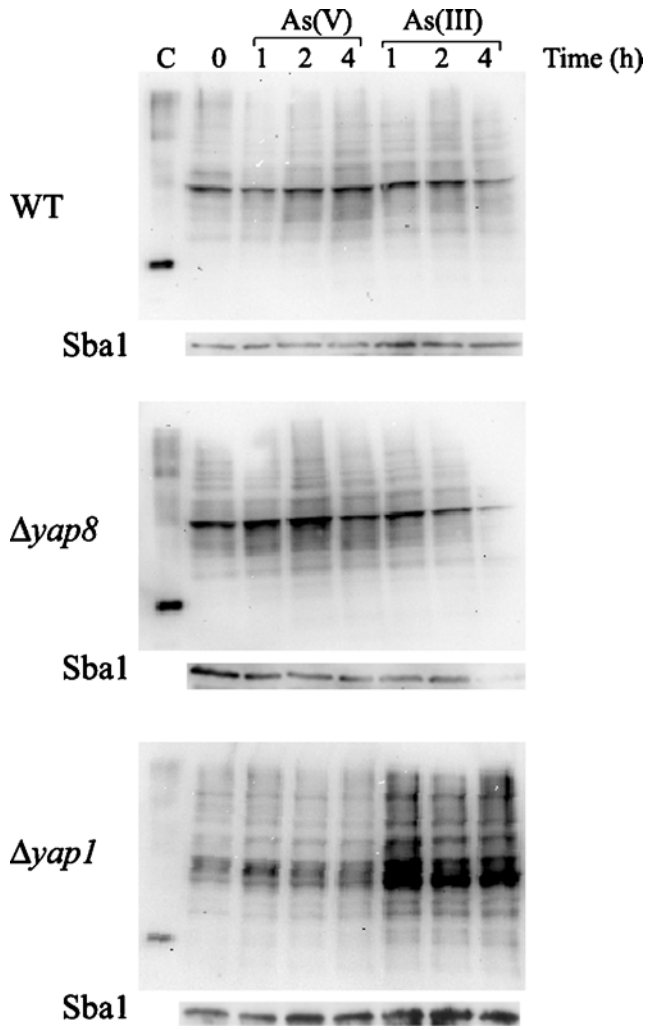


Figure 3 Immunological detection of protein carbonyl groups in the *Saccharomyces cerevisiae* wild-type (WT) and *Δyap1* and *Δyap8* mutant strains

The carbonyl groups introduced in proteins as a consequence of arsenate and arsenite treatment were derivatized to 2,4-dinitrophenylhydrazone (DNP) and were immunoblotted with anti-DNP antibodies. Sba1 was used as an internal loading control. C, OxyBlot™ Protein Standard (Intergen). A representative experiment is shown.

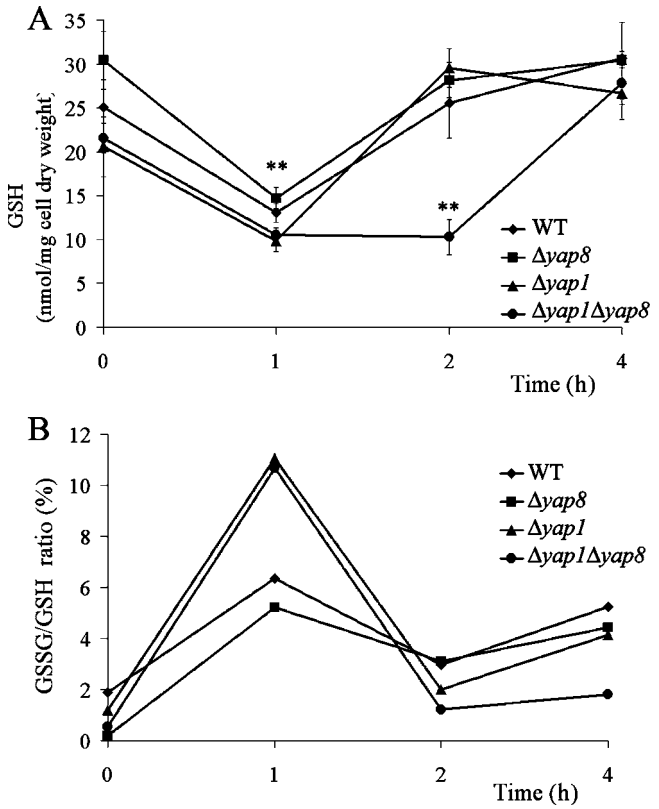


Figure 4 Antioxidant defences during arsenic treatment

(**A**) The wild-type (WT) and *Δyap1*, *Δyap8* and *Δyap1Δyap8* mutant cells were grown to mid-exponential phase and were exposed to 2 mM arsenate. Extracts were obtained at the time points indicated and GSH levels were determined as described in the Experimental section. Values are means ± S.D. for three independent experiments. (**B**) Levels of GSSG were determined in the same extracts after its derivatization to GSH and the GSSG/GSH ratio was calculated. $^{**}P < 0.01$.

carbonylated proteins, most of high molecular mass, were observed in the *yap1* strain. These findings suggest that the effects of oxidative stress generated by arsenic compounds lead to protein oxidation only in the absence of *yap1*.

YAP1 deletion interferes with the redox equilibrium under arsenate

Our transcriptome analysis showed that arsenate up-regulates genes in the cellular pathway of sulphur and methionine metabolism. Furthermore, it has been shown that arsenite-exposed cells channel a large part of assimilated sulfur into glutathione biosynthesis [16]. Yap1 is an important regulator of *GSH1*, which encodes the enzyme responsible for catalysing the condensation of cysteine on to the γ-carbon atom of glutamate in the limiting step of glutathione biosynthesis. This prompted us to evaluate how the requirement of Yap1 couples with the induction of GSH biosynthesis mediated by arsenate and arsenite. It was observed, by measuring the GSH and GSSG contents in the wild-type and mutant strains subjected to arsenate stress, that in all strains the GSH levels diminish about 2-fold during the first 1 h of treatment with 2 mM As(V) (Figure 4A). These values rise gradually over time, reaching physiological levels after 4 h. A similar pattern was observed under As(III) treatment (results not shown). The GSSG/GSH ratio increases after 1 h of treatment and reflects the decrease in GSH (compare Figures 4A and 4B). Notably, the double mutant *Δyap1Δyap8* takes a longer time to recover the GSH levels of the parental strain. Our results show that the redox equilibrium is disrupted in strains bearing *YAP1* deletions, although homoeostasis is rapidly achieved through the enhancement of GSH generation.

Yap1 is maintained in the induced state in long-term arsenate exposure

The biochemical assays clearly show that the wild-type strain, in contrast with the mutant *yap1*, does not suffer the deleterious effects of oxidative stress on exposure to inorganic arsenic. Indeed, the microarrays revealed that many of the antioxidant defences, as well as the arsenic detoxification system, were induced under As(V) treatment (see Supplementary Tables S1 and S2) to facilitate cell adaptation. In order to analyse the c-Myc–Yap1 protein levels in cells induced or not with arsenate, Western-blot analyses were performed. As Figure 5(A) shows, the levels of Yap1 were rather higher in arsenate-treated cells than in cells grown under physiological conditions. Yap1 protein levels peak at about 45 min after arsenate addition and a slight induction

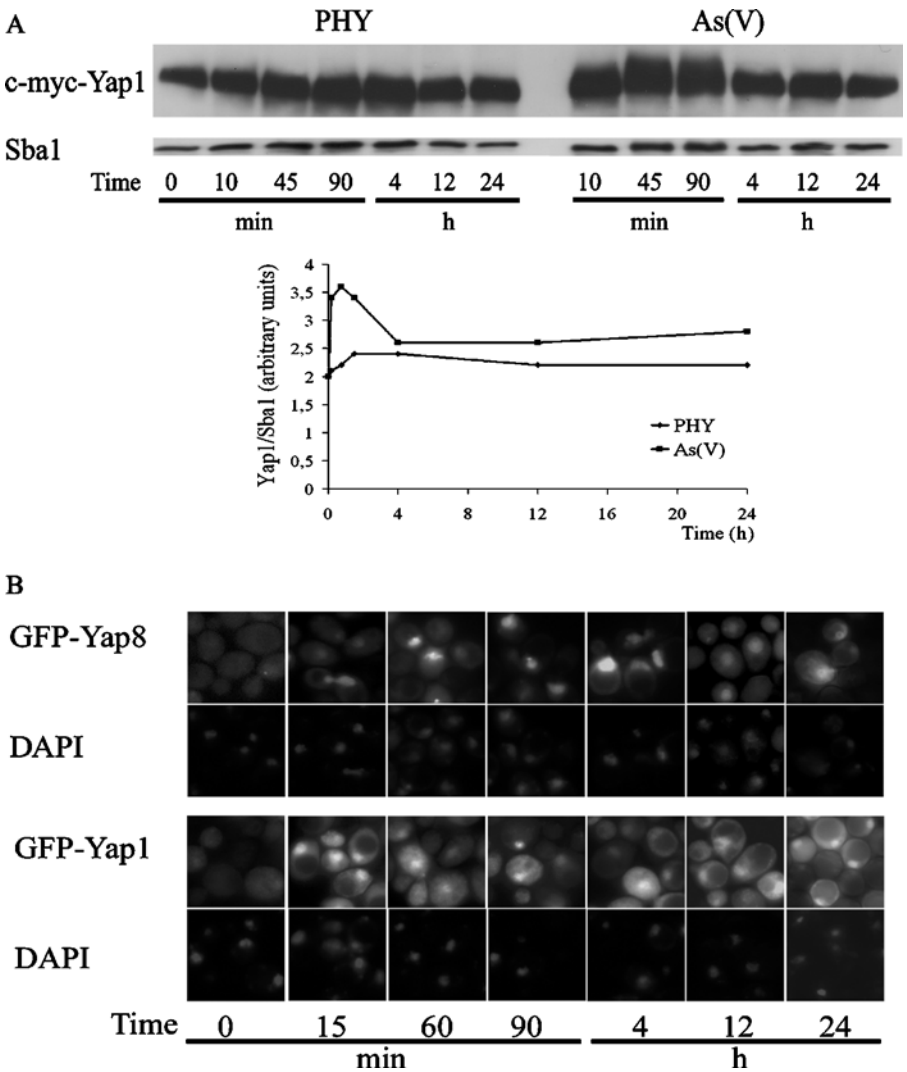


Figure 5 Yap1 is activated in a long-term arsenate exposure

(**A**) Strain BY (EUROSCARF) *yap1* mutant cells transformed with a CEN-plasmid encoding the fusion *c-myc–YAP1* were grown under physiological conditions or in the presence of 2 mM arsenate and sample were harvested at the indicated time points. Protein extraction, separation, transfer and immunoblotting were performed as described in the Experimental section. The protein levels for Sba1 were used as an internal loading control against which all protein levels were normalized (lower panel). (**B**) Kinetics of Yap1 and Yap8 nuclear localization. *Δyap1* and *Δyap8* mutants expressing the fusions *GFP–YAP1* and *GFP–YAP8* respectively, were induced with 2 mM arsenate and analysed for GFP staining at the indicated time points. Representative experiments are shown.

is maintained even up until 12 h of treatment. Furthermore, localization assays revealed that GFP–Yap1, as well as GFP–Yap8, are accumulated in the nucleus until 24 h of arsenate treatment (Figure 5B).

GSH1, *SOD1* and *TRX2* are highly induced under arsenic treatment

To evaluate whether Yap1 activation reflects the induction of the antioxidant cell defences such as *GSH1, SOD1* and *TRX2,* we monitored their transcriptional activation by real-time PCR. Figure 6 reveals that, in the wild-type strain, these genes are highly induced by 2 mM arsenite or arsenate. Strikingly, transcriptional activation of *GSH1*, *SOD1* and *TRX2* is even higher in the *yap8* mutant than in the wild-type strain. Under conditions of exposure to arsenate, all of these genes display a first peak of induction at 90 min, although after 24 h incubation with the metalloid the mRNA levels keep increasing in *yap8* (see Figure 6A). The pattern of mRNA induction in the wild-type and *yap8* strains is very similar up to 4 h exposure to arsenite. However, after this point a strong transcriptional activation of the three genes is observed only in the *yap8* mutant (Figure 6B). Expression of *SOD1* and *TRX2* is completely abolished in the *yap1* mutant (results not shown), a finding consistent with the fact that Yap1 regulates them. Some level of Yap1-independent *GSH1* induction was observed (Figure 6C), suggesting that other factors might be regulating its expression under arsenic stress.

The *yap8* mutant absorbs increased levels of As(III)

As Yap8 regulates As(III) detoxification, we hypothesized that the strong transcriptional activation of the antioxidant genes in the mutant *yap8* was caused by arsenite accumulation. In order to verify this, we used atomic-absorption spectroscopy. After adding 2 mM arsenite to wild-type and *yap8* cultures, the residual amounts of the metalloid in the supernatant at the time points indicated in Figure 7 were determined. We also performed measurements in the *yap1* mutant in order to evaluate the contribution of Yap1 to arsenite detoxification. As Figure 7 shows, arsenite

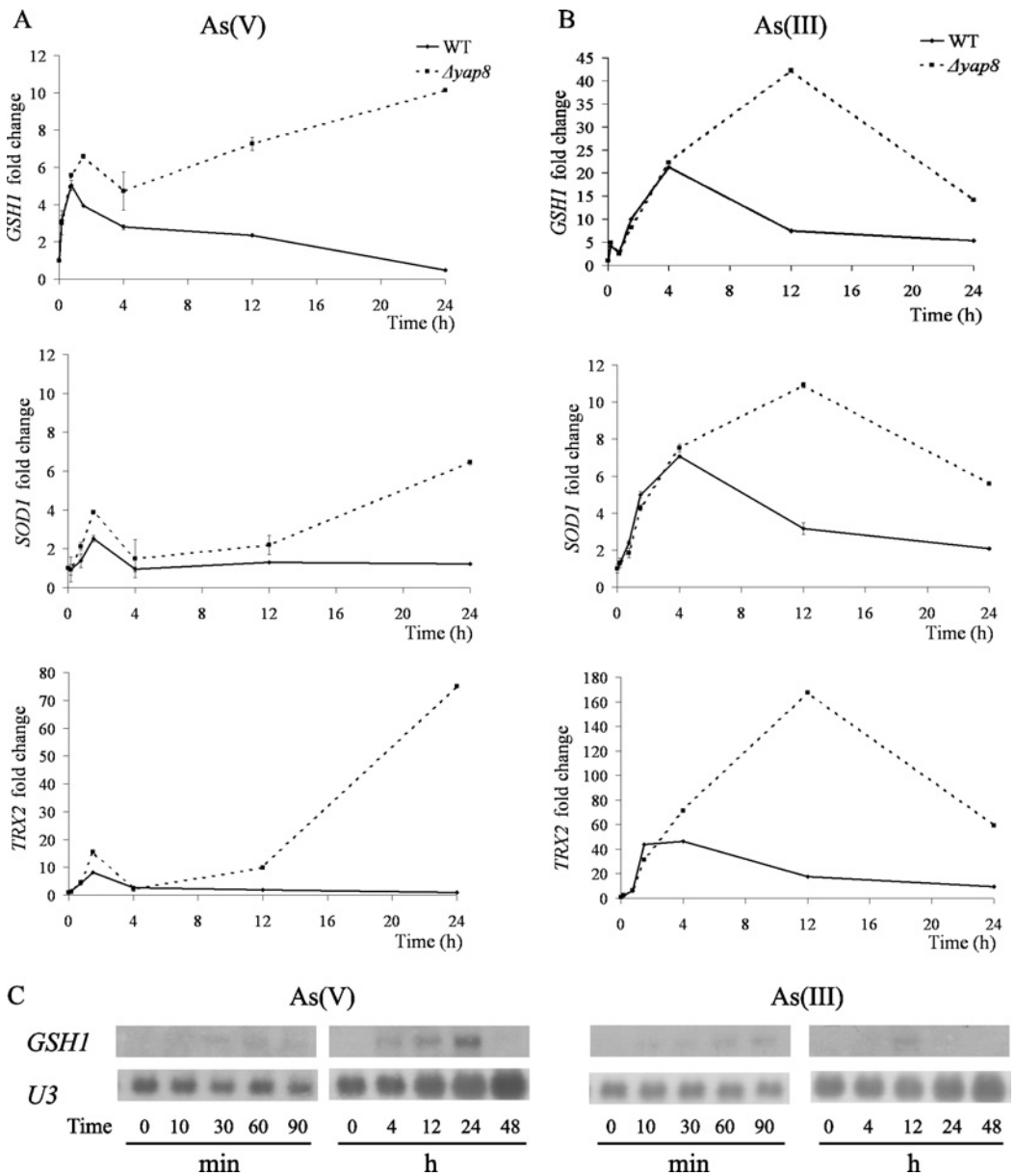


Figure 6 Effect of As(V) and As(III) on the transcriptional activation of *GSH1*, *SOD1* and *TRX2* in the wild-type (WT) and *Δyap8* mutant strains

Cells upshifted to arsenate (**A**)- or arsenite (**B**)-supplemented medium were harvested at the indicated time points. RNA extraction and real-time PCR were performed as described in the Experimental section. (**C**) *GSH1* expression in the *Δyap1* mutant was monitored by Northern blotting as described in the text. The mRNA levels for small nucleolar RNA U3 were used as an internal loading control. Representative experiments are shown.

absorption in *S. cerevisiae* wild-type cells is very low (around 5 %). The *yap8* mutant absorbs higher levels of As(III) (8 %) than those observed in the wild-type. Increased As(III) absorption in the *yap8* mutant may explain why the antioxidant genes are more activated in this strain than in the wild-type (see Figure 6). Surprisingly, *yap1* As(III) absorption is slightly decreased compared with the wild-type, suggesting that either the uptake is compromised in this strain, that extrusion is enhanced or that both events are occurring.

DISCUSSION

It has been accepted that, among the various modes of action for arsenic carcinogenesis in human cells, the oxidative stress relevance is the one that assumes that ROS can directly or indirectly damage DNA and proteins [13,41]. Indeed, the property of the organic arsenicals to inhibit the GSH and thioredoxin reductases, as well as to bind GSH [42,43], perturbs the redox equilibrium of the cytoplasm, leading to the accumulation of ROS. Here we report that Yap1, the major regulator of oxidative stress response in *S. cerevisiae*, is involved in the removal of ROS generated by arsenic compounds. Our data on lipid peroxidation, together with those on intracellular oxidation, indicate that *yap1* and *yap8* mutant cells are more oxidized than those of the wild-type strain upon treatment with arsenic compounds (Figure 2). The increased lipid peroxidation levels that we observed upon As(III) exposure is consistent with the notion that arsenite has the ability to release Fe(II) from its complexes with proteins, potentially stimulating the peroxidation of cellular lipids in yeast [44] (Scheme 1).

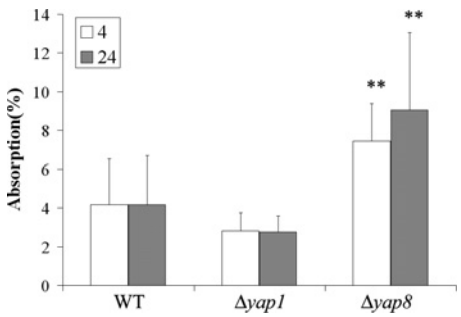
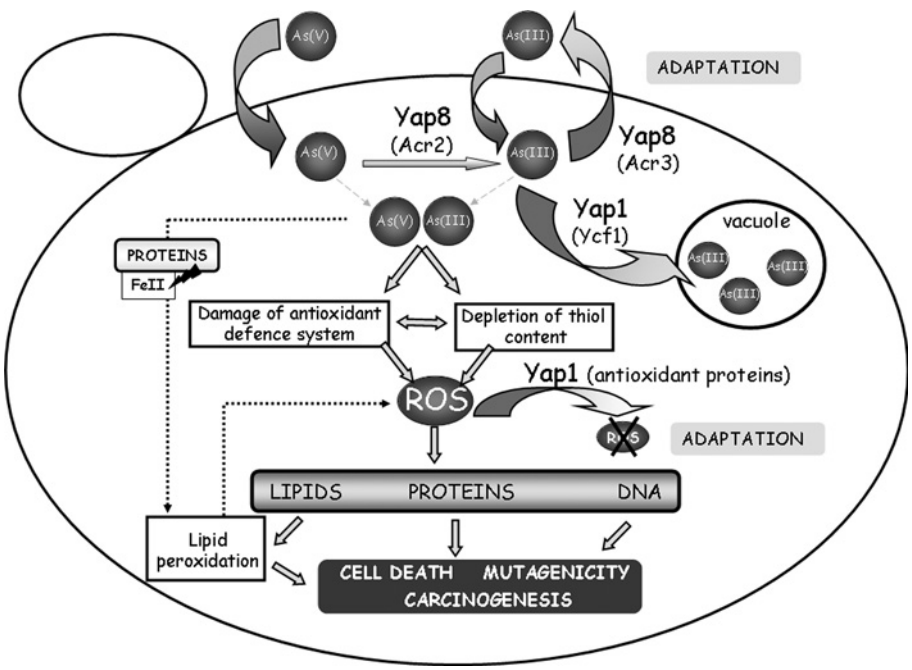


Figure 7 Effect of *YAP1* and *YAP8* deletion on As(III) absorption

Atomic-absorption analyses of As(III) were carried out in buffer solution containing 1 mg (dry weight) of *S. cerevisiae* wild-type (WT) or mutant cells and the amount of As(III) absorbed was estimated by determining the difference between the initial As(III) added (2 mM) and the residual As(III) present in the medium after 4 (white bars) and 24 (black bars) h. The percentage of As(III) absorbed was caculated by determining the ratio of As(III) taken up by cells and the initial As(III). Values are means ± S.D. for three independent experiments. $^{**}P < 0.01$.

This may, in turn, give rise to the highly reactive superoxide radical. Moreover, enhanced lipid peroxidation levels are also in agreement with the assumption that its induction by arsenite may be responsible for the toxic effect of this compound in eukaryotic cells [17]. Both *yap1* and *yap8* strains exhibit high levels of lipid peroxidation and intracellular oxidation; however, *yap1* is the only one displaying high contents of oxidized proteins (Figure 3). The antioxidant genes *GSH1*, *SOD1* and *TRX2* are induced in the wild-type strain their induction being even higher in the *yap8* mutant (see Figures 6A and 6B). The antioxidant machinery is therefore activated by Yap1, which prevents protein damage in these strains.

Redox-inactive toxic metals such as arsenic react with GSH, the reduced form of glutathione, which is the major antioxidant reserve of the cell [45]. We have in fact observed that, during the first 1 h of treatment with arsenate, the levels of GSH are decreased in all strains (Figure 4), with a consequent increase in the oxidized form (GSSG). As GSSG is formed, cells must, in order to maintain redox homoeostasis, induce the generation of GSH. This occurs either via its recycling through the glutathione reductase Glr1 or synthesis *de novo*, in which the activity of Gsh1 is essential. The transcriptomic analyses fully support the activation of both pathways in the wild-type strain (see Table 1 and Supplementary Tables S1 and S2). We show that genes grouped in functional categories related to sulfur metabolism, sulfur-amino-acid biosynthesis and metabolism, as well as *GLR1*, are induced when this strain is treated with 2 mM arsenate for 30 min (Table 1). Indeed, it had already been shown that arsenite-exposed cells channel a large part of assimilated sulfur into glutathione biosynthesis [16]. In the case of *yap1* the recycling pathway is impaired, since *GLR1* is a Yap1 target. Interestingly, even in cells harbouring *YAP1* deletion, the GSH levels upon 2 h of exposure to arsenic compounds increased, a finding consistent with the observation that *GSH1* mRNA levels were not completely abolished in the *yap1* mutant treated with both arsenate and arsenite (Figure 6C). Similarly, under glutathione depletion [46] and cadmium injury [47], *GSH1* has been shown to be regulated by both Yap1 and Met4. Furthermore, the fact that induction of *GSH1* is only partially decreased in *yap1* cells treated with the superoxide-anion generator menadione [48] might suggest that treatment of yeast cells with arsenic compounds also leads to the formation of superoxide radicals, which, in turn, could trigger the Yap1-independent basal transcription of *GSH1*. Yap1 contributes, therefore, to arsenic stress responses by relieving the deleterious effects of ROS in the cells (Scheme 1) through at least the transcriptional activation of antioxidant enzymes encoded by genes such as *TRX2, GSH1* and *SOD1* (Figure 6). Indeed, as suggested by others [17], the enhanced *SOD1* transcription is one of the most important factors responsible for triggering the



Scheme 1 Contribution of Yap1 and Yap8 to arsenic stress responses

Yap8 is the key regulator of this response by mediating the efficient removal of arsenite from the cytoplasm. Yap1 activity is also required at this level, its major contribution being the induction of the antioxidant defences in order to scavenge the ROS generated as a secondary effect of arsenic exposure.

adaptation process to mitigate the toxic effect of arsenite in eukaryotic cells. Genes involved in protein folding are also induced by arsenate stress, a phenomenon that is common to many forms of stress (see Table 1 and Supplementary Tables S1 and S2).

The absence of a significant increase in intracellular oxidation, lipid peroxidation and protein carbonylation, as well the changes in the GSSG/GSH ratio, in the wild-type cells reflects the ability of the wild-type strain to counteract the direct and/or indirect deleterious effects of the metal and to adapt to the stress condition (see Scheme 1). Phenotypic assays reveal, indeed, that the wild-type cells are able to grow in the presence of 2 mM arsenate and arsenite (Figure 1). Furthermore, *YAP8* and *YAP1* are both necessary to trigger the adaptation response, exerting a complementary role, though at different levels. Because *YAP8* is the key regulator of arsenic stress responses by controlling the expression of the arsenite efflux protein encoded by *ACR3*, its absence leads to the accumulation of As(III), enhancing the generation of ROS, as suggested from the atomic-absorption, lipid-peroxidation and intracellular-oxidation assays (see Figures 2, 3 and 7 and Scheme 1).

The *YAP1* gene is shown to be essential in preventing protein oxidation (Figure 3). On the other hand, Yap1 also contributes to the regulated expression of *ACR2* and *ACR3*, through recognizing the *cis*-element GATTAATAATCA positioned in the divergent promoter of these genes (results not shown). It also regulates the expression of the vacuolar pump encoded by *YCF1*, which composes a parallel arsenite detoxification pathway by catalysing the ATP-driven uptake of As(III)–GSH conjugates into the vacuole [1,49]. It is noteworthy that the *yap1* mutant absorbs lower levels of arsenite than does the wild-type strain. Assuming that arsenite vacuolar extrusion mediated by Ycf1 might be at least partially compromised in this mutant, a plausible explanation for the low values observed could be related to an impaired uptake of the metal. It was in fact shown that Hog1 kinase, which in turn modulates the uptake of As(III) dependent on the aquaglyceroporin Fps1, is activated by the metalloid [50]. It is possible that, in the *yap1* strain, this process is not fully operating.

In conclusion, we have identified an oxidative pathway dependent on Yap1 involved in the response of *S. cerevisiae* to arsenic compounds.

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Arsenic stress elicits cytosolic Ca^{2+}-bursts and Crz1 activation in *Saccharomyces cerevisiae*

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ABSTRACT

Although arsenic is notoriously poisonous to life its utilization as therapeutics brings many benefits to human health being therefore essential to uncover the molecular mechanisms underlying arsenic stress responses in eukaryotic cells. Aiming to determine the contribution of Ca^{2+}-signalling pathways to arsenic stress responses, we took advantage of the use of *Saccharamomyces cerevisiae* as a model organism. Here we show that Ca^{2+} enhances tolerance of the wild type and arsenic-sensitive *yap1* strains to arsenic stress in a Crz1-dependent manner, thus providing the first evidence that Ca^{2+}-signalling cascades are involved in arsenic stress responses. Moreover, our results indicate that arsenic shock elicits a cytosolic Ca^{2+}-burst in these strains, without the addition of Ca^{2+} exogenous sources, strongly supporting the notion that Ca^{+2} homeostasis is disrupted by arsenic stress. In response to arsenite-induced increase of Ca^{2+} in the cytosol Crz1 is dephosphorylated, translocated to the nucleus and stimulates CDRE-driven expression of the *lacZ* reporter gene in a Cnb1-dependent manner. The activation of Crz1 by arsenite culminates with the induction of the endogenous genes *PMR1, PMC1* and *GSC2*. Taken together, these data establish that activation of Ca^{2+}-signalling pathways and the downstream activation of Crz1 transcription factor contribute to arsenic tolerance in the eukaryotic model *Saccharomyces cerevisiae*.

INTRODUCTION

Arsenic (As) is a naturally occurring metalloid in the Earth's crust. Highly toxic arsenic ion species are released into the environment primarily through the leaching from geological formations into aquifers, although anthropogenic sources also account for environmental contamination. Although it is used as therapeutics, chronic exposure to arsenic compounds constitutes a global health problem and has been associated with many diseases, including cancer (Tseng 2007). It is therefore important to clarify the cellular and molecular mechanisms involved in the response of eukaryotic cells to arsenic stress. In this context, the remarkably high degree of conservation between *Saccharomyces cerevisiae* and higher eukaryotes makes yeast a valuable model organism to unravel the complex and fundamental mechanisms underlying arsenic stress responses.

Eukaryotic cells, from yeast to humans, respond promptly and precisely to adverse stimuli by a complete reprogramming of gene expression. This process is tightly orchestrated by specific transcription factors, which mediate the induction of genes conferring protective activity. Indeed, microarray transcriptional profiling of yeast cells exposed to arsenic compounds performed by us and others revealed that many cellular pathways, including those involved in oxidative stress defence, redox maintenance, glutathione biosynthesis and arsenic detoxification are enriched in arsenic-treated cells (Haugen *et al.,* 2004; Thorsen *et al.,* 2007; Menezes *et al.,* 2008). The activity of Yap1, a member of the YAP (Yeast AP1-like) family of transcription factors, was shown to be essential to regulate the induction of ROS homeostasis machinery in cells exposed to arsenic stress (Menezes *et al.,* 2004; Menezes *et al.,* 2008).

Although multiple arsenic adaptation pathways, involving transcription factors such as Yap8, Met4, Hog1 and Abf1 have been described (Rodrigues-Pousada *et al.,* 2010), the complex mechanism of arsenic stress responses is still far from being completely understood.

Calcium ion ($Ca^{2+)}$ is an essential element playing a central role as intracellular messenger in eukaryotic cells. The utilization of Ca^{2+} to regulate a wide range of cellular processes, in response to a variety of environmental insults, is a strategy exploited by virtually all eukaryotic organisms. In yeasts, it has been reported that exposure of cells to many stress conditions including iron overload and ethanol shock, disrupts Ca^{2+}-homeostasis (Batiza *et al.,* 1996; Kanzaki *et al.,* 1999; Matsumoto *et al.,* 2002; Peiter *et al.,* 2005; Araki *et al.,* 2009; Popa *et al.,* 2010; Li *et al.,* 2011). However, in some conditions such as H_2O_2, *t*BOOH and aluminium treatment, Ca^{2+} regulates cytotoxicity instead of adaptation (Popa *et al.,* 2010; Li *et al.,* 2011).

The stress-induced increase of cytosolic free Ca^{2+} is triggered either by the mobilization of extracellular sources, mainly via the high affinity Cch1/Mid1 channel (Matsumoto *et al.,* 2002; Peiter *et al.,* 2005; Popa *et al.,* 2010), or the release of vacuolar sources through the Yvc1 ionic channel (Denis and Cyert, 2002). Homeostasis is restored by the sequestration of Ca^{2+} into the vacuole, through the action of the Ca^{2+}-ATPase Pmc1 and the Ca^{2+}/H^{+} exchanger Vcx1 (Palmer *et al.,* 2001). Alternatively, Pmr1 and Cod1 can direct Ca^{2+} to the secretory pathway in the ER and Golgi. Under conditions of high cytosolic Ca^{2+} concentrations, the essential Ca^{2+}-receptor protein calmodulin (CaM) binds to Ca^{2+} undergoing a conformational change that allows the activation of target proteins such as the CaM-regulated kinases Cmk1/Cmk2 and calcineurin (CaN)

(Cyert, 2001). CaN is a highly conserved serine/threonine phosphatase composed by a catalytic and a regulatory subunit, encoded by *CNA1* and *CNB1*, respectively, being the latter essential for enzyme activity and induction by CaM (Cyert *et al.,* 1991; Cyert & Thorner 1992; Cyert 2003). CaN carries out multiple functions in yeast, standing out the regulation of the zinc-finger transcription factor Crz1. Like the mammalian ortholog NFAT (Yoshimoto *et al.,* 2002), upon dephosphorylation by CaN, Crz1 rapidly relocates to the nucleus (Stathopoulos-Gerontides *et al.,* 1999) and mediates transcriptional activation through the CDRE (CaN-dependent Response Element). *PMC1* and *PMR1*, encoding Ca^{2+} transporters, and *GSC2*, encoding a cell wall biosynthetic enzyme, are among the Crz1 target genes (Matheos *et al.,* 1997; Stathopoulos & Cyert 1997; Denis & Cyert 2002).

Interestingly, our recent microarray data suggests that arsenic stress affects the expression of genes involved in Ca^{2+}-signalling pathways and these findings prompted us to investigate the role of Ca^{2+} ion in the yeast stress response to arsenite. Here we report that arsenite disrupts Ca^{2+}-homeostasis and triggers the activation of Crz1, which in turns regulates the induction of genes encoding the Ca^{2+}-transporters Pmr1 and Pmc1 and encoding a protein involved in the biosynthesis of cell wall, Gsc2.

MATERIALS AND METHODS

Bacterial and yeast strains, plasmids and growth conditions

126 The *Escherichia coli* strain XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*
127 *relA1 lac [F´ proAB laclqZΔM15 Tn10 (Tetr)]* (Stratagene, Agilent Technologies,
128 USA) was used as the host for routine plasmid amplification purposes.
129 Outgrowth was performed in SOC medium at 37º C. Plasmids were selected on
130 LB plates supplemented with 100 μg ampicillin ml^{-1}.
131 A list of *S. cerevisiae* strains and oligonucleotide primers used in this work are
132 presented in tables 1 and S1 (see Supplementary material), respectively. The
133 microhomology PCR method (Guldener *et al.,* 1996) was used to generate the
134 double mutant strains as follows: (1) *crz1cnb1*, deletion of *CRZ1* coding region
135 in the *cnb1* mutant; (2) *yap1crz1*, deletion of *CRZ1* coding region in the *yap1*
136 mutant; and (3) YAA3-*yap1*, deletion of complete *YAP1* coding region in the
137 YAA3 strain. The double mutant *yap1cbn1* was generated through the
138 transformation of linearized pLF1 plasmid (encoding a *YAP1* version where the
139 *HIS3* gene was inserted in the internal *Bam*HI site of *YAP1*) in the *cnb1* strain.
140 Gene disruptions were confirmed by PCR analysis of genomic DNA using
141 upstream (A1) and downstream (A4) specific primers (Table S1, Supplementary
142 Material). The pEVP11-APOAEQUORIN (Batiza *et al.,* 1996) was used to
143 measure cytosolic free Ca^{2+} levels. Strains were grown in synthetic medium
144 [SM - 0.67% (w/v) ammonium sulfate/yeast nitrogen base without amino acids,
145 2% (w/v) glucose, supplemented with 20 μg L-histidine-HCl monohydrate ml^{-1},
146 60 μg leucine ml^{-1}, 20 μg uracil ml^{-1} and 20 μg L-methionine ml^{-1}]. Selective
147 synthetic media were prepared omitting the aminoacid corresponding to the
148 auxotrophic marker. For solid media, agar was added to a final concentration of
149 2% (w/v). All spectrophotometric measurements of yeast biomass were
150 performed using a polystyrene 45 mm path length cuvette and the BIO-RAD

SmartSpec^{TM} 3000 Spectrophotometer. Exponential growth phase cells were attained through the dilution of overnight cultures to an optical density at 600 nm (OD_{600}) of 0.1 ± 0.01 in fresh media followed by incubation to an OD_{600} of 0.5 ± 0.05. For each experiment cells were treated with $NaAsO_2$ (Sigma-Aldrich) and/or $CaCl_2$ (Merck) under the conditions indicated in the respective figures. For RNA extraction samples were washed with appropriated buffers and stored at -80ºC. Standard methods were used for genetic analysis, transformation and gene disruption procedures (Ausubel *et al.,* 1995).

Growth assays

Phenotypic growth assays were carried out by spotting 5 µl of sequentially diluted cultures (approx. $5x10^3$, $1x10^3$, $5x10^2$, $1x10^2$, 5x10 and 1x10 cells) in synthetic medium containing up to 1.5 mM $NaAsO_2$ and/or 50 mM $CaCl_2$. Growth was recorded after 2 days of incubation at 30º C. To monitor cell growth in liquid media early exponential phase cultures (OD_{600} 0.5 ± 0.05) were diluted to 0.1 ± 0.01 and were treated with 1.5 mM $NaAsO_2$. The cultures were incubated during 28 h at 30° C with orbital agitation (200 rpm) and the OD_{600} was monitored in intervals of 2 h.

Microarray analysis

Total RNA was isolated as described previously (Puig *et al.,* 2005) from exponentially growing BY4741 yeast cells that were either exposed or not to sodium arsenate 2 mM for 60 minutes; 50 µg of total RNA were sent for labelling and hybridization to Affymetrix Yeast Genome S98 arrays. For further

information about sample preparation visit the Duke Microarray Core Facility at http://www.genome.duke.edu/cores/microarray/. The data were analysed by using both Partek® Genomics Suite™ and dChip softwares. The microarray data have been deposited in the microarray database Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE33427.

In vivo monitoring of Ca^{2+}-pulse induced by arsenic stress

Monitoring of cytosolic Ca^{2+} was performed using the Apoaequorin reporter system (Batiza *et al.,* 1996). For this purpose, the yeast strains BY4742 and *yap1* were transformed with the plasmid pEVP11-Apoaequorin, kindly provided by Dr. Patrick H. Masson. For the luminescence assays, stationary pre-cultures were diluted to an OD_{600} of 0.1 ± 0.01 in fresh synthetic medium and were grown to an OD_{600} of 1 ± 0.1. To reconstitute functional aequorin, 50 µM native coelenterazine (Sigma; dissolved in methanol) were added to the cell suspension and the cells were incubated for 2 h at 30° C in the dark. Cells were harvested by centrifugation and were washed three times with phosphate buffered saline (PBS; 10 mM phosphate, pH 7.4, 138 mM NaCl, 2.7 mM KCl) to remove the excess of coelenterazine. The pellet was resuspended in synthetic medium and the cell suspension was transferred to a 96-wells microplate. Before the induction of arsenic stress, the base-line luminescence was determined by 1 min of recording at 10 s intervals using a Perkin Helmer VICTOR^3 luminometer. $NaAsO_2$, to a final concentration of 1.5 mM, was injected into the samples and light emission was recorded for further 9 min at 10 s intervals. The light emission was reported as relative luminescence units (RLU), expressed as counts per second (cps) OD_{600}^{-1}. Multiple tests were performed

for each condition and a representative experiment is shown.

Protein extraction and immunoblot assays

Strains encoding the *CRZ1-GFP* fusion were grown in synthetic media until exponential growth phase and were induced with 1.5 mM As(III). To specifically inhibit calcineurin function, the immunosuppressant cyclosporin A (Sigma) was used to a final concentration of 10 μg ml^{-1} 30 min prior to cell induction with arsenite. Samples were collected at the time points indicated in the respective figure. Proteins were extracted by the TCA lysis method and 150 μg of total proteins were immunoblotted as previously described (Menezes *et al.,* 2008). Crz1-GFP was detected using the anti-GFP (Serrano *et al.,* 2011) and anti-Rabbit (Santa Cruz) as a primary and secondary antibodies, respectively. Pgk1 was used as the internal loading-control. Protein detection was performed using the kit SuperSignal West Pico Chemoluminescent Substrate (Thermo Scientific).

Fluorescence microscopy

Yeast strains expressing the green fluorescent protein (GFP)-tagged *CRZ1* were grown to early exponential growth phase in synthetic medium and cells were induced with 2.5 mM $NaAsO_2$ for 15 min. DAPI (4´,6-diamidino-2-phenylindole) (Sigma-Aldrich) was added to a final concentration of 5 μg ml^{-1}, 5 min before the end of the incubation with arsenite. Cells were collected, were fixed with 3.7% (v/v) formaldehyde (Sigma-Aldrich) for 10 min at room temperature and were washed twice with PBS. After washing, cells were resuspended in 200 mM DABCO [(1,4-diazadicyclo[2.2.2]octane) - dissolved in

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

β–Galactosidase assays

β-galactosidase activity measurements in cells harbouring the *CDRE-lacZ* reporter construct were performed as previously described (Menezes *et al.,* 2004). For this purpose BY4742, *cnb1* and *crz1* exponential-phase cells (OD_{600} 0.5 ± 0.05), encoding the *CDRE-lacZ* reporter construction, were treated either with 1mM $NaAsO_2$ or 20 mM $CaCl_2$ at 30ºC for 90 min. Cells were harvested by centrifugation, were permeabilized with chloroform and were assayed for enzyme activity using the Biotek Epoch Microplate Spectrophotometer. Miller units were calculated as previously described (Miller 1972). Experiments were performed using biological triplicates and the mean standard deviation (SD) is shown.

Real-time PCR

RNA was extracted from early log-phase cultures that were either untreated or exposed during 30 min to 1.5 mM $NaAsO_2$. DNA was removed by on-column DNAse I digestion (RNase-Free DNase Set; Qiagen). Total RNA (1µg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche Diagnostics). qPCR reactions were performed in the LightCycler 480 Instrument (Roche), using LightCycler 480 Green I Master (Roche) and the

oligonucleotides listed in table S1. 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 480 Software 1. Actin was used as a reference gene. All assays were made in triplicate.

RESULTS AND DISCUSSION

Genome-wide transcriptional analysis reveals that arsenic stress affects the expression of Ca^{2+}-related genes

Aiming to investigate the response of *S. cerevisiae* cells to mid-term arsenic exposure, we compared the mRNA expression profile of wild type cells up shifted to 2 mM Na_2HAsO_4-enriched medium for 60 min. Besides the up regulation of genes included in functional categories previously shown to be targeted by arsenic compounds, such as sulphur/methionine metabolism and redox homoeostasis (Haugen *et al.,* 2004; Thorsen *et al.,* 2007; Menezes *et al.,* 2008), our transcriptomic analysis revealed a significant alteration of the expression profile of genes related to Ca^{2+}-signalling pathways (Table S2, Supplementary Material). In spite of several genome-wide experiments reported, this is the first time that such a response is detected, probably because none of previous works addressed the mid-term response (60 min) induced by arsenate. Among these genes are *CRZ1*, encoding the master transcriptional regulator of Ca^{2+}-mediated signalling; *RCN1* and *CMP2*, encoding proteins related to calcineurin function; *CMD1*, *CMK2* and *RCK2*, encoding calmodulin and proteins directly regulated by calmodulin binding, respectively; and the calcium transporters encoded by *MID1* and *CCH1* (Table

2). Based on these results we hypothesized that Ca^{2+}-signalling pathways could be involved in the response of yeast cells to arsenic stress.

Exogenous Ca^{2+} increases arsenic tolerance in a Crz1-dependent manner

To address the contribution of Ca^{2+}-signalling to arsenic stress responses we first examined the growth phenotypes of the wild type, the arsenic-sensitive mutant *yap1* (Menezes *et al.,* 2004) and the respective isogenic *crz1* and *cnb1* knockout strains in synthetic media supplemented with $CaCl_2$. All the strains revealed similar growth patterns both in the absence and in the presence of $CaCl_2$ concentrations up to 100 mM (Fig. 1a, upper panels). The *crz1* and *yap1crz1* strains did not exhibit any growth defect under Ca^{2+}-replete condition compared to the wild type strain, indicating that cells were not under Ca^{2+} stress. Indeed, all the strains showed normal growth in media containing up to 200 mM $CaCl_2$ (Fig. S1, Supplementary Material). As already described, the *yap1* mutant strain displayed high growth sensitivity to 1 mM As(III), whereas the wild type strain was shown to be resistant to 1 mM arsenite and moderately sensitive to the higher concentrations. Remarkably, arsenic-stressed wild type and *yap1* cells, simultaneously treated with 50 mM $CaCl_2$, were clearly more tolerant to arsenite stress (Fig. 1a, see middle and lower panels). Moreover, acquisition of tolerance mediated by Ca^{2+} was shown to be dose-dependent since media supplementation with 100 mM $CaCl_2$ further enhanced the growth of these strains in the presence of arsenite. Our results therefore show that the increase in the availability of exogenous Ca^{2+} partially relieve arsenic toxicity and suggest that Ca^{2+} may be implicated in the adaptive response to arsenic stress.

301 The zinc-finger transcription factor Crz1 is a central player of the Ca^{2+}-signalling
302 cascade. To determine whether Ca^{2+} contributes to arsenic tolerance through
303 the activation of Ca^{2+}-signalling pathways we monitored, simultaneously, the
304 growth of the respective *crz1* isogenic strains. First, we found that the *crz1*
305 mutant was moderately sensitive to 1 and 1.5 mM As(III) (see Fig. 1a - lower
306 and middle panels, and Fig. 1b and 1c) revealing therefore a link between the
307 Crz1 pathway and arsenic stress responses in yeast. Second, when *CRZ1* was
308 disrupted in the *yap1* strain, the Ca^{2+}-mediated phenotype recovery of the
309 double mutant was compromised in the presence of 1 mM arsenite plus 100
310 mM $CaCl_2$, when compared to the *yap1* single mutant, being completely
311 abrogated in medium supplemented with only 50 mM $CaCl_2$. Finally, the growth
312 recovery of *crz1* by Ca^{2+} was not as efficient as for the wild type strain in all the
313 stress conditions tested. Altogether, these results provide strong evidences
314 pointing out to the importance of Crz1 and Ca^{2+}-signalling pathways to full
315 adaptive *S. cerevisiae* arsenic stress responses.
316 Under moderate arsenic stress conditions (1 mM $NaAsO_2$), Ca^{2+} induced some
317 degree of tolerance even in the absence of Crz1 (Fig. 1a – middle panel)
318 implying that this ion may mediate arsenic tolerance also through the activation
319 of Crz1-independent targets of the Ca^{2+}-signalling cascade. The calmodulin-
320 kinase Cmk2, the related protein-kinase Rck2, the glutamate decarboxylase
321 Gad1 and the endoplasmic reticulum-residing proteins Hph1/Hph2 are potential
322 candidates, due to their protective functions under oxidative and cell wall stress
323 (Cyert 2001; Sanchez-Piris *et al.,* 2002; Heath *et al.,* 2004), which are known
324 cellular damages caused by arsenic stress (Menezes *et al.,* 2008; Thorsen *et*

325 *al.,* 2009). Indeed, we found that *CMD1*, *CMK2* and *RCK2* were up regulated by
326 arsenic in our transcriptomic analysis (Table 2).
327 Since the activation of Crz1 by Ca^{2+} is dependent on CaN function, we included
328 in the phenotypic analysis the *cnb1* mutant, which is defective in the regulatory
329 subunit of the CaN complex. As cited before, inactivation of Cnb1 renders the
330 phosphatase complex non-functional. Curiously, the *cnb1* mutant exhibited
331 increased tolerance to high-dose arsenite stress compared to the wild type
332 strain, which was further increased when $CaCl_2$ was added to the medium (Fig.
333 1a - lower panel). The double mutant *yap1cnb1* displayed similar growth
334 patterns under low-dose arsenite stress (Fig. S2, Supplementary Material).
335 Although *yap1cnb1* was very sensitive to 1 mM arsenite, its growth was rescued
336 through the supplementation of the medium with $CaCl_2$. Under high arsenite
337 dose (1.5 mM) *yap1cnb1* growth recovery occurred only when media was
338 supplemented with 100 mM $CaCl_2$ (Fig. 1a – middle and upper panels).
339 In order to understand the distinct behaviour of *crz1* and *cnb1* strains, we
340 performed epistasis analysis. For this purpose, we constructed the double
341 mutant *crz1cnb1* and compared the growth phenotypes of the three strains
342 exposed to arsenic stress in the presence and absence of $CaCl_2$. The results in
343 Fig. 1d clearly show that *cnb1* mutation is dominant over *crz1* mutation. This
344 response is not specific to arsenic stress conditions since *cnb1* is also dominant
345 over *crz1* in the presence of high $CaCl_2$ concentrations (see Fig. S1,
346 Supplementary Material). These results were not surprising since CaN is
347 upstream of Crz1 in the Ca^{2+}/CaM/CaN/-signalling pathway, regulating its
348 translocation into the nucleus under conditions triggering the mobilization of
349 Ca^{2+} in the cytosol. A plausible explanation for this phenotypic paradox may lie

on the fact that CaN regulates many substrates and therefore carries out multiple functions in yeast (Cyert 2001). Indeed, it was already reported that CaN mutants display defects that are not mimicked by the *crz1* mutant, which reinforces its participation in the regulation of additional yeast proteins (Cyert 2003). Our results suggest that CaN is possibly exerting dual effects: a) a protective function through the regulation of Crz1, and b) the mediation of arsenic toxicity either by activating proteins conferring toxicity or repressing those contributing to tolerance.

Arsenite elicits a transient Ca^{2+} release into the cytosol

The transient increase in free cytosolic calcium is a mechanism used by eukaryotic cells to activate the Ca^{2+}-signalling pathways and thus to regulate many cellular processes in response to specific environmental cues (Viladevall *et al.,* 2004; Tseng 2007; Araki *et al.,* 2009). Given that an excess of extracellular Ca^{2+} favours cell adaptation to arsenic stress and that Crz1 activity is required to arsenic tolerance we next evaluated whether exposure to arsenite was accompanied by an increase of free cytosolic Ca^{2+} in media containing standard $CaCl_2$ concentrations (approximately 0.8 mM) (Abelovska *et al.,* 2007). For this purpose we used the apoaequorin-based methodology, which relies on the induction of aequorin activity by Ca^{2+} in a dose-dependent fashion (Batiza *et al.,* 1996). To spontaneously reconstitute functional aequorin, wild type and *yap1* cells transformed with the pEVP11-Apoaequorin plasmid were incubated in the presence of the coelenterazine cofactor as described under Materials and Methods. The base-line luminescence of both cultures, recorded during 10 min at 30ºC, did not increase over the time (Fig. 2, broken lines). The wild type and

375 *yap1* cells promptly responded to an arsenite shock, displaying a sharp rise
376 followed by a rapid fall in the luminescence, thus reflecting the arsenite-derived
377 fluctuations in free cytosolic Ca^{2+} (Fig. 2, full lines). The Ca^{2+}-pulse amplitude
378 exhibited by the arsenic-sensitive mutant *yap1* was slightly, but reproducibly,
379 higher than that of the wild type strain (compare Fig. 2a and 2b).
380 These results set forth that arsenic compounds disturb Ca^{2+}-homeostasis and
381 further support the hypothesis that the activation of the Ca^{2+}-signalling cascade
382 may represent a mechanism employed by yeast cells to deal with arsenic
383 stress.
384 Under stress conditions, the increase of cytosolic free Ca^{2+} can be generated by
385 the mobilization of extracellular or vacuolar sources. The results on the
386 phenotypic analysis here reported indicate that the enhancement of
387 extracellular Ca^{2+} availability promotes tolerance, suggesting that the arsenic-
388 induced cytosolic Ca^{2+}-burst might result from the mobilization of external
389 sources. The fact that the *mid1* mutant, which is defective in the plasma-
390 membrane high affinity Ca^{2+}-channel, is sensitive to arsenic compounds and
391 that Ca^{2+} media supplementation did not efficiently improve the tolerance of this
392 mutant to 1.5 mM As(III) (Fig. 2c), as it happens with the wild type strain, further
393 supports this hypothesis. Curiously, the *cch1* mutant exhibited growth patterns
394 similar to the wild type strain (Fig. 2c), suggesting that its role seems to be
395 dispensable for the arsenic-mediated activation of Ca^{2+}-signalling. The
396 discrepancy between both mutants, whose genes encode proteins cooperating
397 to form a high-affinity Ca^{2+} influx system, was already described and is
398 attributed to the fact that Mid1 can also operate independently of Cch1 (Popa *et*
399 *al.,* 2010; Li *et al.,* 2011).

Crz1 is induced by arsenite stress

The stress-induced increase of cytosolic free Ca^{2+} leads to the formation of a Ca^{2+}/CaM complex that binds to and activates the serine-threonine protein phosphatase calcineurin. One of the main functions of CaN is to dephosphorylate Crz1, thus triggering its nuclear accumulation when Ca^{2+} cytosolic levels rise. To provide additional evidences corroborating the importance of Ca^{2+}-signalling and Crz1 activity to arsenic stress responses, we monitored Crz1-GFP protein phosphorylation status in the wild type and *yap1* strains and in conditions where CaN activity was specifically inhibited by cyclosporin A (CsA). As shown in Fig. 3a, Crz1 was mainly phosphorylated under the control condition in the wild type and *yap1* mutant strain. Treatment of cells with As(III) induces a partial shift of the protein to a faster migrating form in the SDS-PAGE gel. This form corresponds to the dephosphorylated GFP-Crz1, since it completely disappeared when cells were treated with the calcineneurin inhibitor CsA. To provide further support of CaN-dependent Crz1 activation by arsenic, *in vivo* fluorescence microscopy was used to monitor Crz1-GFP dynamics in wild type, *yap1* and *cnb1* cells subjected to arsenite stress. Crz1-GFP was found dispersed throughout the cytoplasm in the absence of arsenite (Fig. 3b). Exposure to 2.5 mM As(III) induces its rapid translocation into the nucleus of almost all wild type and *yap1* cells within the first 15 min of incubation. Arsenite-induced Crz1-GFP nuclear accumulation was completely abrogated in the *cnb1* strain, which is devoid of the regulatory subunit of CaN. Altogether, the results here reported are consistent with the notion that arsenite

mediates a cytosolic Ca^{2+} release leading to the induction of CaN, which in turn leads to Crz1 dephosphorylation and its consequent nuclear accumulation.

Arsenite stimulates CDRE-dependent gene expression

The Crz1 transcriptional activator regulates the expression of many genes whose products are involved in diverse cellular functions and the DNA motif designated as CDRE (CaN-dependent response element) mediates the activation of the large majority of these genes (Denis & Cyert 2002). To determine whether arsenite stimulates CDRE-driven gene expression we used a reporter construction where four *in tandem* CDRE *cis*-elements were positioned in the promoter controlling the expression of the *lacZ* gene (Araki *et al.,* 2009). The β-galactosidase activity values observed for the wild type strain treated with 20 mM $CaCl_2$ for 90 min revealed a ten-fold increase compared to the control condition (Fig. 4). The absence of both Crz1 and Cnb1 abrogated the induction of the reporter gene demonstrating the Ca^{2+}-responsiveness of the system and its dependence on intact Crz1 and Cnb1 function. Exposure of wild type cells to 1 mM arsenite led to an eight-fold increase of *β*-galactosidase activity compared to the untreated condition (Fig. 4), being a similar pattern of induction observed in the *yap1* strain (data not shown). Remarkably, some degree of *lacZ* activation was noticed in the *crz1* mutant whereas in cells devoid of *cnb1* no *β*-galactosidase activity was detected (Fig. 4). These observations suggest that in response to arsenite an additional activator protein, whose activity seems to be influenced by the absence of CaN activity, may partially contribute to the induction of *lacZ* expression. Taken together, these results

indicate that the CaN-dependent Crz1 activation by arsenite has the potential to induce the expression of endogenous CDRE-regulated genes.

PMR1, PMC1 and *GSC2* are induced by arsenite stress

To verify whether CaN/Crz1 activation by arsenite stimulates the activation of endogenous target genes under the Ca^{2+}-signalling cascade we monitored by qPCR the expression levels of the Crz1-regulated genes *PMR1* and *PMC1*, involved in ion homeostasis, and *GSC2,* encoding a protein implicated in cell wall synthesis (Denis & Cyert 2002). Fully supporting our hypothesis, the treatment of cells with arsenite induced expression of the three genes in the wild type as well as in the *yap1* strain (Fig. 5). Full activation of *PMR1, PMC1* and *GSC2* by arsenite was clearly dependent on Crz1 activity, although significant levels of *PMR1* expression were still observed in the *crz1* strain (Fig. 5a). These data are consistent with the β-galactosidase assay results, showing that CDRE-regulated expression is partially dependent on Crz1 (Fig. 4) and are similar to those described in a previous work (Araki *et al.,* 2009). The comparison of *PMR1* and *PMC1* levels in the wild type and *yap1* strains revealed that expression of these genes was enhanced in the latter (Fig. 5a/b), suggesting that either the maintenance of Ca^{2+}-homeostasis in *yap1* cells requires increased amounts of Pmr1 and Pmc1 or that Yap1 exerts a negative effect on the expression of these genes. The evidence showing that *yap1* cells exhibited increased Ca^{2+}-pulse amplitude (Fig. 2) support the first possibility. Collectively, these results reinforce the hypothesis that arsenic stress induces a Ca^{2+}-cytosolic pulse activating the Ca^{2+}-signalling pathways, which in turn culminates with the induction of at least *PMR1, PMC1* and *GSC2.*

CONCLUDING REMARKS

The deleterious effects and widespread distribution of arsenic metalloids in Earth's crust led organisms to develop complex adaptation mechanisms and protection systems that are highly conserved between mammals and yeasts. However, the mechanisms underlying arsenic tolerance/toxicity are still far from being deciphered. Yet, despite the toxic effects of arsenic exposure there has been a resurgence of interest in the utilization of arsenic trioxide in the treatment of acute promyelocytic leukaemia (APL) (Kanzaki *et al.,* 1999; Lallemand-Breitenbach *et al.,* 2012). The mechanisms by which ATO selectively induces apoptosis of cancer cells are not yet fully understood, however, it was shown that ATO triggers endoplasmic reticulum (ER) stress responses and disturbs calcium Ca^{2+}-homeostasis (Binet *et al.,* 2010). Thus, to improve the effectiveness of arsenic-derived drugs in cancer therapy is essential to recapitulate the cellular and molecular processes affected by the treatment.

In this work we took advantage of the use of the eukaryotic model yeast *Saccharomyces cerevisiae* to investigate how Ca^{2+}-signalling pathways contribute to arsenic stress responses in eukaryotic cells. We report for the first time that an excess of extracellular Ca^{2+} relieves arsenic toxicity in two different genetic backgrounds of *S. cerevisiae*. Furthermore, we show that tolerance acquisition mediated by Ca^{2+} is dependent on the function of Crz1, directly implicating the Ca^{2+}-signalling cascade in this process. Upon arsenic stress, the burst of Ca^{2+} in the cytosol triggers the CaN-dependent Crz1 dephosphorylation and nuclear accumulation, thereby leading to the induction of CDRE-driven expression and the *PMR1, PMC1* and *GSC2* endogenous genes.

Although required to Crz1 activation, Cnb1 seems also to mediate arsenic toxicity, either by activating proteins conferring toxicity and/or repressing those contributing to tolerance. Further work is in progress in order to clarify this issue.

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LEGENDS TO FIGURES

Figure 1. Exogenous Ca^{2+} supply improves tolerance of the wild type and *yap1* strains to arsenite stress in a Crz1-dependent fashion. a) Wild type BY4742, *yap1* and the respective *crz1* and *cnb1* knockout strains were grown to early exponential phase and were spotted onto synthetic medium supplemented with As(III) and/or $CaCl_2$. Growth was recorded after 48 h incubation at 30^{o}C. A representative experiment is shown. b and c) Exponentially growing wild type and *crz1* cells were diluted to OD_{600} 0.1±0.01, induced or not with 1.5 mM As(III) and growth was monitored during 28 h at 30^{o}C. The growth curves show average values from at least three independent experiments. d) *CRZ1/CNB1* epistasis analysis. Wild type BY4742, *crz1*, *cnb1 and crz1cnb1* knockout strains were grown to early exponential phase and the spot assays were performed as described above. WT – wild type, SM – synthetic medium.

Figure 2. Arsenite stress elicits a transient cytosolic Ca^{2+}-pulse. Wild type BY4742 (a) and *yap1* (b) cells expressing coelenterazine-reconstituted aequorin were injected or not with 1.5 mM As(III) directly in the luminometer plates and luminescence was recorded during 10 min. The arrows indicate the time of arsenite addition. Each determination was repeated at least 3 times with no significant variations. The broken lines correspond to the base-line luminescence. RLU – Relative Light Units, expressed as counts per second/OD, SM – synthetic medium. c) Mid1 is required to Ca^{2+}-mediated enhancement of arsenic tolerance. Wild type BY4741 and the isogenic *mid1* and *cch1* strains were grown to early exponential phase and were spotted onto synthetic medium

supplemented with As(III) and/or $CaCl_2$. Growth was recorded after 48 h incubation at 30^{o}C. A representative experiment is shown.

Figure 3. Arsenite stress triggers Crz1 activation. a) Wild type and *yap1* cells expressing *CRZ1-GFP* were grown to early exponential phase and 30 min prior induction with 1.5 mM As(III) were treated or not with cyclosporin A. Samples were collected at the indicated time points and proteins were analysed by immunoblotting using an anti-GFP antibody. Pgk1 protein levels were used as a loading control. b) Wild type, *yap1* and *cnb1* cells expressing *CRZ1-GFP* were grown to early exponential phase, induced for 15 min with 2.5 mM As(III) and analysed under a fluorescence microscope. A representative experiment is shown. SM – synthetic medium, CsA – cyclosporin A.

Figure 4. Exposure to arsenite induces CDRE-driven expression. Wild type, *crz1* and *cnb1* cells expressing the *CDRE-lacZ* reporter construct were up-shifted to 1 mM As(III) or 20 mM $CaCl_2$ for 90 min at 30ºC. Cells were collected, permeabilized and *β*-galactosidase activity was monitored. Measurements were performed in triplicate and the mean standard deviation is shown. WT – wild type, SM – synthetic medium.

Figure 5. *PMR1, PMC1* and *GSC2* are induced by arsenite stress. Early exponential phase wild type, *yap1* and *crz1* cells were up-shifted to 1.5 mM As(III) and harvested after 30 min incubation at 30ºC. The relative expression of *PMR1* (a), *PMC1* (b) and *GSC2* (c) was monitored by qPCR, using the relative quantification method with efficiency correction and *ACT1* as a reference gene.

570 Measurements were performed in triplicate and the mean standard deviation is
571 shown. SM – synthetic medium.
572

573 Table 1: *S. cerevisiae* strains used in this work

Strain	Genotype	Source
BY4742	*MAT*α *his3 leu2 lys2 ura3*	EUROSCARF
crz1	*MAT*α *his3 leu2 lys2 ura3 YNL027W::kanMX4*	EUROSCARF
cnb1	*MAT*α *his3 leu2 lys2 ura3 YKL190W::kanMX4*	EUROSCARF
crz1cnb1	*MAT*α *his3 leu2 lys2 ura3 YKL190W::kanMX4 YNL027W::HIS3MX4*	This study
yap1	*MAT*α *his3 leu2 lys2 ura3 YML007W:: kanMX4*	EUROSCARF
yap1crz1	*MAT*α *his3 leu2 lys2 ura3 YML007W::kanMX4 YNL027W::HIS3MX4*	This study
yap1cnb1	*MAT*α *his3 leu2 lys2 ura3 YKL190W::kanMX4 YML007W::HIS3MX4*	This study
BY4741	*MAT*a *his3 leu2 met15 ura3*	EUROSCARF
mid1	*MAT*a *his3 leu2 met15 ura3 YNL291C::kanMX4*	EUROSCARF
cch1	*MAT*a *his3 leu2 met15 ura3 YGR217W::kanMX4*	EUROSCARF
YAA3	*MATa his3::CRZ1-GFP-HIS3 leu2 ura3 met15*	*(Araki et al., 2009)*
YAA3-*yap1*	*MATa his3::CRZ1-GFP-HIS3 leu2 ura3 met15 YML007W::kanMX4*	This study
YAA4	*MATa his3::CRZ1-GFP-HIS3 leu2 ura3 met15 YKL190W:: kanMX4*	*(Araki et al., 2009)*
YAA5	*MAT*α *his3 leu2 lys2 ura3 aur1::AUR1-C-4xCDRE-lacZ*	*(Araki et al., 2009)*
YAA6	*MAT*α *his3 leu2 lys2 ura3 YNL027W::HIS3MX4 aur1::AUR1-C-4xCDRE-lacZ*	*(Araki et al., 2009)*
YAA7	*MAT*α *his3 leu2 lys2 ura3 YKL190W:: kanMX4 aur1::AUR1-C-4xCDRE-lacZ*	*(Araki et al., 2009)*

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576 Table 2: Ca^{2+}-related genes whose expression is affected by arsenic

Category	Gene	Function	Fold change
Ca^{2+}-Signalling	***CRZ1***	Transcription factor that activates transcription of genes involved in stress response	2.09
Calcineurin complex	***RCN1***	Protein involved in calcineurin regulation during calcium signalling	2.78
	CMP2	Calcineurin A; one isoform (the other is CNA1) of the catalytic subunit of calcineurin	1.9
Ca^{2+}-binding	***CMD1***	Calmodulin; Ca^{2+} binding protein that regulates Ca^{2+} independent and dependent processes	1.37
Calmodulin binding	***CMK2***	Calmodulin-dependent protein kinase	
	RCK2	Protein kinase involved in the response to oxidative and osmotic stress	
Ca^{2+}-transport	***MID1***	N-glycosylated integral membrane protein of the ER membrane and plasma membrane	-1,64
	CCH1	Voltage-gated high-affinity calcium channel	-1.77

577 The average fold induction of triplicate samples is represented. A complete list of all Ca^{2+}-
578 related genes whose expression is altered by arsenic compounds is shown in Table S2.

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582 **REFERENCES**

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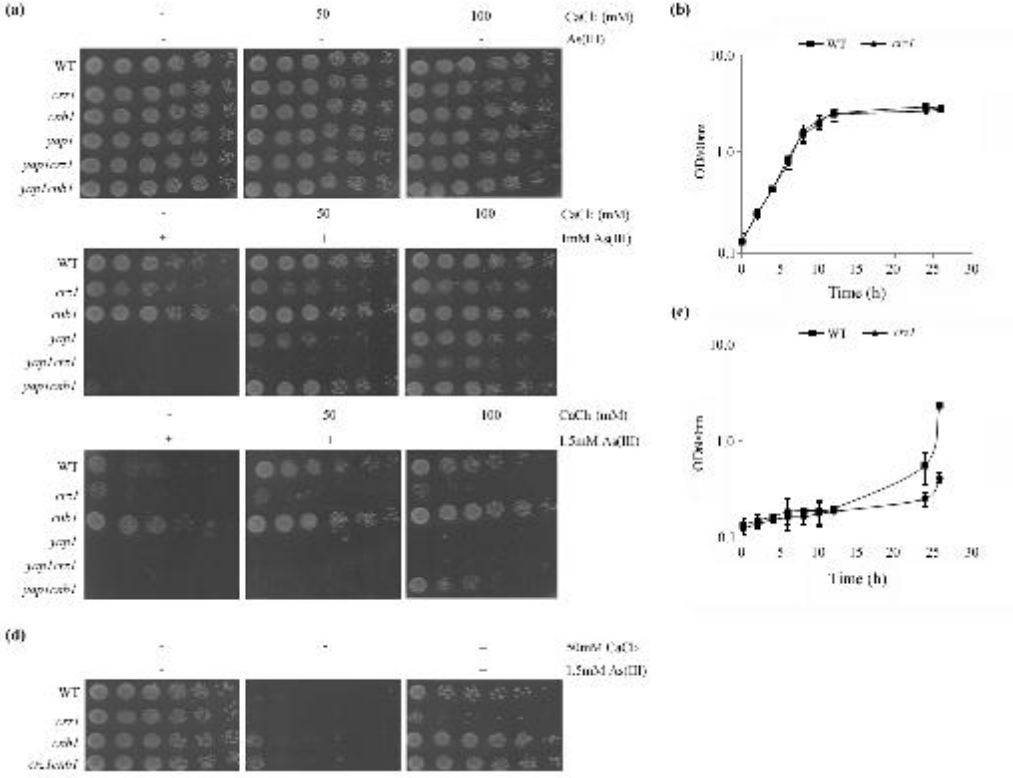
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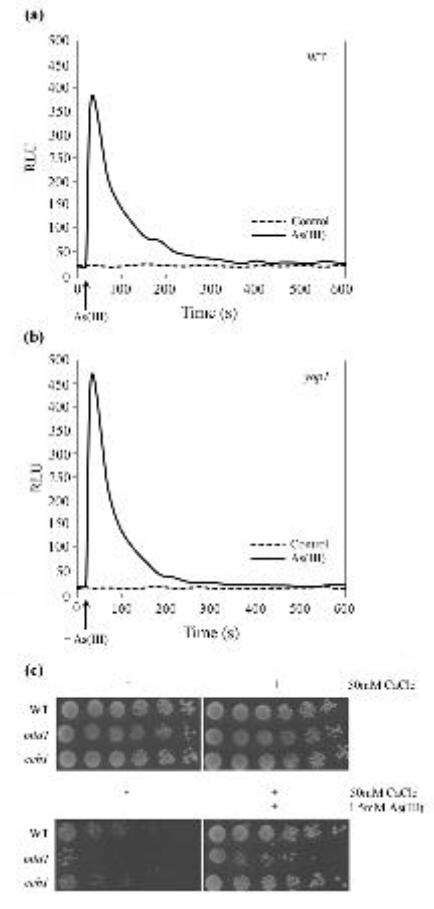
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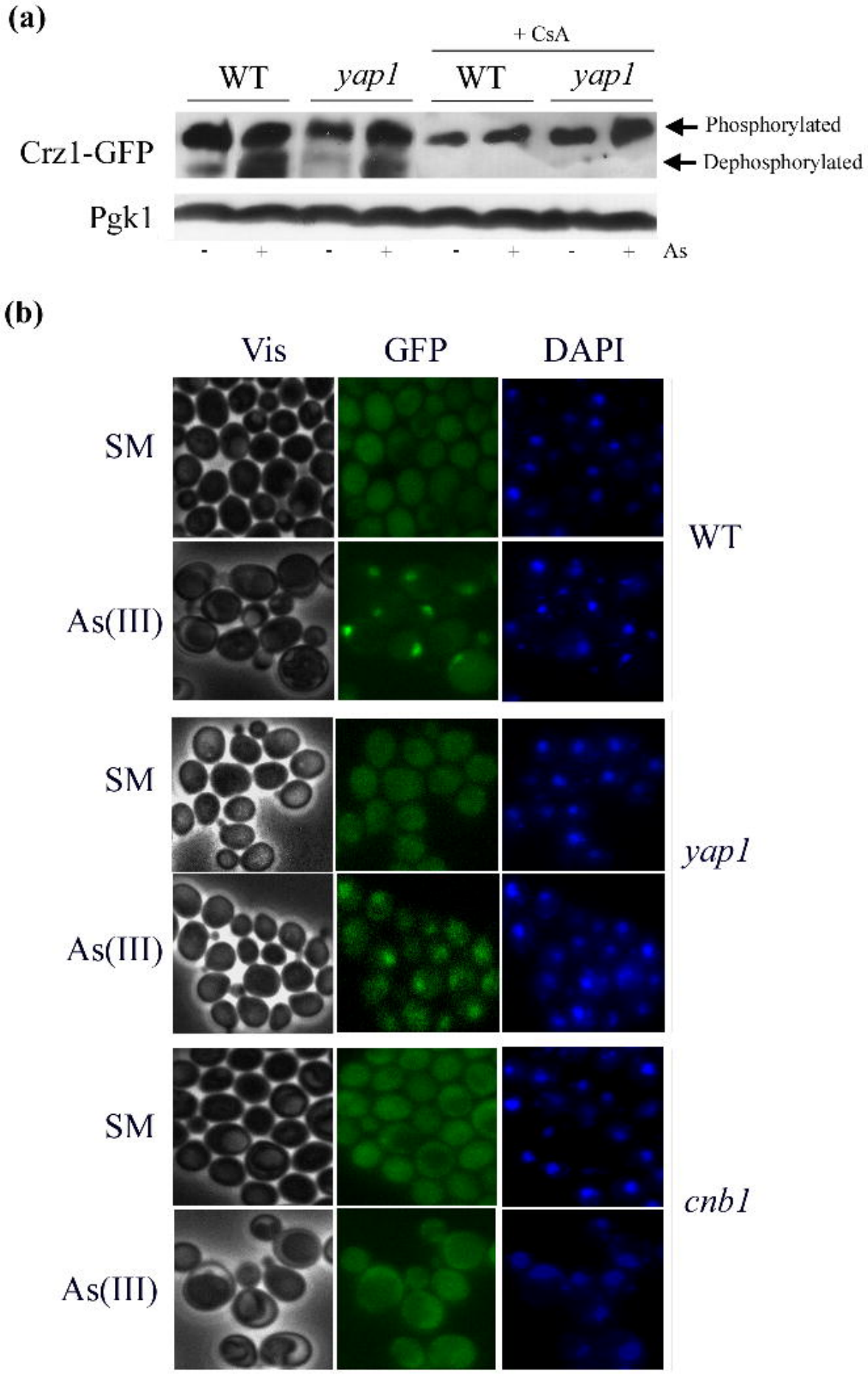
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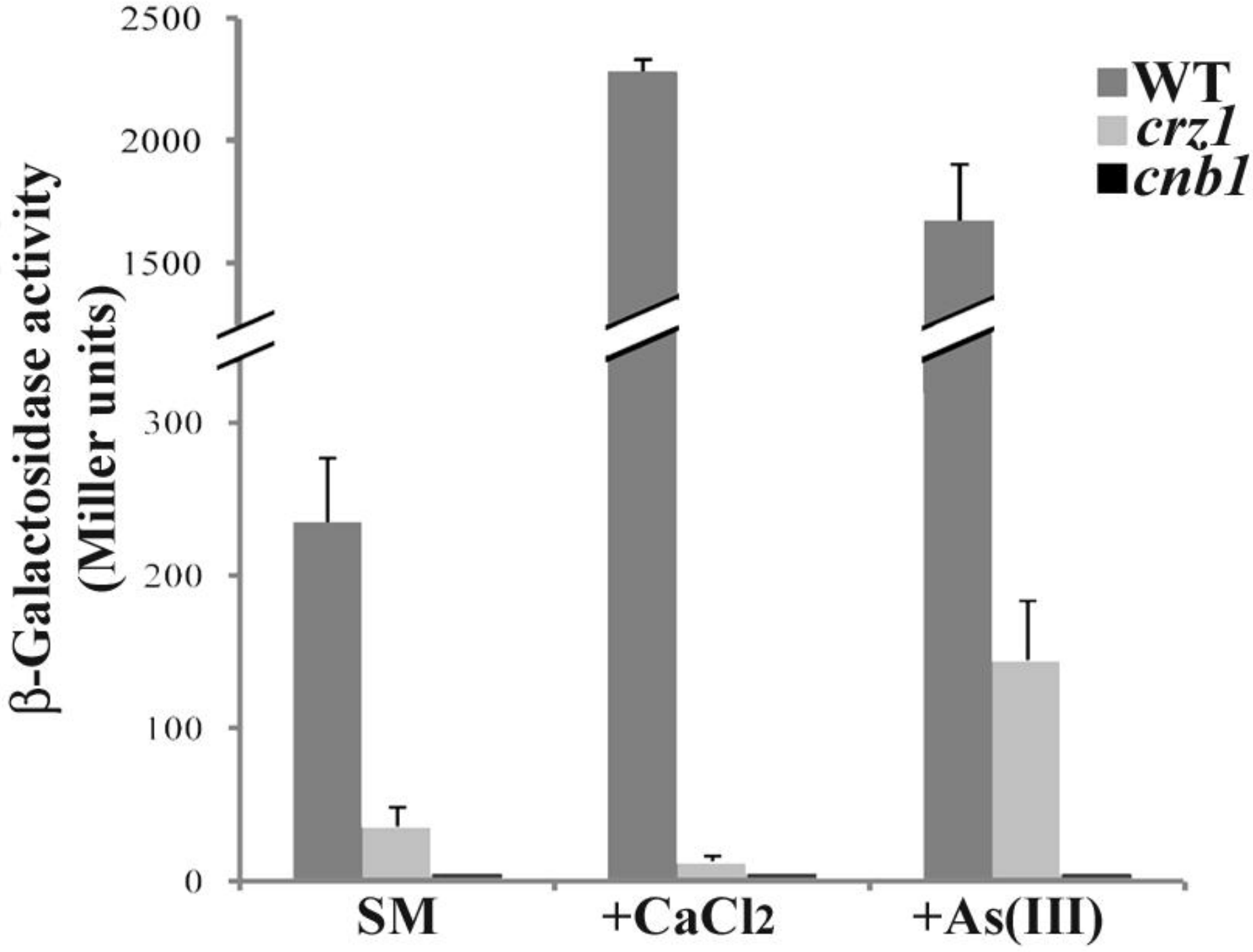
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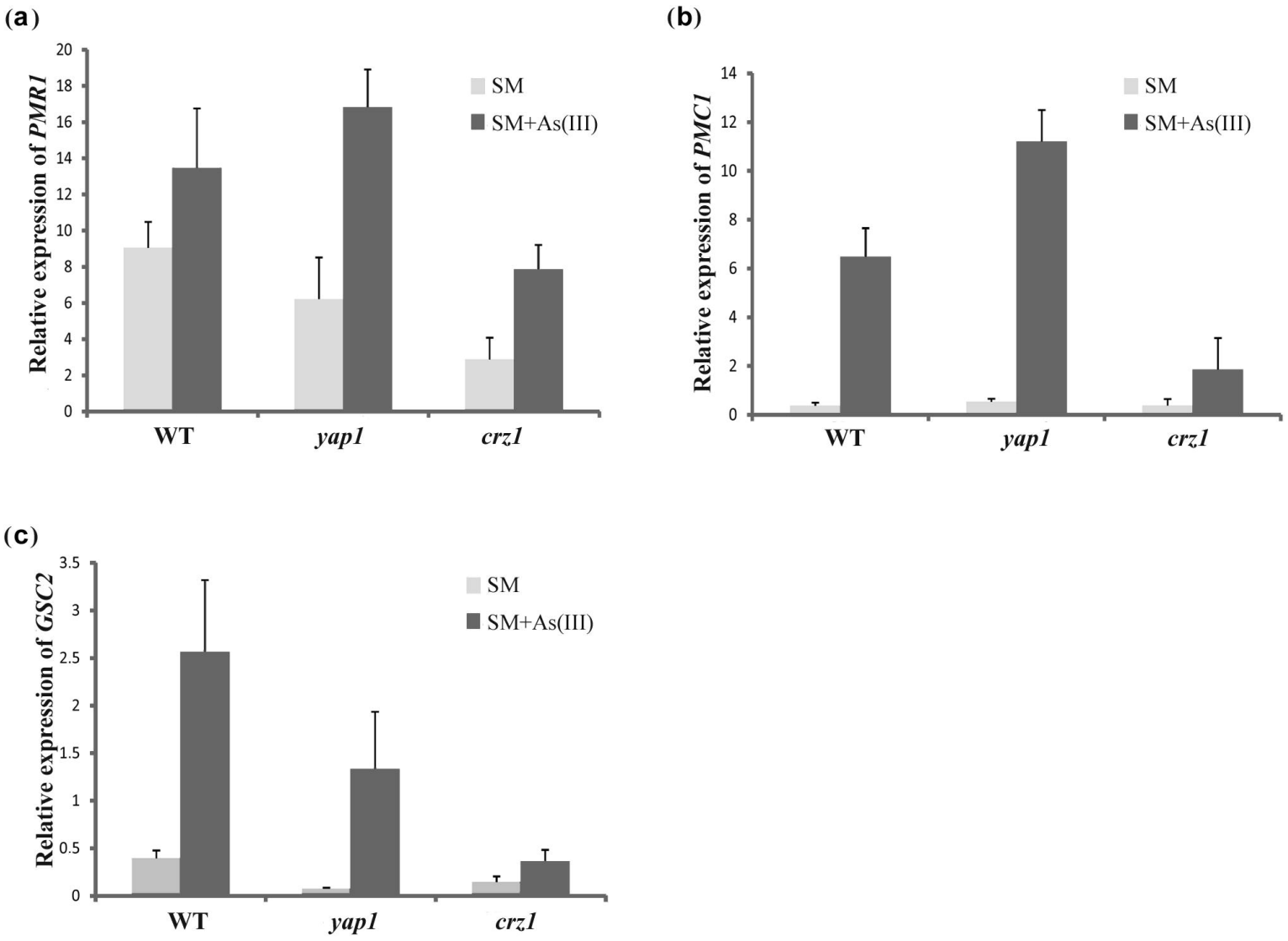
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Relative expression of PMC1
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(c)
Relative expression of GSC2
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Review Article

Iron and Neurodegeneration: From Cellular Homeostasis to Disease

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Accumulation of iron (Fe) is often detected in the brains of people suffering from neurodegenerative diseases. High Fe concentrations have been consistently observed in Parkinson's, Alzheimer's, and Huntington's diseases; however, it is not clear whether this Fe contributes to the progression of these diseases. Other conditions, such as Friedreich's ataxia or neuroferritinopathy are associated with genetic factors that cause Fe misregulation. Consequently, excessive intracellular Fe increases oxidative stress, which leads to neuronal dysfunction and death. The characterization of the mechanisms involved in the misregulation of Fe in the brain is crucial to understand the pathology of the neurodegenerative disorders and develop new therapeutic strategies. *Saccharomyces cerevisiae*, as the best understood eukaryotic organism, has already begun to play a role in the neurological disorders; thus it could perhaps become a valuable tool also to study the metalloneurobiology.

1. Iron Neurotoxicity

Iron (Fe) is the most important element for almost all types of cells, including brain cells. It is an essential cofactor for many proteins involved in the normal function of neuronal tissues, such as the non-heme Fe enzyme tyrosine hydroxylase required for the synthesis of myelin and the neurotransmitters dopamine, norepinephrine, and serotonin [1]. In a normal brain, Fe appears widely distributed by region and cell-type and it accumulates progressively during aging and neurodegenerative processes [2]. Fe is an originator of reactive oxygen species (ROS). Ferric iron (Fe^{3+}) can be reduced to ferrous iron (Fe^{2+}) by the superoxide radical ($O_2^{\bullet -}$) ($Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$). Fe^{2+} can also react with H_2O_2 generating the highly reactive hydroxyl free radical ($^{\bullet}OH$) ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$, Fenton reaction) [3]. The combination of these reactions results in the so-called Haber-Weiss reaction ($O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$), which together with dopamine oxidation can trigger neurotoxicity [4]. Therefore, the control of Fe homeostasis is essential to keep a healthy brain.

1.1. Iron Homeostasis. In mammals, the regulatory mechanism for Fe homeostasis is mediated by the iron-regulatory proteins IRP1 and IRP2, which posttranscriptionally modulate the expression of specific mRNAs in response to intracellular Fe [5, 6], mainly transferrin (Tf) and ferritin. Tf is an Fe-binding blood plasma glycoprotein that controls the level of free Fe, and ferritin is an Fe storage protein composed of H and L subunits that assemble to form a hollow sphere in which ferric iron (Fe^{3+}) precipitates are sequestered [7]. The ferritin subunits have different functions. The H chains are involved in the rapid oxidation of Fe^{2+} to Fe^{3+}, and the L chains function in the nucleation of Fe^{3+} within the protein shell. While the L-rich ferritins are associated with iron storage, the H-chain ferritins are associated with responses to stress [8].

In Fe-depleted cells, IRPs bind to the IREs cis-elements in the 5′UTR (untranslated region) of ferritin and in the 3′UTR of TfR1. By binding to the IRE in the 5′UTR, of ferritin, IRPs prevent translation, whereas by binding to IRE in the 3′UTR of TfR1, the IRPs protect the transcript from degradation [9]. In Fe-replete cells, IRPs do not bind to IREs, and

ferritin and other transcripts are freely translated, whereas TfR1 undergoes cleavage and subsequently degradation [6, 10, 11].

Although Fe metabolism in mammals is mainly regulated at the level of absorption, changes in gene expression in response to Fe overload have been observed in a variety of eukaryotes from yeast to mammals [12, 13].

Ferroportin (Fpn), the basolateral membrane Fe exporter, is the only Fe exporter to date identified in mammals [14–16]. Fpn mediates the release of the Fe in conjunction with ceruloplasmin (Cp), which must oxidize the Fe^{2+} transported by Fpn to Fe^{3+} before release into the extracellular medium [17]. Fpn expression has also been detected on the blood-brain barrier (BBB) endothelial cells, neurons, oligodendrocytes, astrocytes, the choroid plexus and ependymal cells. Since Cp is essential for stabilization of Fpn, under conditions of Cp deficiency or malfunction Fpn is not expressed, which results in a decreased Fe efflux potentiating cellular Fe overload [18]. These observations indicate that Cp plays a major role in maintaining Fe homeostasis in the brain and in protecting it from Fe-mediated free radical injury.

The Fe uptake pathway starts in the intestines, where Fe^{3+} is reduced to Fe^{2+} that then is transported to the blood by Fpn. In the blood, Cp oxidizes Fe^{2+} to Fe^{3+} and promotes its binding to the serum iron carrier, Tf [19]. In order to enter the brain, Fe needs to cross two distinct barriers, the BBB and BCSF (blood-cerebrospinal fluid) [20]. The most common pathway for Fe transference across the BBB is through the TfRs expressed in the endothelial cells. The circulating Fe bound to Tf is captured by TfR, entering the brain by endocytosis and then is translocated across the endosomal membrane, probably through the divalent metal transporter 1 (DMT1) [21]. In addition to the Tf-TfR pathway, it has been suggested that the lactoferrin receptor-lactoferrin (LfR-Lf) pathway might also play a role in Fe transport across the BBB. Fe^{2+} in the cytoplasm can also be transported inside the mitochondria by mitoferrin or participate in electron exchange reactions [22, 23]. Figure 1 summarizes the brain Fe uptake pathways.

Fe-related neurodegenerative disorders can result from both iron accumulation in specific brain regions or defects in its metabolism and/or homeostasis.

As the brain ages, Fe accumulates in regions that exhibit pathologic characteristics of Alzheimer's disease (AD) [24], Parkinson's disease (PD) [25], or Huntington's disease (HD) [26, 27]. In younger individuals, the largest amounts of Fe are in the oligodendrocytes whereas in older individuals over 60 years old most of the Fe is found in the microglia and astrocytes of the cortex, cerebellum, hippocampus, basal ganglia, and amygdala [27]. In these regions Fe is either bound to neuromelanin, a dark brown pigment that accumulates essentially iron, or to ferritin [28]. Interestingly, neurons express mostly H-ferritin, microglia express mostly L-ferritin, and oligodendrocytes express similar amounts of both subunits [29, 30]. Additionally neurons excrete the nonrequired Fe through the carrier Fpn (Figure 1).

It has been widely accepted that abnormal high concentrations of Fe contribute to neurodegenerative processes; however, a major question has not yet been answered. Is the excessive Fe accumulation in the brain an initial event that causes neurodegeneration or a consequence of the disease process?

Fe accumulation has been shown to lead to neuronal death [31]. Available Fe interacts with molecular oxygen and generates reactive oxygen species (ROS) through Fenton and Haber-Weiss reactions [32, 33], which leads to oxidative stress. Mitochondrial dysfunction has also been raised as a common cause for a number of neurodegenerative diseases. Since mitochondria has an important role in the Fe-S clusters formation [34], malfunction can result in a low Fe-S cluster synthesis and consequent activation of DMT1 and decrease of Fpn1, Fe accumulation, and oxidative stress [4]. Oxidative injury induces lipid peroxidation, nucleic acid modification, protein misfolding and aggregation, and cell dysfunction and death [35].

1.2. Alzheimer's Disease (AD). AD is the most common cause of age-related neurodegeneration and is characterized by the progressive loss of memory, task performance, speech, and recognition of people and objects. AD is characterized by the accumulation of aggregates of insoluble amyloid-β protein (Aβ), and neurofibrillary tangles (NFTs) consisting of precipitates/aggregates of hyperphosphorylated tau protein [36].

In AD, Fe accumulation has been observed in and around the amyloid senile plaques (SP) and neurofibrillary tangles (NFTs) [37]. The excessive Fe can lead to alterations in the interaction between IRPs and their IREs [38] and disruption in the sequestration and storage of Fe by ferritin [39]. Further studies have suggested that high Fe toxicity may be due to the propensity of Fe^{2+} to generate ROS [40], and *postmortem* analysis of AD patients' brains has revealed activation of two enzymatic indicators of cellular oxidative stress: heme oxygenase-1 (HO-1) [41] and NADPH oxidase [42]. In addition, other evidence suggests that in AD the Fe metabolism is disrupted. Tf is not found in the oligodendrocytes but rather trapped within senile plaques and ferritin is expressed within reactive microglial cells that are present both in and around the senile plaques [43]. A decade ago Rogers et al. [44] provided another link between iron metabolism and AD pathogenesis by describing the presence of an IRE in the 5′UTR of the amyloid precursor protein (APP) transcript. APP 5′UTR is responsive to intracellular iron levels, which regulate translation of APP holo-protein mRNA by a mechanism similar to the translation of ferritin-L and -H mRNAs via IREs in their 5′UTRs. Recently, Duce et al. [45] have described that APP is a ferroxidase that couples with Fpn to export Fe. In AD APP ferroxidase activity appears inhibited, thereby causing neuronal Fe accumulation.

1.3. Parkinson's Disease (PD). PD is a progressive disorder that manifests as tremor at rest, bradykinesia, gait abnormalities, rigidity, postural dysfunction, and loss of balance [46]. It is the most prevalent neurodegenerative disorder after AD affecting about 2% of people over 65 years old. PD is characterized by the loss of the *substantia nigra* dopaminergic neurons [47] and the deposition of intracellular inclusion bodies known as Lewy bodies. The principal protein

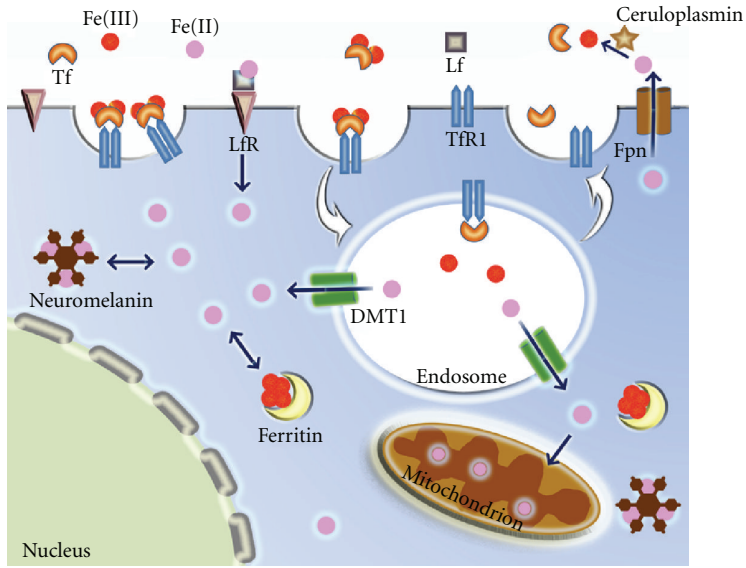

Figure 1: The brain iron homeostasis. Iron (Fe) binds to transferrin (Tf), enters the brain through the transferrin receptors (TfR) by endocytosis, and translocates across the endosomal membrane through the divalent metal transporter 1 (DMT1). Lactoferrin receptors (LfR) provide another pathway to transport Fe from Fe containing lactoferrin across the cell membranes. Inside the cell Fe binds to H-ferritin and accumulates around the neuromelanin. Ferroportin (Fpn) transports Fe^{2+} outside the neuron that is oxidized to Fe^{3+} by ceruloplasmin promoting its binding to Tf.

component of these bodies is α-synuclein (α-syn) that is ubiquitously expressed in the brain [48].

Several studies have confirmed an increase of Fe in the *substantia nigra* of most severe cases of PD [49–51]; however, there are still some conflicting reports about the stage during disease progression at which nigral Fe changes occur.

Nevertheless, there is a general agreement that total nigral Fe levels increase in PD, possibly leading to nigrostriatal dopamine neuron degeneration as a result of its ability to produce ROS and cause lipid peroxidation [52, 53].

The elevated Fe content, besides contributing to the increase of oxidative stress, also enhances α-syn aggregates [54]. It has been shown that α-syn harbors an IRE in its 5′-UTR. Thus high intracellular Fe might also regulate α-syn aggregation through the IRE/IRP system, therefore, causing the death of dopaminergic neurons [55]. As Fe deposits are commonly found in the Lewy bodies, Fe might play a role on the pathogenicity of α-syn in PD.

1.4. Huntington's Disease (HD). HD is a neurodegenerative disorder characterized by progressive motor, cognitive, and psychiatric deterioration. Typically, onset of symptoms is in middle-age (30 and 50 years old), but the disorder can manifest at any time between infancy and elderliness. HD is caused by a dominant glutamine expansion (CAG repeat coding) within the N-terminal of the huntingtin protein that initiates events leading to neuronal loss primarily within the striatum and cerebral cortex. Full-length huntingtin is large (~350 kD), but it is the smaller N-terminal fragments that are the main mediators of disease progression [56]. These fragments have aberrant interactions with themselves and other biomolecules that lead to the molecular hallmarks of HD including aggregates, transcriptional repression [57], oxidative damage, and metabolic dysfunction [58].

For individuals with HD, increased Fe levels have primarily been observed in the basal ganglia, namely, in the *striata* and the *globus pallidus* [59]. In addition, ferritin-Fe levels are increased in *striata* of early clinical HD patients as measured by magnetic resonance imaging (MRI) [60]. Fe levels increase early stage in HD and continue to increase with age, which suggests that Fe may play a role in the progression of the disease. However, the mechanisms involved in this process are not yet understood. Although both AD and PD are characterized by Fe accumulation, the Fe regulation patterns seem to be different from HD [59]. For instance, Parkinson's disease is characterized by Fe accumulation in the *substantia nigra*, which has not been observed in HD. It is possible that in HD Fe accumulation occurs because after neuronal loss, cells with higher Fe content replace the dead cells. Thus Fe accumulation in HD is most probably a secondary effect of the disease [60].

1.5. Other Neurological Disorders. The accumulation of Fe has also been implicated in a series of other neurological diseases, such as Neuroferritinopathy, Hallervorden-Spatz syndrome, and Aceruloplasminemia that are characterized by mutations in genes that encode for ferritin light polypeptide (*FTL*), pantothenate kinase (*PANK2*), and ceruloplasmin, respectively.

Neuroferritinopathy is dominantly inherited and is a late-onset disease of the basal ganglia that presents extrapyramidal features similar to HD and PD. It is caused by a single adenine insertion at position 460-461 that is predicted to change the C-terminal residues of the gene encoding L-chain ferritin [61]. Brain histochemistry of patients with neuroferritinopathy showed abnormal aggregates of ferritin and Fe and low serum ferritin concentrations. The C-terminus of the aberrant L chain might interfere with the formation of

the hollow sphere allowing inappropriate release of Fe from the loaded ferritin [62].

Another evidence for the involvement of Fe in neurodegeneration is provided by the study of Hallervorden-Spatz syndrome (HSS), also referred to as neurodegeneration with brain-iron accumulation 1 (NBIA) or pantothenate-kinase-2-associated neurodegeneration (*PANK2*) [63]. HSS is an autosomal recessive disorder characterized by dystonia, pigmentary retinopathy in children and neuropsychiatric defects in adults. The HSS patient's MRI has a characteristic pattern in the *globus pallidus*, known as "the eye of the tiger" because of its appearance [64]. Zhou et al. [63] identified the genetic basis for this neurodegenerative disease in which Fe accumulation is most dramatic by detecting the underlying mutations in the gene that encodes for pantothenate-kinase. This enzyme is essential for the coenzyme A biosynthesis [65], which in turn catalyzes the phosphorylation of pantothenate (vitamin B_5), *N*-pantothenoyl-cysteine, and pantetheine [63]. The product of this reaction, 4′-phosphopantothenate, is then converted to 4′-phosphopanthetheine in a reaction that consumes cysteine. HSS results from 4′-phosphopantothenate deficit, which is caused by genetic defects in *PANK2*. Given that cysteine is consumed in the conversion of 4′-phosphopantothenate, an absence of functional *PANK2* might explain the observed accumulation of cysteine in the degenerating brain areas of HSS patients. Consequently the cysteine Fe-chelating properties might account for the observed regional Fe accumulation, and cysteine-bound Fe may promote Fe-dependent oxidative damage in these regions [66]. Even though *PANK2* is not directly involved in Fe metabolism, its absence may contribute for Fe accumulation in the brain, leading to neuronal death via oxidative stress.

Finally, aceruloplasminemia, an autosomal recessive disorder caused by mutations in the ceruloplasmin gene, also results in Fe overload in the brain characterized mainly by retinal neurodegeneration [17]. Cp is a multicopper ferroxidase responsible for the Fe homeostasis by promoting Fe incorporation into Tf, therefore, playing a key role in releasing Fe from the cells [67]. Consequently, mutations in the ceruloplasmin gene may cause Fe metabolism misregulation in the brain. Due to the low release of cellular Fe and the high nontransferrin-bound Fe uptake, the intracellular Fe concentration becomes abnormally high. This induces oxidative stress and formation of ROS triggering a cascade of pathological events that lead to neuronal death.

1.6. Fe-Chelation Therapies. Oxidative stress, protein aggregation, and active redox Fe have been considered promising pharmacological targets for the treatment of AD and PD. BBB permeable Fe chelators can be used as potential therapeutic agents in the treatment of neurodegenerative diseases. A promising Fe chelator is desferrioxamine (Desferal), which has been shown to prevent up to 60% of dopaminergic neurons from death in a rat model of PD [68]. The main disadvantage of desferrioxamine is that it cannot cross the BBB, due to its size and hydrophobicity [65]. Clioquinol, a small lipophilic Fe chelator that can cross the BBB, has also proved to have beneficial effects in patients with AD [69]. However, clioquinol is not iron selective and has very toxic effects. Aroylhydrazones are the new nontoxic lipophilic Fe chelators that can form a neutral complex with Fe and diffuse out of the membrane [70]. Other important class of compounds proposed for therapy is the polyphenols that have antioxidant properties and can bind Fe [71]. A major limitation is their capacity to be absorbed at the gastrointestinal tract and subsequently be transported through the BBB.

The development of an effective non-toxic therapeutic agent for such complex brain disorders still represents a challenging task.

2. The Yeast Model

In the last decade, the budding yeast *Saccharomyces cerevisiae* has been used as a model system to gain insights about the mechanisms of neurodegenerative disorders such as Parkinson's, Huntington's, and Alzheimer's [72]. Yeast cells are generally used to study key proteins involved in the etiology and/or pathology of these diseases. When a yeast homologue exists, the corresponding gene can be easily disrupted or overexpressed to determine the loss or gain of function phenotypes, respectively. When a yeast homologue is not present, the human gene can be expressed in yeast and any relevant phenotype that results from this expression can be analyzed. The latter has been called humanized yeast models [73]. Despite their simplicity, yeast cells possess most of the same basic cellular machinery as neurons in the brain, including pathways required for protein homeostasis and energy metabolism. Also their easy genetic manipulation makes these cells an ideal tool for molecular biology.

Many of the genes and biological systems that function in yeast Fe homeostasis are conserved throughout eukaryotes to humans [74]. *S. cerevisiae* expresses three genetically distinct transport systems for Fe, two reductive systems and one nonreductive system. The reductive Fe uptake system consists in a low-affinity pathway defined by Fet4, that can also transport other metals and in a high-affinity pathway that is mediated by a protein complex composed of a multicopper ferroxidase Fet3, the mammalian Cp homologue, and a permease Ftr1. The Fet3-Ftr1 complex is specific for Fe and is regulated both transcriptionally and posttranscriptionally by this metal [75–77]. The nonreductive Fe uptake system is mediated by the ARN family (Arn1-4) of membrane permeases that transport siderophore-Fe^{3+} complexes [78, 79]. Additionally Harris et al. [80] showed for the first time that Fet3 can functionally replace ceruloplasmin in restoring Fe homeostasis.

Moreover, cells are able to spare Fe through the regulation of Tis11 homologues and Cth1/2-mediated degradation of mRNAs coding for Fe-binding proteins, thereby facilitating the utilization of limited cellular Fe levels [81, 82].

Since *S. cerevisiae* lacks the Fe storage protein, ferritin, during Fe overload this is sequestered into the vacuole by the Ccc1 transporter, which is under the control of the Yap5 transcription factor [13]. On the other hand, Fet5/Fth1 complex mobilizes Fe out of the vacuole for use during Fe limitation [83].

Given the similarities between yeast and mammals and the availability of humanized *S. cerevisiae* strains, yeast could potentially become an effective model to dissect the molecular pathway associated with the misregulation of Fe homeostasis in the neurodegenerative diseases.

One good example of the use of yeast to study Fe accumulation in a neurodegenerative disease was first reported for Friedreich's ataxia (FRDA). FRDA is an autosomal recessive mitochondrial disorder that causes progressive damage to the nervous system, resulting in gait disturbance, speech problems, heart disease, and diabetes. It is caused by GAA triplet expansion in the first intron of the frataxin gene (*FA*) [84].

A gene with high sequence similarity to *FA* was initially identified in yeast, the yeast frataxin homologue, *YFH1* [85] and later it was shown that the two proteins were both located in the mitochondria. Moreover, human *FA* could complement for the absence of the yeast *yfh1* [86]. However, *FA* function was only discovered when Lodi and coworkers [87] showed that the *YFH1* knockout strain led to an excessive Fe accumulation in the mitochondria resulting in the generation of ROS and consequently oxidative damage. The yeast frataxin homologue provided the evidence that FRDA is indeed a mitochondrial disorder. The yeast model allowed a better understanding of the FDRA pathophysiology and provided a tool for assaying therapeutic targets.

3. Concluding Remarks

In this paper, we have summarized the role of Fe, a redox-active transition metal, in neurodegenerative disorders. Despite a considerable investigation already performed, it is still not clear whether excessive Fe accumulation in the brain is an initial event that causes neuronal death or is a consequence of the disease process. The growing evidence suggests that the abnormal high Fe levels in the brain may have genetic causes, as found in patients with aceruloplasminemia, or sporadic causes that can disrupt the normal mechanisms of Fe transport into the brain. In addition, elevated Fe levels generate ROS and increase the levels of oxidative stress, which is considered one of the pathways leading to neuronal death. A new study from Lei et al. [88] shows that loss of Tau impairs the Fpn Fe export by preventing the proper trafficking of APP ferroxidase to the neuronal surface, leading to Fe accumulation, which results in degeneration of dopaminergic neurons in PD. These findings suggest the involvement of a new mechanism associated with Tau's role in PD. However, the precise role of Fe transport proteins in the brain is not completely understood, which impairs the success of therapeutic strategies to prevent the damaging effects of the Fe in the brain.

Finally, we believe that the use of the yeast neurodegenerative disease models might provide valuable insights into key aspects of the Fe pathology in the brain and pave the way towards the discovery of promising therapeutic targets.

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Review Article

Oxidative Stress in Alzheimer's and Parkinson's Diseases: Insights from the Yeast *Saccharomyces cerevisiae*

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Alzheimer's (AD) and Parkinson's (PD) diseases are the two most common causes of dementia in aged population. Both are protein-misfolding diseases characterized by the presence of protein deposits in the brain. Despite growing evidence suggesting that oxidative stress is critical to neuronal death, its precise role in disease etiology and progression has not yet been fully understood. Budding yeast *Saccharomyces cerevisiae* shares conserved biological processes with all eukaryotic cells, including neurons. This fact together with the possibility of simple and quick genetic manipulation highlights this organism as a valuable tool to unravel complex and fundamental mechanisms underlying neurodegeneration. In this paper, we summarize the latest knowledge on the role of oxidative stress in neurodegenerative disorders, with emphasis on AD and PD. Additionally, we provide an overview of the work undertaken to study AD and PD in yeast, focusing the use of this model to understand the effect of oxidative stress in both diseases.

1. Introduction

Misfolded proteins are typically insoluble and tend to form long linear or fibrillar aggregates known as amyloid deposits. Amyloid-like protein fibrils are a well-known pathological hallmark of age-related neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD). Alzheimer's and Parkinson's diseases are the most common forms of dementia, currently affecting 30 and 4 million people worldwide, respectively. In AD, the beta-amyloid (Aβ) peptide accumulates mainly extracellularly, whereas in PD, the α-Synuclein (α-Syn) protein accumulates, within neurons, inside the Lewy bodies (LB) and Lewy neurites (LN). Although there is a plethora of factors interfering in those pathological depositions, it is clear that oxidative stress may play a crucial role in neuronal death in neurodegenerative disorders [1–3].

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen and are produced in all aerobic cells. Oxidative stress occurs when the generation of ROS in a system exceeds that system's ability to neutralize and to eliminate them. All organisms have developed adaptive responses to oxidative stress that involve defensive enzyme and molecular chaperones—the expression of both being orchestrated by stress-responsive transcription factors—as well as antioxidant molecules [4]. Excessive production of ROS, and the consequent disruption of cellular redox balance, drives the oxidation of biological macromolecules, such as DNA, proteins, carbohydrates, and lipids, potentially leading to failure of biological functions [4].

Many ROS possess unpaired electrons and are therefore free radicals. The generation of free radicals is closely linked with the involvement of trace metals, particularly copper and irons [5, 6]. To cope with this potential hazard, the concentration of cytosolic free metals is accurately controlled through regulation of their uptake, storage, and mobilization, in order to maintain redox-active metals in normal physiological limits [7, 8]. Inside cells, "free pools" of copper and iron are avoided through their effective sequester by metal-binding proteins [5, 9]. The chelatable redox-active

iron constitutes the so-called labile iron pool (LIP), which serves as a transient source of iron [5, 9, 10]. Nevertheless, whenever cells are subjected to stress conditions, an excess of superoxide anion radical acts as an oxidant of Fe-S clusters of several enzymes, releasing "free iron." The released iron can in turn participate in Fenton type reactions, producing the highly reactive hydroxyl radical [11].

During the oxidative burst triggered during inflammatory processes, cells of the immune system produce both superoxide anion and nitric oxide (NO) free radicals. Nitric oxide is produced by the NO synthase family of enzymes. NO may directly react with its biological targets, as it is known to regulate the catalytic activity of various enzymes primarily by interacting with Fe-S clusters, oxidized copper centres, heme, and tyrosyl radicals [12]. NO also reacts with superoxide ion (O_2^-) or oxygen to form the nitrogen radical peroxynitrite ($ONOO^-$). Reactive nitrogen species (RNS) are highly reactive towards biological macromolecules and are thought to be responsible for NO-mediated cell death.

The aim of this paper is to provide an overview on the role of oxidative stress in neurodegenerative disorders, with emphasis on AD and PD. Despite the absence of a nervous system in yeast, several studies have shown that this eukaryotic unicellular organism is a suitable model system to understand the molecular mechanisms underlying neurodegenerative diseases. The knowledge from those studies is summarized herein. Finally, we discuss how yeast models have been or may be used to extend our understanding on the role of oxidative stress in AD and PD.

2. Oxidative Stress and Neurodegenerative Diseases

The human brain is responsible for approximately 20% of our body oxygen consumption and thus subjected to a high metabolically derived level of ROS [13, 14]. An increasing body of evidence suggests that oxidative stress is involved in the etiology and pathogenesis of neurological disorders. The lipid bilayer of the brain is rich in polyunsaturated fatty acids and oxygen and is therefore highly susceptible to lipid peroxidation, a complex process involving the interaction of polyunsaturated fatty acids with free radicals that results in production of reactive electrophilic aldehydes. Lipid peroxidation occurs in several neurodegenerative diseases [15]. Evidence of oxidative stress in these diseases is further supported by increased DNA (and often RNA) base oxidation products and oxidative protein damages in specific regions of the brain [4]. The destruction of cellular components can induce a diversity of cellular responses through generation of secondary reactive species and ultimately lead to cell death via apoptosis and necrosis [16–18]. Mitochondrion is the center of ROS production. About 90% of mammalian oxygen consumption is mitochondrial, making mitochondria particularly important in neurons due to their high demands for energy. This fact, together with the observation that mitochondrial perturbation occurs in multiple neurodegenerative disorders [19], suggests that neurodegenerative diseases are mitochondrial diseases.

3. Alzheimer's Disease and Oxidative Stress

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disease caused by severe neurodegeneration in the hippocampus and neocortical regions of the brain of affected individuals [20].

AD pathological hallmarks include extracellular amyloid plaques and intracellular aggregates (neurofibrillary tangles). The major component of the amyloid plaques is the amyloid peptide Aβ, which results from the proteolysis of the amyloid precursor protein (APP). APP is a ubiquitously expressed transmembrane protein exerting a critical role in neuron growth and survival [21, 22]. APP proteolysis to form the Aβ peptide involves the sequential action of aspartic protease BACE1 (β-secretase) and of γ-secretase, a multiprotein complex [23]. The length of Aβ peptide may range from 39 to 43 aminoacid residues, due to different γ-secretase cleavage sites. Aβ appears to be unfolded, under physiological conditions [24]. In amyloid plaques, the most frequent species are Aβ40 and Aβ42, the latter being the most prone to aggregation [23]. Neurofibrillary tangles are composed of hyperphosphorylated tau protein, a microtubule-binding protein thought to be involved in microtubules stabilization and in regulation of axonal transport in the brain [25].

The causes of Alzheimer's disease are not well understood, except for a small percentage of cases that are linked to familial genetic mutations [26]. Several hypotheses have been put forward with the aim to explain the cause of the sporadic form of the disease. One widely discussed of those hypotheses assumes that amyloid deposits of Aβ peptides are the causative agents of AD [27]. The "amyloid" theory is further supported by the link between mutations in the APP gene and some inherited forms of the disease [26].

Aβ toxicity is dependent on Aβ's conformational state, peptide length, and concentration [28, 29]. Moreover, it has been described that Aβ toxicity is also related to Aβ's ability to form hydrogen peroxide and free radicals [30, 31]. These findings are supported by the significant lipid peroxidation, protein oxidation, and DNA oxidation observed in AD brains [29, 32, 33]. In addition, two factors reinforce the role of oxidative stress in AD pathogenesis: pro-oxidants increase Aβ production, whereas several antioxidants, namely vitamin E, melatonin, and several free radical scavengers, can protect neurons from Aβ-induced toxicity [34].

Interestingly, the Aβ peptide is not toxic in the absence of redox metal ions, and many recent studies implicate biometals in the development or progression of Alzheimer's disease [6, 35–37]. Accordingly, sophisticated techniques have shown an overaccumulation of copper, iron, and zinc within the amyloid plaques compared with the surrounding tissues [38]. Aβ has high affinity for redox-active metals being able to reduce them and consequently lead to the formation of hydrogen peroxide and oxidized amyloid [6]. Butterfield and Bush proposed that a single methionine residue (Met35) of Aβ42 is critical for the oxidative and

neurotoxic properties of this peptide [39, 40]. Substitution of Met35 renders the Aβ peptide nonoxidative and nonneurotoxic [40]. The sulphur atom of Met35 is highly susceptible to oxidation, forming the sulfide radical $MetS^{\bullet+}$ and reducing copper(II) to its high-active low-valency form [5, 40]. The $MetS^{\bullet+}$ radical is able to undergo very fast reactions with superoxide ion, leading to the formation of methionine sulfoxide (MetO). In AD senile plaques, a significant fraction of Aβ has Met35 in the form of MetO [41].

Another well-studied source of oxidative stress in AD is mitochondria damage and its consequent functional abnormality that favors the production of ROS. Indeed, it was shown that neurons in AD exhibit a significantly higher percentage of damaged mitochondria compared to an aged-matched group [42]. Furthermore, mitochondrial dysfunction has been widely implicated in the etiology of AD, since early impairments of mitochondrial function and oxidative stress may precede Aβ overproduction and deposition [43]. Also inflammation can induce oxidative damage in AD, especially via microglia, leading to increased ROS and RNS formation and the resulting damage to lipid, proteins, and nucleic acids [44–46].

4. Parkinson's Disease and Oxidative Stress

Parkinson's disease is an age-related neurodegenerative disorder affecting the central nervous system. It is characterized by the progressive degeneration of nigrostriatal dopaminergic neurons within the *substantia nigra pars compacta*, which is the pathological process responsible for the motor symptoms attributed to PD [47]. The pathological hallmark of the disease is the presence of proteinaceous cytoplasmic inclusions designated as Lewy bodies and Lewy neurites. These are predominantly composed of the presynaptic protein α-Synuclein (α-Syn) [48] together with proteasomal and lysosomal subunits as well as molecular chaperones [49].

The ubiquitous α-Syn brain protein is implicated in both hereditary and sporadic PD. Its encoding gene, *SNCA,* was the first genetic determinant associated with the disease and, for this reason, much of the work on PD converges on α-Syn [50]. α-Syn was shown to interact with lipids and membranes, accelerating amyloid fibril formation [51], and it has been proposed to regulate the dynamics of synaptic vesicles at the synapse [52]. Indeed, α-Syn exhibits a remarkable conformational plasticity being its structure largely dependent on the surrounding environment. The monomeric α-Syn is a typical natively unfolded protein under physiological conditions [53, 54]. However, under specific conditions, such as the increase of its intracellular levels, α-Syn can adopt different conformations, including several α-helical and β-sheet species folded to different degrees in both monomeric and oligomeric states [55].

Although PD is a recognized multifactorial disease, a large body of evidence has implicated oxidative stress in the pathogenesis of PD. The conclusive connection between PD and oxidative stress is supported by the increased oxidative damage of sugars, lipids, nucleic acids, and proteins observed in *postmortem* dopaminergic neurons within the *substantia nigra pars compacta* of PD brains [6, 56, 57].

Auluck et al. proposed that the impairment of α-Syn function leads to its local accumulation, favoring the formation of toxic oligomeric species that interfere with ER-to-Golgi trafficking, mitochondria turnover—through the abrogation of mitophagy—and generate oxidative stress. Moreover, the abnormal interaction of α-Syn with membranes has been implicated in the cytoplasmic retention of catecholaminergic neurotransmitters yielding cytotoxicity through the generation of dopamine adducts and ROS [52]. This effect is potentiated in the presence of iron-rich environments, as it is the case of Lewis bodies in the neurons decorating the *substantia nigra* of PD patients [58–61]. Indeed, it is known that dopamine is able to coordinate iron and regenerate Fe^{2+}, possibly providing an equally important source of hydroxyl radical production [62].

Mitochondria have been claimed as dominant sites for oxidative stress-driven initiation and propagation in PD. The direct implication of this organelle in PD was first suggested by the use of the mitochondrial complex I (CI) inhibitor MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [63, 64]. This chemical mimics human PD in animal models and is associated with development of Parkinsonism in humans subjected to accidental exposure [65]. Further corroborating the relevance of mitochondria in PD, it was shown that the well-known CI inhibitor rotenone induces death of dopaminergic cells [66–69]. In addition, depletion of the antioxidant peptide glutathione (GSH) in PD cells, which may be caused by a decrease in its synthesis and recycling [70], has been associated with a decrease of mitochondrial CI activity, resulting in mitochondrial dysfunction [71, 72]. Moreover, defective mitochondrial CI function is observed in the *substantia nigra* of PD brains [73, 74]. Recent studies have also demonstrated that α-Syn monomers and oligomers associate with the inner mitochondrial membrane where they can physically associate with CI, thereby interfering with the mitochondrial function and increasing free radicals production [72, 75]. Further highlighting the relevance of mitochondrial dysfunction and oxidative stress in the pathological process of the disease, several genes associated with familial cases of PD were shown to encode either mitochondrial proteins or mitochondria-associated proteins [6, 76]. Among the latter is DJ-1, a protein that shares structural homology with the *Escherichia coli* chaperone Hsp31 and is thought to have a protective role against oxidative stress [77]. Under conditions of oxidative stress, DJ-1 is relocated to the mitochondria, affecting the sensitivity of specific neuron populations to compounds targeting mitochondrial CI, namely, rotenone, paraquat, and MPTP [6, 78–80].

Several evidences support as well an unbalanced generation of RNS as a feature of PD pathology. First, nitrated α-Syn accumulates in LB of PD cells. Secondly, the treatment of GSH-depleted dopaminergic cells with RNS inhibitors prevents mitochondrial CI inhibition [6], indicating that RNS itself has a role in mitochondrial dysfunction and ROS generation in PD. Lastly, glial cells within the *substantia nigra* exhibit increased NO levels [81], possibly due to the accumulation of interferon-γ (IFN-γ) [82], a cytokine which was shown to promote induction of RNS in brain.

5. *S. cerevisiae* as Model Organism to Study Neurodegenerative Diseases

Budding yeast *Saccharomyces cerevisiae* has been introduced as an experimental organism in the midthirties of the 20 century. Since then, its potential as a model organism has been exploited in many areas of biology [83]. Despite lacking the physiological complexity of the mammalian nervous system, yeast was recently used in the study of neurodegenerative disorders, such as Alzheimer's and Parkinson's Diseases. This became possible due to the development of powerful yeast genetic tools as well as the high conservation of fundamental biological processes and pathways associated with neurodegeneration including protein folding, cellular trafficking, and secretion [84]. It is noteworthy that about one-fifth of yeast genes are members of orthologous gene families associated with human diseases [85]. This is an important aspect to consider when studying human diseases in yeast. If a homologue of the gene implicated in the disease is present in yeast genome, a unique opportunity to directly study its function is offered, either through its deletion or overexpression. Otherwise, if the disease-associated gene does not have a yeast counterpart, functional analysis can still be performed via heterologous expression [86, 87]. Equally important "humanized" yeasts are being used as platforms for high-throughput screenings of compounds with therapeutic potential.

6. Yeast as a Model for Studying Alzheimer's Disease

Yeast models have been extensively used to study several molecular aspects of AD, even though yeast lacks for some AD-associated genes. Studies in yeast have been mainly focused on the *in vivo* APP processing, Aβ oligomerization, and toxicity.

The usage of heterologous expression of human secretases in yeast has greatly contributed to the understanding of human APP processing. It has allowed the discovery of BACE1 inhibitors and prompted the study of the individual function of each component of the γ-secretase complex [88].

Growing evidence suggests that the oligomeric forms of the Aβ peptide, rather than amyloid fibrils, are the most toxic forms [89–92]. These findings have shifted the focus of investigation towards the earliest stages of Aβ oligomerization. As a result, the following described yeast systems were developed and are now useful tools not only in the study of Aβ-oligomerization, but also in the understanding of the molecular events triggered by aggregation as well as in the screening of potential therapeutic compounds that affect the aggregation process.

The first yeast study on Aβ oligomerization used a two-hybrid approach to analyze Aβ dimerization. Protein-protein interactions were measured by fusing the Aβ peptide to a LexA DNA-binding domain and also to a B42 transactivation domain, and then monitoring the expression of a *lacZ* reporter driven by a LexA-dependent promoter [93]. The authors showed that Aβ peptide was able to form dimers, *in vivo*, in the yeast cell nucleus.

Bagriantsev and Liebman and von der Haar et al. implemented a different yeast model system that may constitute a valuable tool to seek for agents that interfere with the initial steps of Aβ42 oligomerization [94, 95]. In this study, the ability of Aβ42 peptide to aggregate was monitored by fusing it to the middle and C-terminal domain of Sup35. The Sup35 yeast translational termination factor can undergo spontaneous conversion into a prion state, losing its function [96]. Sup35 loses the ability to aggregate when its prion-forming (N-terminal) domain is deleted. However, the insertion of Aβ peptide sequences in place of the original prion domain of Sup35 protein restores its ability to aggregate [94, 95]. Using this reporter system, it was possible to confirm *in vivo* the impact of point mutations, previously shown to inhibit Aβ42 aggregation *in vitro* [94, 97]. Furthermore, it was shown that the Hsp104 yeast chaperone, a chaperone known to rescue proteins from the aggregated state in other yeast models of neurodegenerative diseases [98, 99], appears to have a contrary function in AD, protecting Aβ-fusion protein from disaggregation and degradation [94, 95].

Oligomerization of Aβ was also the subject of a third yeast study, by the use of a reporter consisting of Aβ fragment fused to GFP [100]. The assay was based on the premise that aggregates of the fusion protein suppressed green fluorescence. The Aβ-GFP fusion was shown to cause slight but significant yeast growth reduction and to induce a heat shock response (HSR), as indicated by the cotransformation of yeast with Aβ-GFP and HSE2 element fused to a downstream *lacZ* gene. The authors put forward the hypothesis that HSR could arise from Aβ inducing ROS and/or the presence of misfolded proteins and suggested that HSR might be a target for further studies seeking for inhibitors of Aβ effects [100].

Recently, Treusch et al. engineered a yeast model for studying Aβ toxicity [101]. The overexpression of a construct harboring the Aβ42 fragment fused at the N-terminus to an endoplasmatic reticulum targeting sequence was driven by a galactose-inducible promoter. Aβ oligomers localized to secretory compartments and, like in neurons, contributed to toxicity in yeast. A screen for genetic modifiers allowed the identification of 40 genes that were able to modulate Aβ toxicity. Among those, 12 had homologues in humans, 3 being related to clathrin-mediated endocytosis, and 7 functionally associated with the cytoskeleton. Interestingly, all the former genes behaved as Aβ toxicity suppressors and had been previously shown to be or interact with validated risk factors for AD. The authors further showed that Aβ affects clathrin-mediated endocytosis and proposed that Aβ oligomers may interact with transmembranar receptors and prevent their correct destination [101].

7. Yeast as a Model for Studying Parkinson's Disease

As a common feature of sporadic and familial cases of PD, the understanding of the pathological processes associated with α-Syn has attracted special attention. In order to gain insight into α-Syn pathobiology, Outeiro and Lindquist

exploited a myriad of advantages of using *S. cerevisiae* as a model organism, by developing a powerful "humanized" yeast system. As a means to study the α-Syn dynamics *in vivo*, the authors overexpressed in yeast cells a construct harboring the wild type or the mutant versions of human *SNCA* gene fused to GFP [102]. This pioneering system faithfully reproduces several features of PD in yeast, allowing to thoroughly investigate the pathological processes involved in the disease. Three strains, designated as NonTox, InTox, and HiTox, were created to express α-Syn at different levels [52]. As it happens in complex eukaryotic models, the appearance of cytoplasmic foci, cytotoxicity, and α-Syn-decorated vesicle accumulation was shown to be dose dependent [102, 103]. Moreover, high doses of α-Syn lead to increased toxicity, accumulation of cytoplasmic lipid droplets, vesicle trafficking defects, ER stress, activation of the heat-shock response, impairment of the ubiquitin-proteasome pathway, and mitochondrial dysfunction in the HiTox strain, therefore recapitulating the pathological features displayed by PD patients whose genome encodes duplications or triplications of *SNCA* locus [52].

The α-Syn yeast model developed by Outeiro and Lindquist has been the basis of several genome wide and high-throughput analyses aimed at unveiling the intricacies of PD. The systematic screening of a galactose-inducible overexpression library in the InTox strain revealed the Rab GTPase Ypt1 (Rab1) as suppressor of α-Syn toxicity [104], reinforcing the role of α-Syn in vesicle formation and delivery. In addition, an unbiased genome-wide screen for modifiers of α-Syn toxicity was performed in the InTox strain, allowing the identification of the polyamine transporter Tpo4 [105] and highlighting the significance of polyamine pathway in PD pathogenesis. Using the ResponseNet algorithm to integrate α-Syn mRNA profiling and genome-wide genetic data, it was found that trehalose might be involved in the protection pathway against α-Syn toxicity possibly promoting misfolded protein clearance. In addition, mitochondrial dysfunction and oxidative/nitrosative stress also appeared as consequences of α-Syn overexpression in yeast [106]. Comparison of the transcriptome of HiTox and NonTox strains provides further evidence supporting the assumption that mitochondrial dysfunction and oxidative stress are associated to conditions in which α-Syn is expressed at high levels. It has also been verified that mitochondria morphology is affected and ROS is accumulated in the HiTox strain further suggesting that high levels of α-Syn elicit global mitochondrial dysfunction [107]. This may suggest that α-Syn accumulation is the origin of oxidative damage of specific neuronal cells in PD.

More recently, overexpression of α-Syn in yeast revealed that the knockout of genes encoding lipid elongases, namely, *ELO1, ELO2, and ELO3*, impairs cell growth, dramatically decreases the survival of aged cells, and leads also to ROS accumulation and aberrant protein trafficking [108]. A similar strategy, using a different plasmid to drive galactose-inducible α-Syn expression in distinct *S. cerevisiae* backgrounds, disclosed the significance of fatty acid synthase activity and intracellular redox status in the mechanisms of α-Syn toxicity [109].

α-Syn-humanized yeasts have also been exploited to search for compounds with therapeutic potential. In this context, the HiTox strain was used in a high-throughput chemical screen to identify agents capable of rescuing the robust toxicity of this strain. A class of small molecules of 1,2,3,4-tetrahydroquinolinones were identified and shown to revert the formation of α-Syn foci, to reestablish ER-to-Golgi trafficking, to ameliorate mitochondria damage, to limit ROS production, and consequently to reduce α-Syn toxicity not only in yeast but also in other more complex PD models [107].

8. Concluding Remarks

Although AD and PD have been extensively studied, the exact mechanism of disease progression or pathogenesis remains largely unknown. As outlined in this paper, several *in vivo* and *in vitro* studies point towards a role of oxidative stress in AD and PD pathogenesis. Nevertheless, whether it is a primary cause or simply a consequence of the neurodegenerative process is still an unanswered question. In addition, specifically concerning AD, there are quite a few contradictory reports regarding the role of oxidative stress in the disease. Indeed, it has been described that oxidative stress may as well lead to an increase in Aβ [14, 110], and *in vivo* studies showed a negative correlation between oxidative stress and Aβ, indicating an antioxidant role for Aβ [111].

Yeast can be a powerful tool as a means to clarify several of these issues. Within this context, yeast models of AD may in the future be used to monitor Aβ oligomerization and toxicity under an oxidative environment or in the absence of ROS (hypoxia). Interestingly, a yeast model consisting of Aβ peptide fused to GFP has been successfully used to test whether folate, an antioxidant, was able to prevent Aβ aggregation [112]. To better understand the relationship between oxidative stress and α-Syn aggregation, in the pathological processes triggering PD, it would be interesting to assess both the behavior of α-Syn in the "humanized" NonTox strain under oxidative environments and in the InTox and HiTox strains under hypoxia conditions.

Future studies combining yeast and animal models of AD and PD will certainly provide valuable insights into the role of oxidative stress in these neurodegenerative diseases.

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