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Perturbation of Calcium Homeostasis, and a Novel Function for the Ubiquitin Ligase (E4) Ufd2 in Yap8 Stability

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Crz1 marked with GFP of *S. cerevisiae* (Rita T. Ferreira *et al.*, unpublished)

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Contents

Dedication

“You need to be able to present your work for the amount of time you have been allocated, for instance 1 min. We will discuss this at a later stage.”

Claudina Rodrigues-Pousada (July 2013)

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I am indebted to many people who contributed with their time, expertise and passion during my doctoral studies.

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December 21st 2015 *Rita T. Ferreira*

Thesis Publications

This thesis is the description of the peer reviewed original research published in the **OpenMicrobiology** (formerly SGM Open) and **Biology Open (BiO)** online Open Access journals:

E4-Ubiquitin ligase Ufd2 stabilizes Yap8 and modulates arsenic stress responses independent of the U-box motif.
Rita T. Ferreira, Regina A. Menezes and Claudina Rodrigues-Pousada
Biology Open, AOP 27 July 2015.
DOI:10.1242/bio.010405, *in press*.

Arsenic stress elicits cytosolic Ca^{2+} bursts and Crz1 activation in *Saccharomyces cerevisiae*.
Rita T. Ferreira, Ana R. Courelas Silva, Catarina Pimentel, Liliana Batista-Nascimento, Claudina Rodrigues-Pousada and Regina A. Menezes
Microbiology SGM Open, AOP 25 June 2012.
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Foreword

This thesis is intended to summarize most of the subjects I have been involved with during the four year period from April 2011 to March 2015. The contributions of yeast to molecular and cell eukaryotic biology led us to investigate basic biological processes occurring in response to arsenic toxicity. Using a simple and fast growing organism tractable to genetic and biochemical approaches, this thesis asks how do cells respond to arsenic challenges. Implications for the field of yeast stress response are discussed, as well as the limitations of the work and avenues for future research.

We have worked on transcriptomic analysis of *S. cerevisiae* response to arsenic, that was pointing out to a link between this cytotoxic agent and the genetic regulation of Ca^{2+}-signaling pathways. Our results revealing the perturbation of calcium homeostasis were published in Microbiology of the Microbiology Society (previously the Society for General Microbiology) in a paper that I co-authored (Chapter 2). We have moved to investigate the post-translational mechanisms controlling the levels of the transcription factor Yap8. This study has shown that the ubiquitin chain elongation enzyme Ufd2 is required for Yap8 stabilization critical to arsenic tolerance and detoxification. The results were published in Biology Open in a paper that I authored (Chapter 3). Although the outcomes of the two studies above seem to be unrelated, both address two different ways of stress response: one through the cellular effects of arsenic toxicity; and another through the understanding of the fundamental basis of Yap8 regulation with a pivotal role in arsenic elimination by yeast cells. We have finally tried to document the roles of ubiquitin and SUMO (small ubiquitin-related modifier) in modulating the response to arsenic. Preliminary data suggesting that Lys62 and Lys198

of Yap8 may be modified by SUMO were collected in a manuscript in preparation that I authored (Chapter 4).

In addition, I have worked on the Yap8 stability studies involving the multiprotein transcriptional mediator (Silva A.R. *et al*., unpublished); results from cycloheximide chase assays revealed that loss of the RNA polymerase II mediator subcomplex Cdk8 (cyclin-dependent kinase 8) stabilized the transcription factor Yap8 (data not shown).

Summary

Human exposure to elevated levels of inorganic arsenic (As) poses the greatest threat to global public health. However, arsenic trioxide (ATO) is presently the most active antineoplastic agent in the treatment of acute promyelocytic leukaemia (APL) (Trisenox®, Teva Pharm). Despite the success of this drug, the actual mechanism of action is complex and is not completely understood. We have therefore investigated the mechanisms by which the cell responds to arsenic that perturb homeostasis, and how homeostasis can subsequently be restored, while also focusing on the mechanistic understanding of individual protein regulation important for the cell to thwart the cytotoxic effects of arsenic.

Using *S*. *cerevisiae*, also known as baker's yeast—a model system relevant to human biology, we have firstly performed DNA microarrays to obtain novel molecular biological pathways that are mapped by arsenic. The resulting analysis revealed a transcriptional response to arsenic stress of five 'core' components of calcium signaling and calcium transport (CaM, CaN, Crz1, Mid1 and Cch1). Bioluminescence spectroscopy and genetic analyses showed that arsenic induces a transient increase in cytoplasmic calcium ($[Ca^{2+}]_{cyt}$), followed by a calcineurin/Crz1-dependent gene expression to restore homeostasis and cell wall integrity. A comprehensive protein-protein interaction study based on two-hybrid system revealed that at biochemical level, the E4-Ubiquitin ligase Ufd2 interacts with the AP-1-like transcription factor Yap8 to perform a new cellular role by stabilizing and activating Yap8, without the U-box ligase activity of Ufd2. The *in silico* analysis revealed putative sites of Yap8 to be targets of the ubiquitin and small ubiquitin-related modifier (SUMO), as well as their possible interconnection. Site-

directed mutagenesis analyses suggested that all six lysines (K46,47,62,90,96,198) control Yap8 stability, and K62 and K198 as potential SUMOylation sites that control Yap8 activity.

This study contains fundamental biological information that might possibly offer the context for a more reasoned and informed approach to the effects of arsenic toxicity.

Keywords: Arsenic (As), acute promyelocytic leukaemia (APL), yeast *Saccharomyces cerevisiae*, yAP-1 transcription factor Yap8, Ca^{2+}-Calcineurin-Crz1, yeast E4-Ufd2 (ubiquitin chain assembly factor), SUMO (small ubiquitin-related modifier)

Sumário

A exposição humana a elevados níveis de arsénio (As), sob a sua forma inorgânica, constitui uma ameaça à saúde pública global. Por outro lado, o trióxido de arsénio (TOA) é, presentemente, o agente antineoplásico mais ativo no tratamento da leucemia promielocítica aguda (LPA) (Trisenox®, Teva Pharm). Apesar da sua eficácia terapêutica estar comprovada, o exato mecanismo de ação é complexo, não sendo completamente conhecido. É preciso, portanto, investigar ao nível da célula os mecanismos de resposta ao arsénio que perturbam a homeostase e como esta pode ser recuperada, e ao mesmo tempo, compreender a regulação individual das proteínas importantes no combate aos efeitos tóxicos do arsénio.

Utilizámos a levedura *S. cerevisiae* ("baker's yeast"), um sistema modelo relevante no campo da biologia humana, e utilizámos a tecnologia de DNA microarrays para obter novas vias e alvos moleculares e biológicos mapeados pelo arsénio. A análise revelou uma resposta, ao nível transcricional, de cinco componentes centrais para a sinalização e o transporte de cálcio (CaM, CaN, Crz1, Mid1 e Cch1). As análises de espectroscopia de bioluminescência e de genética mostraram que o arsénio é responsável por um aumento da concentração de cálcio no citoplasma ($[Ca^{2+}]_{cyt}$), acompanhado da expressão de genes-alvo do Crz1 e da calcineurina (CaN). O estudo de interações proteína-proteína baseado no sistema de dois híbridos revelou que, ao nível bioquímico, o enzima E4-Ufd2 ligase de ubiquitina interage com o fator de transcrição Yap8 do tipo AP-1 para desempenhar uma nova função celular, estabilizando o Yap8 e ativando-o sem recorrer ao seu domínio de atividade como ligase, designado de "U-box". As análises *in silico* revelaram os locais na proteína Yap8 de possível ligação à ubiquitina e ao pequeno modificador relacionado

com a ubiquitina (SUMO), e uma possivel conexão entre ambos. As análises de mutagénese sítio-dirigida sugeriram que as seis lisinas (K46,47,62,90,96,198) controlam a estabilidade da proteína Yap8, e que as lisinas K62 e K198 como potenciais locais de SUMOilação controlam a sua atividade.

Este estudo contém informação biológica fundamental, podendo vir a facultar um contexto mais fundamentado e informado dos efeitos tóxicos do arsénio.

Palavras-chave: Arsénio (As), leucemia promielocítica aguda (LPA), levedura *Saccharomyces cerevisiae*, fator de transcrição Yap8 (de tipo yAP-1), Ca^{2+}-Calcineurina-Crz1, E4-Ufd2 (fator de elongação de ubiquitina) de levedura, SUMO ("small ubiquitin-like modifier"–pequeno modificador de tipo ubiquitina)

Abbreviations

ABC ATP-binding cassette
ACT1 ACTin gene
AD Activation Domain
ADP adenosine diphosphate
AML Acute Myeloid Leukaemia
ALD AdrenoLeukoDystrophy
AP-1 Activating Protein-1
APL Acute Promyelocytic Leukaemia
AQP Aquaglyceroporin
ARE AP-1 Recognition Element, *see* AP-1
ARR1-3 ARsenicals Resistance
As Arsenic
As(III) Arsenite
As(V) Arsenate
ATO Arsenic trioxide
ATP Adenosine TriPhosphate
BiO Biology Open (Journal)
bZIP Basic-region Leucine ZIPper
Ca Calcium
$[Ca^{2+}]_{cyt}$ Cytosolic calcium ion concentration
$[Ca^{2+}]_{I}$ intracellular Ca^{2+} concentration, *see* Ca
CaM Calmodulin
CaMK Ca^{2+}/CaM-dependent protein kinases, *see* CaM
CaN Calcineurin
cCRD C-terminal cysteine rich domain
cDNA Complementary DNA, *see* DNA
CDRE Calcineurin-dependent response element
Cmk2 Ca/CaM-dependent protein kinase 2, *see* Ca, *see* also CaM
CML Chronic Myeloid Leukaemia
CN Calcineurin
CFTR Cystic Fibrosis Transmembrane (conductance) Regulator
Cnb1 CaN regulatory subunit B, *see* CaN
Co-IP Co-immunoprecipitation
c.p.s. Counts per second
Crz1 CaN-responsive zinc finger transcription factor, *see* CaN
CsA Cyclosporine A
DABCO 1,4-Diazabicyclo[2.2.2]octane
DAPI 4',6-Diamidino-2-phenylindole
DBD DNA binding domain, *see* DNA
DMSO Dimethyl sulfoxide
DNA DeoxyriboNucleic Acid
ER Endoplasmic Reticulum
EUROSCARF EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis
Fps1 Aquaglyceroporin, plasma membrane channel
Fw Forward (oligonucleotide)
Gad1 Glutamate Decarboxylase 1
GFP Green Fluorescent Protein
GSC2 Glucan Synthase of Cerevisiae
GSH Glutathione
GTP Guanosine-5'-triphosphate
HA Hemagglutinin
HECT Homologous to E6-carboxy terminus
HBAg Hepatitis B surface antigen
Hph1/2 Homologous integral ER membrane proteins, *see* ER
Hog High osmolarity glycerol
HSFs Heat Shock Factors
Hxts Hexose transporters
IP Immunoprecipitation
MAPK Mitogen-Activated Protein Kinases
Mid1 Mating pheromone-induced death
MYC MyeloCytomatosis (oncogene)
mRNA messenger RNA, *see* RNA
NFATc Nuclear Factor of Activated T cells
NES Nuclear Export Signal
NDB ID Nucleic Acid Database Identification
NLS Nuclear Localization Signal
OATPs Organic Anion Transporting Polypeptides
OD Optical Density
ORF Opening reading frame
PAGE Polyacrylamide gel electrophoresis

PBS Phosphate-Buffered Saline
PCR Polymerase Chain Reaction
PDB ID Protein Data Bank Identification
Pgk1 3-Phosphoglycerate kinase
Pho Phosphate transporters
P_i inorganic Phosphate
Pmc1 Plasma membrane calcium
PML Promyelocytic leukaemia protein
Pmr1 Plasma membrane ATPase-related, *see* ATP
ppb parts per billion
PTM post-translational modification
qPCRs quantitative PCRs, *see* PCR
Rad23 RADiation sensitive
RARα Retinoic Acid Receptor alpha
RING "really interesting new gene"
RLU Relative Luminescence Units
ROS Reactive Oxygen Species
r.p.m. Rotations per minute
RTG Retrograde
RT-PCR Reverse-Transcriptase PCR, *see* PCR
Rv Reverse (oligonucleotide)
SC Synthetic Complete
SCF Skp1, Cullin, F-box
SD Synthetic defined/minimal medium *or* sd/SD, standard deviation
SDS Sodium Dodecyl Sulfate
SEAR South East Asia
SGD *Saccharomyces* Genome Database
SGM Society for General Microbiology
SMT3 Suppressor of Mif Two 3
SPB Spindle Pole Body
STRE STress Responsive Elements
SUMO Small ubiquitin-like modifier *or* small ubiquitin-related modifier
SV-40 Simian Vacuolating virus 40 *or* Simian Virus 40
TCA Trichloroacetic acid
TFs Transcription factors
Ub Ubiquitin
Ubc Ubiquitin-conjugating
Ufd2 Ubiquitin fusion degradation 2
Ulp Ubiquitin-like protease
UPP Ubquitin-proteasome pathway
UPS Ubiquitin-proteasome system
UV Ultraviolet
WT Wild-type
Yap Yeast specific AP-1-like activator proteins, *see* AP-1
Ycf1 Yeast cadmium factor-1
YPD Yeast Peptone Dextrose
YRE Yeast Response Element

latin

c.a., circa, approximately
cf., confer with, consult
e.g., exempli gratia, for example
et al., et alia., and other people
i.e., id est, that is to say
vs*.,* versus, against, turned

amino acids

A, Ala, alanine
C, Cys, cysteine
D, Asp, aspartate
E, Glu, glutamate
F, Phe, phenylalanine
G, Gly, glycine
H, His, histidine
I, Ile, isoleucine
K, Lys, lysine
L, Leu, leucine
M, Met, methionine
N, Asn, asparagine
P, Pro, proline
Q, Gln, glutamine
R, Arg, arginine
S, Ser, serine
T, Thr, threonine
V, Val, valine
W, Trp, tryptophan
Y, Tyr, tyrosin

1
Introduction

1.1
Historical Aspects, and Biochemistry of As

Arsenic (As) compounds are extremely toxic with a long history of use as a poison for life. Nevertheless, arsenic has been also used in medicine, and its earliest use goes back to the times of ancient Rome, when Galen and Hippocrates used it to treat ulcers [1]. In the 1800s and early 1900s, arsenic was commonly used to treat diseases, such as psoriasis and syphilis. Through the systematic chemical modification of arsenic derivate, before the “pre-antibiotic era”, the discovery of salvarson (arsphenamine) made it the main medicine used against syphilis [2].

As (atomic number, 33; relative atomic mass, 74.92) has chemical and physical properties intermediate between a metal and a nonmetal, and is often referred to as a semi-metal or metalloid. It belongs to Group VA of the Periodic Table, and can occur in four oxidation states (+5,+3,0,-3) [3]. Nevertheless, it is generally found in the environment in two biologically important oxidation states—the pentavalent arsenic [As(V), arsenate] and trivalent arsenic [As(III), arsenite]. Of the inorganic arsenic compounds, arsenic trioxide (ATO, As_2O_3)—the form of arsenite used in cancer therapy [3], sodium arsenate (Na_3AsO_4), and arsenic trichloride are the most common found in nature [4].

Metalloids belong to the group of elements which also includes transition metals. Heavy metals affect cells and living organisms in various ways; some heavy metals have essential functions (e.g., iron, cobalt, zinc, copper,

and manganese) and are toxic only in an overdose, whereas others are xenobiotic and highly toxic (e.g., arsenic, cadmium, lead, and mercury) [5]. Thus, all cells and organisms maintain metal homeostasis within physiological or sub-toxic levels, respectively, and utilize metal detoxification mechanisms. (For arsenic detoxification in yeast and mammals, see Section 1.6.)

1.2 Molecular and Cellular Effects of As

In general, the toxicity of a given metal depends on its physicochemical properties and ligand preferences: 'soft' transition metals (like cadmium and mercury) prefer sulfur as their ligand; while 'hard' transition metals (like chromium and manganese) and metalloids (arsenic, antimony and selenium) favor oxygen in their higher oxidation states and sulfur in their lower oxidation states; lead, iron, cobalt, nickel, copper and zinc may use oxygen, sulfur or nitrogen as ligands [6].

At the molecular level, an important feature of As(III) chemistry lies in its high reactivity, forming strong bonds with functional groups, such as thiolates of cysteine residues or the imidazolium nitrogens of histidine residues, and therefore its ability to bind to and affect the activity of many proteins in the cell [7]. Hence, the biochemical effects of the arsenic trivalent form (principally, arsenite and arsenic trioxide) may be mediated by reactions with closely spaced cysteine residues on critical cell proteins [8]. On the other side, As(V)—as a chemical analogue of inorganic phosphate (P_i), can uncouple the oxidative phosphorylation in mitochondria by displacing phosphate in ATP synthesis [9]. However, As(III) is generally more toxic than As(V), and both of them are more toxic than the organic arsenic compounds [10].

At the cellular level, the well known carcinogenic effect of arsenic-containing compounds results from their ability to cause oxidative stress by generating reactive oxygen species (ROS); to cause epigenetic changes in DNA repair and DNA methylation, causing chromosomal abnormalities; as

well as to inhibit protein function/activity leading to cell proliferation or cell death [6, 11, 12]. Indeed, arsenic facilitates profound cellular alterations, including induction of apoptosis, inhibition of proliferation, stimulation of differentiation, and inhibition of angiogenesis via numerous pathways [13].

Despite arsenic's cytotoxic effects, ATO is currently used as a mitochondria-targeting drug in acute promyelocytic leukaemia (APL) capable of triggering apoptosis in leukaemia cells (see Section 1.4.1), though the mechanisms by which ATO kills cancer cells are not fully understood [14, 15].

1.3 As Poisoning to Humans

Arsenic is a metalloid element, which forms a number of poisonous compounds [16]. As previously referred, the two oxidation states of inorganic arsenic, As(V) and As(III), are most commonly found in nature, namely in rocks and soil. It is widely distributed throughout the earth's crust, and is found in groundwater supplies in many countries of the world. South East Asia (SEAR) countries have been identified as the most highly affected geographical areas by arsenic contamination [17]. Furthermore, antrophogenic sources such as industrial and urban pollution are also responsible for introducing this metalloid into the environment. For example Asturias, an autonomous region in Northern Spain with a large industrial area, registers high lung cancer incidence and mortality [18].

Exposure of humans to arsenic occurs by inhalation as well as consumption of contaminated water and food (for a review see Ref. [19]). The environmentally relevant concentrations of arsenic could reach >10 µg/l, as reviewed by Dopp *et al*. [20].

Acute and chronic exposure is considered to be the greatest single-cause of ill-health wide world. Both short- and long-term exposure cause health problems such as vomiting, esophageal and abdominal pain, and bloody 'rice water' diarrhea [1]. In addition, inorganic arsenic is also associated with renal, hepatic, pulmonary and adrenal tumors [21]. In addition, prenatal

exposure to arsenic environmental contaminant has been documented to cause genome-wide changes in human newborns [22].

Recently, a journal article review [23] reported that four million people in Argentina are exposed to arsenic contamination from drinking waters of several center-northern provinces, associated with an increased risk of serious chronic diseases, showing the need for adequate and timely actions.

1.4
Applications of As in Medicine

As aforementioned (Section 1.2), arsenic has long been known to act as a carcinogen; paradoxically, it has also been demonstrated that arsenic can have anti-cancer activity in some cases [24, 25]. In traditional Chinese medicine, ATO was recorded in the Compendium of Materia Medica by Li Shi-Zhen. In Western medicine, arsenicous oxide (Fowler's solution) was used as a treatment of choice for chronic myeloid leukaemia (CML) in the 19^{th} century. Osler stated in his textbook of medicine: '*There are certain remedies that have an influence upon the disease. Of these, arsenic, given in large doses, is the best. I have repeatedly seen improvement under its use*.' (1892). Nevertheless, due to toxic side effects of long-term dose of oral arsenic in most patients, and with the advent of modern radiotherapy and chemotherapy, the arsenic treatment for CML was given up in Western medicine [26]. Noticeably, the discovery of the therapeutic effect of As_2O_3 in APL has revived this ancient drug [27]. (See section below.)

1.4.1
Targeted Therapy by ATO: the Current Front-line Therapy for APL

As we have seen above, arsenic has been successfully used in the molecularly targeted therapy of APL (the M3 subtype of AML), which was once a lethal disease, yet nowadays the majority of patients with APL can be successfully cured by molecularly targeted therapy [28].

APL is characterized by a balanced reciprocal chromosomal translocation fusing the promyelocytic leukaemia (*PML*) gene on chromosome 15 with the retinoic acid receptor α (*RARα*) gene on chromosome 17 [27]. It has been found that ATO or all-trans-retinoic acid (ATRA) alone exerts therapeutic effect on APL patients with the *PML*-*RARα* fusion gene, and the combination of both drugs can synergistically act to further enhance the cure rate of the patients [28].

In addition, ATO induces apoptosis via changes in the mitochondrial membrane potential; an important aspect of ATO treatment of malignant cells is that elevated intracellular levels of hydrogen peroxide (H_2O_2) lower mitochondrial membrane potential, which leads to cytochrome *c* release, and subsequent activation of the caspase pathway [29].

Trisenox® (Teva Pharm) (www.trisenox.com) injection is indicated for induction of remission and consolidation in patients with APL who are refractory to, or have relapsed from, retinoid and anthracycline chemotherapy, and whose APL is manifested by the presence of the t(15;17) translocation or *PML*/*RARα* gene expression [30].

The excellent improvement in the survival rate of APL patients is an example of the advantage of modern medicine. Nevertheless, despite the satisfactory advancements regarding the efficacy of ATO-based therapy for APL patients, there is still a large research effort to improve current knowledge of chemotherapy with ATO [31].

1.5 Budding Yeast as an Eukaryotic Model System

The budding yeast *Saccharomyces cerevisiae* is perhaps the most useful yeast species owing to its use since ancient times in Egyptian methods of baking and brewing [32].

In biology, this unicellular organism allows the examination of evolutionarily conserved aspects of eukaryotic cells since it maintains many of the same ‘core’ cellular signaling mechanisms, and compartmentalizes the metabolic pathways into different organelles as it occurs in human cells (see

Figure 1.1). As an eukaryote, *S. cerevisiae* shares the complex internal cell structure of higher eukaryotes, however without the very large fraction of genome containing non-coding DNA–commonly referred to as “junk DNA”, that is found in humans [33]. Apart from an even diploid set of chromosomes, yeast cells harbor numerous proteins similar to human cells that make them in particular suitable for recapitulating the fundamental aspects of eukaryotic biology [34].

Small, newly budded *S. cerevisiae* cells grow until they achieve a critical size, when they can then produce new buds themselves [35]. Furthermore, these cells can be transformed allowing for either the addition of new genes, or the deletion through homologous recombination. In addition, the possibility of working with an haploid state facilitates the study of multiple processes, and allowed the generation of collections of haploid knockout strains [e.g., the collection from European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF)].

Yeast model system has been useful for the current knowledge of fundamental mechanisms of eukaryotes, such as cell division, DNA replication, metabolism, protein folding and intracellular transport [36].

Important advances in the neurodegenerative diseases field [37] allowed the identification of novel disease drug targets that rescued phenotypes of yeast cells and patient neurons in similar ways, and those targets would not have been identified without yeast genetics (cf. also Section 1.5.2).

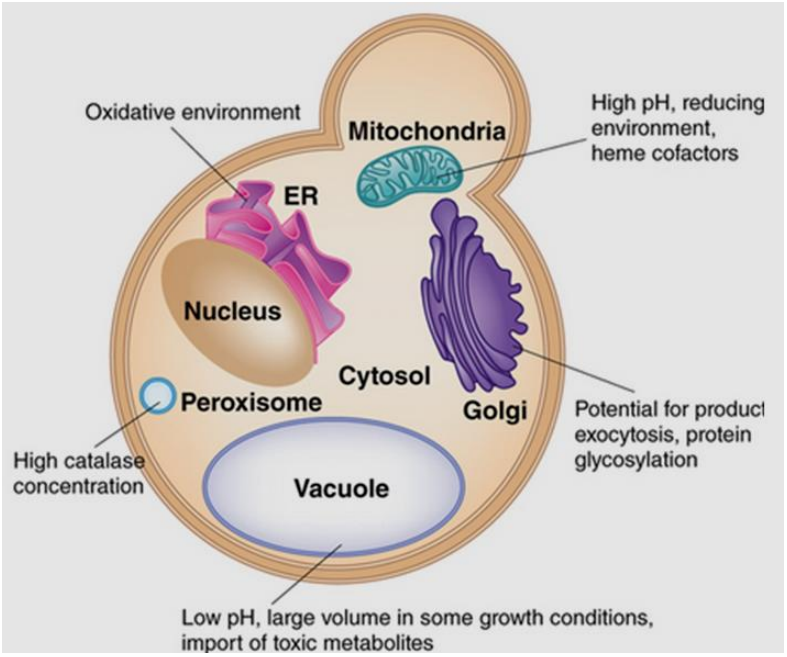

Figure 1.1 Budding yeast compartmentalizing metabolic pathways in essential 'core' organelles found in most eukaryotic cells: nucleus, endoplasmic reticulum (ER), mitochondria, Golgi apparatus, peroxisome, and vacuole. (After Ref. [38].)

1.5.1
The Yeast Genome Sequencing Project

In April 1996, the complete genomic sequence of the *S. cerevisiae* strain S288C [39], that was determined through a worldwide effort, was released to the public. It was a collaboration of more than 600 scientists from over 100 laboratories to sequence the *S. cerevisiae* genome, reported as "the largest decentralized experiment in modern molecular biology", resulting in a unique data resource, and representing the first complete set of genes from a eukaryotic organism [40]. The sequence of the yeast *S. cerevisiae* consists of 12,068 kilobases, nearly 6,000 ORFs, and is c.a. a factor of 250 smaller vs. the human genome [41]. Notably, the resulting knowledge of the entire yeast genes made possible the identification of many mammalian orthologs.

Indeed, during the course of the yeast genome sequencing project, Rodrigues-Pousada C. and coworkers discovered the peroxissomal ABC

transporter protein Pat1 as revealing great functional similarities to the human ALD protein [42, 43]. Almost 200 genes are orthologs to those that cause disease in humans (e.g., the yeast *YCF1*, and human *CFTR*); and around 71 human genes complement yeast mutations, which is important in order to unravel the molecular basis of many human diseases ([39, 44-46], cf. also Table 1 in Appendix).

The *Saccharomyces* Genome Database (SGD) provides scientific high-quality, curate data about the genes and gene products of *S. cerevisiae* [47].

1.5.2 Yeast Applications: Biomedical Research, Food and Wine, and Chemical Industries

Currently, a large network of scientists takes advantage of the simple yeast assay system that helps them speed up the discovery of more complex biological processes of the eukaryotic biology, and limiting the use of animal models.

Yeast cells—which share the 'core' cell biology of human cells, provide a powerful experimental tool to study the intricacies of eukaryotic biology given its genetic tractability, and remarkable conservation of fundamental cellular processes with higher eukaryotes [48, 49]. They have been extensively used as living test tubes to learn about the underpinnings of human diseases from neurodegenerative, prions, cancer and other age-related diseases [50-54]; to renal disorders [55]; as well as physiological processes such as angiogenesis, cell death or DNA repair [56].

Very recent systematic humanization of yeast genes also determined conserved functions and genetic modularity [49]. In addition, yeast cells allow a global knowledge of toxicological and resistance mechanisms upon exposure to weak acids used as food preservatives, herbicides or in pharmacology [57].

It is also noteworthy the impact of these 'microbial cell factories' on energy technology [58], wine industry [59], industrial wheat straw

hydrolysates (WSH) [60], but also on food and beverage production processes [61].

1.5.2.1

Biopharmaceuticals from Health-Care Industries

Since the 1980s, development of the pharmaceutical industry has been marked by the rapid expansion of biotherapeutics [62].

The first recombinant protein expressed in *S. cerevisiae* was human interferon, followed by the production of the first genetically engineered vaccine – hepatitis B surface antigen (HBAg). HBAg has been extracted from yeast cells and sold under various names. The greatest value of *S. cerevisiae* passes through the production of human insulin, presumably; its production covers nearly half the insulin needed by the diabetic patients worldwide. In addition, the first authorized product for human therapeutics is a commercially available recombinant human albumin, Recombumin® (approved for the production of childhood vaccines for rubella, measles, and mumps) [63]. (see Table 2 in Appendix.)

1.5.3

Stress Responses in Yeast

All organisms are exposed to a variety of stressful conditions [64], including heat shock, osmotic stress, glucose starvation, exposure to metals (e.g. cobalt) or metalloids like arsenic, or to inhibitors of energy metabolism (lonidamine and levamisole, see Ref. [65]). All of these stresses disrupt the cellular homeostasis and integrity. Thus, cells have evolved adaptive responses to protect and stabilize proteins until more favorable conditions are achieved for cell proliferation. In particular, many metals cause oxidative stress in cells, interfere with protein function and activity, and/or impair DNA repair mechanisms (either directly or indirectly). Upon metal exposure, cells arrest cell cycle progression, alter gene expression and metabolism, and adjust transport processes to ensure cellular and genetic integrity [66].

The simplest eukaryotic organism *S. cerevisiae* has proven, over the years, to be an excellent experimental model to dissect the transcriptional mechanisms involved in the maintenance of homeostasis upon exposure to unfavourable and stress conditions (for oxidative stress, cf. Ref. [67]). The hallmarks of stress signals common to all living cells (from bacteria to man) include damaged cellular proteins, repression of the synthesis of house-keeping genes and induction of the heat shock proteins. Heat shock factors (HSFs), that are well conserved from bacteria to man, were first discovered by researchers while they were studying the puffing pattern of *Drosophila* chromosomes [68]. Despite their requirement for the damage control, namely in cell wall remodeling [69], they are also present during normal conditions, known as molecular chaperones, which assist the correct folding of the proteins in the cell. Furthermore, similar to mammalian HSF1, yeast HSF is activated by multiple stresses, including heat shock, oxidative stress, and carbon source starvation [70]. Recent studies have also provided new clues about the role of the heat shock response in the complex discover of potential anticancer drugs that allow the control of protein homeostasis [71].

In yeast cells (and in other eukaryotic cells), regulation of adaptive responses to metals largely occurs at the transcriptional level through a transcriptional reprogramming regulated by specific transcription factors (TFs), i.e., proteins that bridge sensors of cytotoxic oxidants and the transcriptional activation of particular sets of genes. In the yeast *S. cerevisiae*, there are three types of TFs: the HSFs (cited above), the partially redundant Msn2 and Msn4, and the basic leucine zipper (b-ZIP) transcription factors of Yeast AP-1-like (Yap) family [72] (details of Yap family will be discussed in the Section 1.5.3.1). Msn2 and Msn4 are two important players in stress responses, which bind to the ‘stress response elements’ (STREs) present in the promoter regions of their stress-responsive genes, following a variety of stresses. STRE was the first common *cis*-regulatory element to be identified in a large set of genes showed a response to almost of all of the stressful conditions, termed by general stress response. Both recognize the 5’-CCCT DNA sequence, present on the promoter genes of target genes [73]. The STRE was the first common *cis*-element identified in a set of genes

responding to an array of stress conditions. In the yeast genome, over 186 genes (c.a. 3% of the yeast genome) are potentially regulated in a STRE-dependent manner [74]. Msn2/Msn4 together with Hot1 are required for the induction of subsets of high osmolarity glycerol (HOG) pathway-dependent genes [75].

To summarize, the yeast *S. cerevisiae* displays a privileged position as a model organism for interpreting the mechanisms of response to chemical stresses with impact in environmental health, pharmacology, and biotechnology, and limiting the use of animal models [76].

1.5.3.1

AP-1 Transcription Factors in Yeast, and Yap Family

The best characterized AP-1 factor in *S. cerevisiae* is the Gcn4 protein which is involved in the expression of more than 500 genes [77]. In functional terms, Gcn4 is remarkably similar to the oncoproteins Jun and Fos; Gcn4, Jun, and Fos have the same DNA binding specificity, and they are functionally interchangeable for transcriptional activation from AP-1 sites in yeast cells and mammals. Moreover, Gcn4 resembles mammalian AP-1 factors in mediating a protective response against UV which involves the Ras signal transduction pathway, and a translational control mechanism that leads to increased AP-1 transcriptional activity.

Since the release of the yeast genome sequence, Rodrigues-Pousada C. and other scientists have been involved in the functional analysis of the yeast specific AP-1-like activator proteins (Yaps). The Yap family consists of eight transcription factors, named **Yap1** to **Yap8**, belonging to the basic region leucine zipper (bZIP) super-family. Indeed, the bZIP proteins form one of the largest families of transcription factors in eukaryotic cells. Despite relatively high homology between amino acid sequences of bZIP motifs, these proteins recognize diverse DNA sequences [78, 79].

The bZIP transcription factors possess a great homology within the bZIP domain, and specifically Yap1 and Yap2 display a great C-terminal homology (see Figure 1.2). With the respect to structural similarities, indeed Yap1 and Yap2 share the highest similarities in their structures, and to a

lesser extent to Yap3; Yap4 is most homologous to Yap6; so is Yap5 and Yap7; whereas Yap8 is the most divergent family member. Although they display relatively high homology between the amino acid sequences of their bZIP motifs, these proteins recognize a diversity of DNA sequences [78]. They specifically bind to the Yap recognition element (YRE), and they are implicated in various stresses although with a moderate interplay between the transcriptional regulators [79, 80]. Despite homologues for Yap1 exist in other eukaryotes, none has been described for the remaining family members [81].

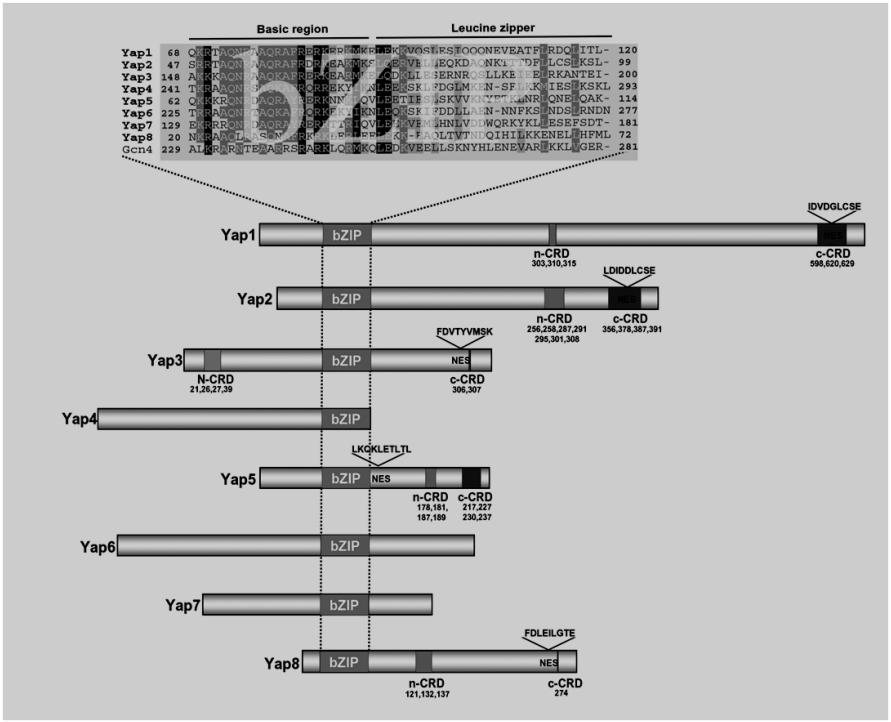

Figure 1.2 Structural features of eight Yap family members of *S. cerevisiae*. Sequence alignment of Yap basic region and leucine zipper (bZIP) with the true yeast AP-1 protein, Gcn4. Representation of the entire Yaps showing the positioning of cysteine-rich domains (n-CRD and c-CRD), and nuclear export signal (NES). (After Ref [72], adapted.)

Recently, Amaral *et al.* [82] gave new insights into the DNA-binding region of Yap8 transcription factor by demonstrating that Ser29, Leu26, and

Asn31 of the basic region are relevant to Yap8 specificity, with the latter two amino acid residues displaying importance for the stability of the Yap8-DNA-complex. The three amino acid residues at sites 29, 26 and 31 are of highly conserved positions in the other Yap family of transcriptional regulators and Pap1 (of fission yeast). Therefore, a homology model of the complex Yap8bZIP-DNA was built based on the 2.0Å resolution crystal structure of the bZIP motif of Pap1, an AP-1-like transcription factor of fission yeast *Schizosaccharomyces pombe* (view the NDB ID associated with the structure: PD0180, and PDB ID: 1GD2).

The general picture of functions of Yap members, emerging over the years, is that Yap1 has a central regulatory role of different stress responses, being particularly important for the adaptive response to oxidative stress [83, 84] (for more details, see next Section), and the mediator of stress-induced transcriptional activation of ABC protein genes (together with Yap2) [85]. Yap2 (Cad1) regulates the cadmium stress response, as well as it confers resistance to 1,10-phenantroline, cerulin, and cycloheximide [80, 86], and is involved in pleiotropic drug resistance (together with Yap1) [79]. Yap4 and Yap6 mediate the response to osmotic shock [87]; *YAP4* expression is also induced under pro-oxidant conditions [88] regulated by Yap1 and Msn2. Yap5 is involved in remodeling gene expression in response to iron bioavailability [89]. Yap8 (together with Yap1) responds to arsenate and arsenite compounds [90, 91] (see Section 1.6.1). With respect to Yap3 and Yap7, their functions have not been described so far (Yap7 has a paralog, Yap5, which arose from the whole genome duplication) [72, 79, 92]. Members of the Yap family carry out distinct biological functions, however, in some cases overlapping. (e.g., example, mutations in the *YAP4* gene affect chromosome stability suppressing the cold-sensitive phenotype of the *yap1* mutant, see Ref. [79]).

As AP-1-like factors, the Yap1, Yap2 and Yap8 members of Yap family are controlled by similar common redox mechanisms, ‘shuttling’ between the cytoplasm and the nucleus under physiological conditions (Figure 1.3, Ref. [93]. The exact mechanism of nuclear export lies on the binding of Crm1 (Xpo1) to Yaps nuclear export signal (NES) domain, which occurs only under

physiological growth [94]. Nonetheless, when cells are treated with various oxidants [95, 96] or with arsenic [97], Yap1 and Yap8 accumulate in the nucleus, respectively.

The molecular mechanisms responsible for Yap1 nuclear accumulation in response to oxidative stress [96] are well understood: the association of Crm1 with the CRD of Yap1 is inhibited by the formation of intramolecular disulfide linkages. In the case of H_2O_2, these linkages involve cysteine residues in the N- and C-terminal CRDs, whereas treatment with the thiol oxidant diamide induces disulfide linkages involving only the C-terminal CRD [96]. With respect to Yap8, arsenic is known to directly modify the sulphydril groups of reactive protein cysteines located in the *c*CRD (C-terminal cysteine responsive domain), and masking the NES preventing recognition by the Crm1 export factor. (In addition, Yap1 accumulates in the nucleus in response to carbon stress in *S. cerevisiae* [98].)

With respect to Yap8 protein, Cys132, Cys137, and Cys274 are crucial for its Crm1-dependent nuclear export, and arsenite and arsenate compounds directly bind to and modify these cysteine residues and hence the NES [97]. (Attention will be drawn to additional aspects of Yap8 post-translational regulation in the Chapters 3 and 4.)

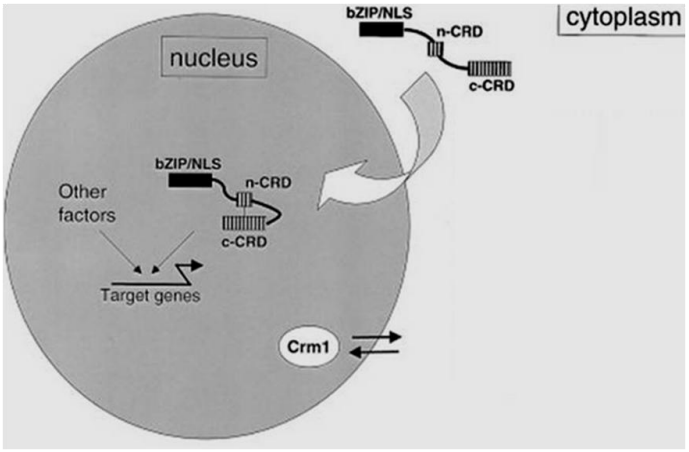

Figure 1.3 Common redox control of AP-1-like factors in yeast and beyond. Under non-stressed conditions, yAP-1 factors are continually imported into the nucleus, and are immediately exported by the nuclear export factor Crm1. [After Ref. [93] (adapted).]

On the other hand, Yap4 and Yap6 are known nuclear-resident proteins, being their localization unaffected by environmental changes [99]; whereas Yap5 was suggested to constitutively localize to the nucleus [100].

To date, such a characterization in detail of AP-1-like factors has been described only in *Saccharomyces cerevisiae*, however, a family of Yap proteins also exists in *Candida glabrata* [79].

1.5.3.1.1

Transcription Factor Yap1 and Multidrug Resistance

Yap1, the first member of the Yap family to be described [101], as well as the best studied one, was initially identified by its ability to bind to and activate the SV-40 AP-1 recognition element (ARE: TGACTAA).

The key function of Yap1 transcription factor consists of the activation of the antioxidant machinery preventing protein damage [91] (see also Section 1.6.1). Yap1 regulates yeast response to H_2O_2, as well as to several unrelated chemicals and metals or metalloids. Activation by H_2O_2 involves Yap1 Cys303-Cys598 intra-molecular disulfide bond formation directed by the H_2O_2 sensor Orp1/Gpx3. The electrophile N-ethylmaleimide (NEM) activates Yap1 through covalent modification of Yap1 C-terminal Cys598, Cys620, and Cys629, in an Orp1 and Yap1-oxidation-independent manner. Also, menadione (a superoxide anion generator and a highly reactive electrophile) operates both modes of Yap1 activation. The reactivity of Yap1 C-terminal domain towards other electrophiles (4-hydroxynonenal, iodoacetamide) and metals (cadmium, selenium) is a common mechanism for sensing thiol reactive chemicals via thiol chemical modification. Within Yap1, two redox centers are relevant for H_2O_2 and thiol-reactive chemical signaling: one triggered by reactive oxygen species (ROS) (e.g., hydroperoxides and the superoxide anion); and the other triggered by chemicals with thiol reactivity (e.g., electrophiles and divalent heavy metals cations) [102].

The Yap1 transcription factor has a unique role in the metabolic stress response [103], and the acquisition of stress tolerance as *YAP1* gene overexpression renders yeast cells resistant to multiple metabolic inhibitors.

It has to be mentioned that Yap1 was found to be a major determinant of tolerance to polyamine toxicity involving the drug: H+ antiporter Qdr3 and the transcription factor Gcn4 as well; Yap1 is also accumulated in the nucleus of the cells exposed to spermidine-induced stress [104].

Finally, the critical role of Yap1 in cobalt stress response [105] (via the regulation of the high affinity phosphate transporter Pho84), as well as cadmium stress response [106] (involving repression of the low affinity iron transporter gene *FET4*) has been reported.

1.6 As Uptake, Transport, and Efflux in Yeast and Mammals

Arsenic is highly toxic and can only permeate cells through the transporters that evolved for accumulation of essential ions and nutrients using the molecular mimicry. The sequence of events during arsenic uptake, transport and efflux in yeast and mammals is schematized in Figure 1.4.

As mentioned in Section 1.2, As(V) behaves as a chemical analogue of P_i. Thus, it is imported by phosphate transporters (Pho) (the counterpart in mammals is the high-affinity phosphate transporter NaPiIIb). Once As(V) is imported, the first step of its removal from the cytoplasm consists of its two-electron reduction to As(III) with glutathione serving as a source of reducing potential. As(V) is reduced to As(III) by the arsenate reductase Acr2/Arr2 (in yeast) or CDC25 phosphatases (in mammals). The inorganic trivalent As(III) is transported into the mammalian cells via multiple pathways: the aquaglyceroporins (AQPs), the glucose permeases (GLUTs), or the organic anion transporting polypeptides (OATPs). As(III) is imported in the absence of glucose by the hexose transporters (hxt), but it is also transported by Fps1- which can import and export (bidirectional). The main difference resides on the fact that As(III) is methylated in mammals, but not in yeast. The export of As(III) is made by the yeast As(III)-efflux protein Acr3/Arr3, or MRP1 in mammals. As(III) is also transported into the vacuole as a glutathione-conjugated substrate by the Ycf1 ABC-transporter. The ABC transporters from the ABCB (MDR1/P-gp), and ABCC (MRP1 and MRP2)

subfamilies are the major pathways of As(III) extrusion. Both MRP1 and MRP2 are able to transport inorganic and monomethylated forms of As(III) conjugated with glutathione. In addition, MRP2 mediates efflux of seleno-bis(S-glutathionyl) arsenium ion. The exact form of As(III) recognized by MDR1/P-gp is uncertain, but it is not glutathione-S-conjugate [107].

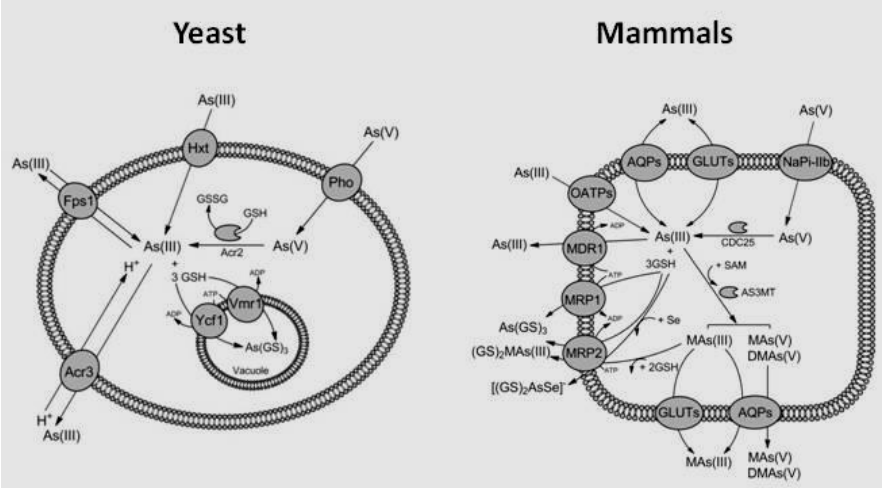

Figure 1.4 Arsenic transport in *S. cerevisiae*, and mammals. [After Ref. [107] (adapted).]

In addition to eukaryotes, prokaryotic cells have also developed mechanisms to take up and detoxify arsenic compounds [7], being the oxidation state essential for the type of transporter used in this process.

1.6.1
Yap8, Transcriptional Regulation of *ACR* genes, and Contribution of Yap1 in the Response to Arsenic

In *S. cerevisiae,* the bZIP transcription factor Yap8 (also known as Arr1/Acr1) of the Yap family is the major player in arsenic stress responses [72]. It regulates transcriptional activation of the arsenicals resistance cluster, which consists of the *ACR2/ARR2* and *ACR*3/*ARR3* genes. As we have seen in previous Section 1.6, the arsenate reductase Acr2, and the plasma membrane arsenite efflux transporter Acr3 (respectively encoded by

the above genes) represent the main pathway of arsenic detoxification. Since Yap8 transcription factor regulates As(III) detoxification, the *yap8* mutant strain consequently absorbs increased levels of As(III) [92].

The Yap1 transcription factor also regulates the *YCF1* gene expression. In addition, Yap1 overproduction restores arsenite resistance to the ABC transporter deficient mutant *ycf1* by activating *ACR3* expression [108].

Most importantly, Yap1 promotes cellular antioxidant defenses through the scavenging of ROS that are generated as a side effect of exposure to arsenic [91]. Yap1 and its target genes *TRX2*, *GSH1* and *SOD1* are highly induced under these conditions indicating that activation of Yap1 is essential to favour cell adaptation by preventing the ROS accumulation. Genome transcriptional profiling of the wild-type strain stressed with arsenate reveals that genes of the functional categories related with sulphur and methionine metabolism, and with the maintenance of the cell redox homeostasis are highly activated. As a consequence, the *yap1yap8* double mutant exhibits a more sensitive phenotype to arsenic stress than single *yap1* or *yap8* mutant. Nevertheless, *YAP8* gene overexpression does not alleviate the *yap1* mutant sensitivity to arsenic, as well as *YAP1* gene overexpression is not able to rescue the sensitive phenotype of the *yap8* mutant either [72].

The work from Menezes *et al.* [91] has revealed increased levels of intracellular oxidation and lipid peroxidation in the single *yap1* and *yap8*, and the double *yap1yap8* mutants; changes in protein carbonylation and the redox equilibrium were also discussed, mostly in the *YAP1* deletion strain lacking the antioxidant defenses causing protein damage.

1.6.2
As Stress, and Other Affected Signaling Pathways in Yeasts

Yeast responds to arsenic though many mechanisms, such as adjusting sulfur metabolism to enhance GSH biosynthesis; enhancing arsenic cellular export through intra-cellular chelation; sequestering arsenic in the vacuole; regulating cell cycle progression; as well as enhancing proteasomal degradation of misfolded and damaged proteins [109].

Although Yap8 and Yap1 appear as the key responsive transcription factors in controlling arsenic detoxification and redox homeostasis, respectively, additional regulators also contribute to cell tolerance. The transcription factor Rpn4 strongly mediates the yeast adaptation to arsenic stress as revealed by expressing profiling [90]. In addition, the transcriptional regulator Met4 (together with Yap1) controls the assimilation of sulphur into glutathione biosynthesis in order to compensate its continuously oxidation during the detoxification process [110]. The mitogen-activated protein kinase Hog1 is also implicated in arsenic resistance, being required to full activation of *ACR3* and *YCF1*, and to control the As(III) uptake through phosphorylation of the aquaglyceroporin Fps1 [111, 112].

Furthermore, it is now well understood that multiple pathways involved in mitochondrial biogenesis protect *S. cerevisiae* from arsenic-induced toxicity, as the case of the RTG (retrograde) genes involved in mitochondria-to-nucleus signaling, and the targets of the multifunctional transcription factor Abf1 [113].

Arsenic toxicity in yeast can also be determined through the balance between chronic activation of general stress factors Msn2/Msn4 in combination with lowered/inhibited TORC1 kinase activity [114].

Recent analyses from the laboratory of Rodrigues-Pousada C. [115] have shown that arsenic disrupts the cellular iron homeostasis in yeast by inducing conditions of iron deficiency as consequence of a destabilization of the high-affinity iron uptake system (this perturbation was also observed in mammals).

1.7
Ca^{2+}-Signaling in Eukaryotes

Calcium ions (Ca^{2+}) are ubiquitous, universal, and versatile intracellular messengers [116]. An increase in the cytosolic Ca^{2+} concentration activates many proteins, including the eukaryotic calmodulin (CaM), and Ca^{2+}/CaM-dependent protein phosphatase calcineurin (CaN or CN) [117].

In yeast, many downstream transcriptional and translational events are known to be controlled by the Ca^{2+}-mediated activation of CaM. In yeast, as in other organisms, CaM is essential for life [118]. The phosphatase CaN is conserved from yeast to humans (except in plants) [119], and many target proteins of CaN have been identified. However, the most prominent and best-investigated targets are: (1) the nuclear factor of activated T cells (NFAT), which regulates, for example, the cardiac hypertrophic response in mammals [120]; and (2) the yeast zinc-finger transcription factor (Crz1) that activates the transcription of genes whose products promote cell survival during environmental stress conditions [121].

It has been shown that the functions of the signaling of calcineurin-Crz1 (see next section)- ranging from ion homeostasis through cell wall biogenesis to the building of filamentous structures, are conserved in different organisms. Moreover, frequency-modulated gene expression through Crz1 was discovered as a striking mechanism by which the cell can coordinate its response to a signal [119].

The new findings concerning **CaN-Crz1 signaling** in yeast exposed to **arsenic stress** will be discussed in Chapter 2.

1.7.1
Ca^{2+}-Signaling and Transport in Yeast

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 *S. cerevisiae* cells exposed to diverse environmental cues [122]. Ca^{2+} enters the cytoplasm across the plasma membrane complex Cch1/Mid1 or the vacuolar transient receptor potential-like channel Yvc1 (Figure 1.5). Once in the cytosol, Ca^{2+} is sensed through its receptor, calmodulin; and once activated, calmodulin transduces the Ca^{2+}-signaling to calcineurin. Dephosphorylation by calcineurin results in nuclear translocation of Crz1/Tcn1, and transcriptional activation of its target genes. Then, Ca^{2+} homeostasis can be restored via Pmc1 (a Ca^{2+} ATPase), as well as by the vacuolar H^{+}/Ca^{2+} exchanger Vcx1. Alternatively, Ca^{2+} is directed to the

secretory pathway in the ER and Golgi apparatus by Pmr1 (a plasma membrane ATPase related) [123].

Crz1 phosphorylation responds to calcium, and regulate more than 100 different targets [124], majorly involved in: (i) cell wall, and lipid biosynthesis, (ii) ion, and small molecule transport, and (iii) vesicle trafficking [125].

CaM also participates in the Ca^{2+}-dependent stress response pathways through activation of the CaM kinases-Cmk1, and Cmk2. Although their physiological roles are not well understood (like CaN), these enzymes seem to participate in a number of stress responses, sometimes acting additively with CaN to promote survival, as it occurs during prolonged incubation with mating pheromone, thermo-tolerance acquisition, and cellular growth in presence of weak organic acids [123]. Regarding CaN, it carries out different functions in yeast, regulating a stress-activated transcriptional pathway, Ca^{2+} homeostasis, and the G2-M transition of the cell cycle [126]. Also, there are evidences suggesting additional, and non-protective roles for CaN, that can result in cell damage. For example, it was already discussed that the yeast cells become more sensitive to oxidative stress, in the presence of Ca^{2+}, probably due to the CaN-dependent dephosphorylation of Yap1, which leads to Yap1 degradation, and consequently damaged antioxidant gene expression [127].

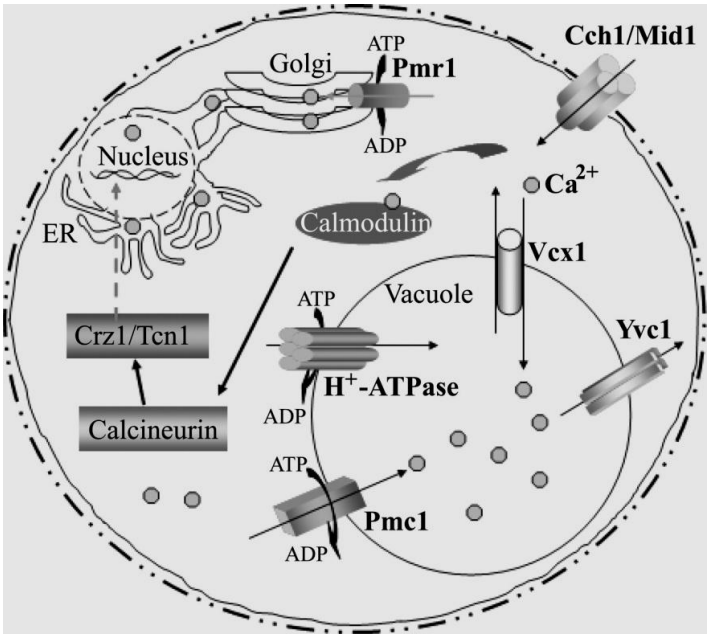

Figure 1.5 Ca^{2+} transport in *S. cerevisiae*. (After Ref. [122].)

1.8
Protein Modifications by Ub, and Programmed Protein Degradation

1.8.1
Ubiquitin-Proteasome System

The story of protein degradation started with an observation by Aaron Ciechanover in Avram Hershko's laboratory (in the late 1970s), followed by studies on the role of ATP in this process- mainly contributed by the laboratory of Irwin Rose [128].

The Ubiquitin-Proteasome System (UPS) is a central proteolytic pathway present in every eukaryotic cells, which regulates vital physiological events. Protein quality control, protein trafficking, cell division and differentiation, and signal transduction- are all controlled processes (to some extent) by the UPS [129].

Protein degradation is a central regulatory mechanism controlling cell processes like gene expression and cell cycle progression, and is also

critical in the protein quality control process (as mentioned above)- which importantly prevents accumulation of aberrant proteins. For those proteins in which refolding is no longer possible, the cells count on proteolytic systems to eliminate unstable proteins, and to recycle their amino acids [130]. Furthermore, also the properly folded proteins- whose levels must be down-regulated, such as cell cycle regulators [131], or transcription factors [132] represent substrates of the proteasome. An important aspect is that, regulation of transcription controls cell development, differentiation, and maintains homeostasis- which supports the importance of the proteasome.

The Ubiquitin-Proteasome Pathway (UPP) was initially recognized as a selective degradation pathway [133], and a large number of studies were devoted to characterize protein ubiquitination (or ubiquitylation) as a signal for protein degradation. However, it is increasingly realized that Ubiquitin conjugation to proteins can be used for many other purposes. Furthermore, in every virtually eukaryotic cells, there are many Ubiquitin (Ub) and Ubiquitin-like proteins (Ulps) [134] that control the activities of many proteins. (For Ulps, see Section 1.8.2.).

A large portion of the proteasomes localizes to the nucleus, therefore protein degradation can occur in the nucleus; and proteasome-dependent protein degradation of nuclear proteins has proven to be instrumental in nuclear function [135]. Degradation processes both in the cytoplasm and the nucleus can occur through two pathways: (1) an N terminus-dependent pathway [136], (2) and a lysine-dependent pathway. These pathways are respectively characterized by the site of initial ubiquitination of the protein- the N terminus, or an internal lysine [137]. Nevertheless, a study has been characterized that the lysine-dependent pathway is the more active pathway within the cytoplasm, whereas in the nucleus the two pathways are both active in protein degradation.[138].

Finally, it has to be emphasized that yeast cells posses the vacuole (analog of mammalian lysosome), required for the autophagic vacuolar (lysosomal) system, and the proteasome, also a large multi-catalytic complex (like in mammals) for the polyubiquitinated proteins degradation [139].

1.8.1.1 The Ubiquitination Cascade

Ubiquitination of substrates recognized by the 26S proteasome, a multisubunit enzyme complex (molecular mass ~ 2000 kDa), is catalyzed by three classes of enzymes: Ub-Activating enzymes (E1), Ub-Conjugating (E2) or Ubc enzymes, and Ub-Ligating (E3) enzymes (Figure 1.6, Ref. [140]). Ubiquitin-binding domains (UBDs) are a collection of modular protein domains that bind to the Ub, in a non-covalent fashion. The preferences of UBDs for ubiquitin chains, of specific length and linkage (see once again Figure 1.6), originate from multimeric interactions, and UBDs synergistically bind multiple Ub molecules, and from contacts with regions that link Ub molecules into a polymer. It was reported that the sequence context of UBDs, and the conformational changes that follow their binding to Ub greatly contribute to ubiquitin signaling *in vivo* [140].

The selectivity of the UPS for a particular substrate also relies on the interaction between a E2 (of which a cell contains relatively few), and a E3 (of which there are possibly hundreds) [141]. Also, it is well understood that the lysine residue- by which the Ub chains are linked together, is an important signal that determines the fate of the substrate; Lys48 and Lys63 are two of the key sites for polyubiquitin conjugation (Figure 1.6), and these linkages are major signals that target polyubiquitinated proteins for destruction (although they are differentially processed by the proteasome) [142]. Other Lys-linked polyubiquitination (or polyubiquitylation) types include Lys6, Lys11, Lys27, Lys29, and Lys23 (Figure 1.6). In contrast, monoubiquitination (or monoubiquitylation) is the substrate modification with a single Ub molecule on a single Lys residue (Figure 1.6); monoubiquitination also has signaling roles (e.g., DNA repair) that are distinct from those of polyubiquitination [143].

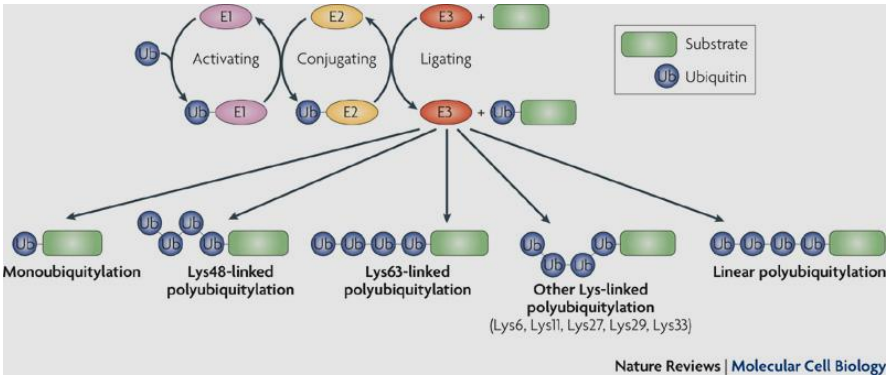

Figure 1.6 The activity of three enzymes (E2, E2, and E3) required for ubiquitylation. (After Ref.[140]).

Like Ub-Conjugating enzymes, there are multiple classes of Ub-Ligating enzymes. These classes include the HECT (homologous to E6-associated protein C-terminus)-type, RING (really interesting new gene)-type, and **U-box** (a modified RING motif without the full complement of Zn^{2+}-binding ligands)-type, all named after the domain in the E3 enzyme that interacts with the E2 enzyme [141]. In addition, E3s can be (i) simple- one protein chain recruits and interacts with both the E2 and the substrate, or (ii) complex- multiple proteins are required to facilitate the ligase function. E3s can facilitate their function either through one protein chain, or as part of a protein complex. Some E3 ligases contain a substrate recruitment domain, as well as a respective E2 binding domain, and are functional as a single entity [144]. However, in other cases, additional proteins are necessary to form a complex where one subunit will recruit the substrate, and another will bind the E2. The primary example of complex E3s is the SCF (Skp1, Cullin, F-box) family [145]– discovered through genetic requirements for cell cycle progression in budding yeast, which requires at least four different protein subunits for ligase function.

The majority of Ub-Ligating enzymes fall into RING-type family [146]. The RING domains comprise approximately 70 amino acid residues, and are characterized by conserved Zn^{2+}-chelating sites that are necessary for domain stabilization. The **U-box domain** is structurally similar to the RING domain, but does not contain Zn^{2+}-binding sites; instead, hydrogen bonding

or salt bridges provide structural stabilization to the 70 residues of the domain [147]. Although HECT and RING domain E3 ligases have been studied extensively, little is known about the **U-box proteins**. To date, the U-box family of E3 enzymes is the smallest class of ligases (with relatively few E3s have been characterized). Nevertheless, it is notable that the U-box containing E3 ligases have been shown to play diverse roles in RNA splicing [148], collaborating with molecular chaperones [149], as well as the ER-associated protein degradation [150].

It has to be mentioned that the two E2 enzymes- Ubc4 and Ubc5, were shown to be extremely necessary for most of the Ub-dependent protein degradation processes in yeast [151].

In some cases, selective protein degradation by the UPS requires multiubiquitylation, which can subsequently require the additional activity of certain **Ub-chain elongation factors- the E4s,** that indeed represent a distinct and novel class of enzymes [152]. Yeast **Ufd2 (ubiquitin fusion degradation protein-2)**, was the first E4 enzyme to be described [153]- that binds to the Ub moieties of preformed conjugates, and catalyses Ub-chain elongation, and in association with E1, E2, and E3 enzymes. Ufd2 belongs to the UFD (Ub fusion degradation) pathway in *S. cerevisiae* (like Ufd1, Ufd3, Ufd4, and Ufd5) [154]. Together with an orchestra of Ub-binding factors, the **E4-Ufd2 enzyme** co-operates with in an escort pathway to transfer and deliver polyubiquitinated substrates to the 26S proteasome [155]. Surprisingly, Ufd2 can have other functions beyond degradation; one example is the Ufd2-mediated stabilization of the Yap8 transcription factor in the arsenic stress response of *S. cerevisiae*. These findings will be discussed in the Chapter 3.

Finally, post-translational modifications modulate the degradation of aggregates-prone proteins by the UPP. It became clear that ubiquitin generally determines whether a protein is degraded via the UPP. Interestingly, phosphorylation appears to precede and regulate additional posttranslational modifications, including ubiquitination, **SUMOylation**, and acetylation, facilitating the clearance by the proteasome, as well as by the lysosome [156]. (For SUMO, cf. Section 1.8.2.1.)

1.8.2
Yeast Ubiquitin-Like Proteins (Ulps), and Cognate factors

1.8.2.1
SUMO

Several ubiquitin-like protein (Ulp) modifiers have been identified for yeast, vertebrates, and plants. One of the most challenging classes are the 'small ubiquitin-like modifier' (**SUMO**) proteins- coupled to numerous targets in the cell since they can modulate their localization, stability, or activity, as well as they can affect protein-protein interactions [157].

SUMOylation (or sumoylation) [158] is a dynamic, and a reversible process. It is similar to ubiquitination, yet biochemically distinct. Remarkably, SUMOylation can also be a signal for polyubiquitylation and proteasomal degradation [159].

There are at least three SUMO isoforms (SUMO1,-2,-3) in mammalian cells but only one in *S. cerevisiae* (Smt3). In yeast, SUMO is a small protein of around 100 amino acids in length encoded by *SMT3* gene. SUMO conjugation requires the E1 heterodimer (Uba2/Aos1), and E2 (Ubc9) enzymes (see Figure 1.6), as well as E3 proteins (Siz1 or Siz2 in yeast) to form a covalent bond with target proteins [158]. DeSUMOylation– the cleavage of SUMO form from its target proteins is mediated by **SUMO specific proteases**, of which **Ulp1** and **Ulp2** have been identified in yeast [160, 161] (for more details, see Chapter 4).

Major targets of SUMO in *S. cerevisiae* are the septins Cdc3, Cdc1, and Sep7, which form a 10-nm filamentous ring that encircles the yeast bud neck [162]. SUMO interferes with processes such as transcription, nuclear-cytoplasmic transport, chromatin integrity and dynamics, and cell cycle control. All these events occur via alterations of the molecular interactions patterns of SUMOylated proteins [138, 163].

Preliminary studies on post-translational regulatory mechanisms by SUMO were undertaken in *S. cerevisiae*, in order to augment our knowledge on the Yap8 regulation (crucial for arsenic detoxification, as referred in Section 1.6.1). (See Chapter 4.)

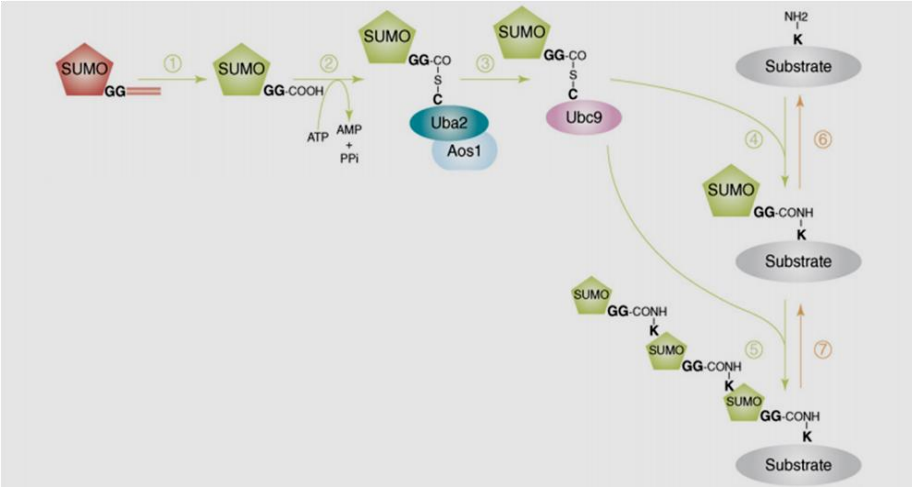

Figure 1.7 SUMOylation and deSUMOylation pathway, and its enzymes. The sequential reactions involved in SUMOylation (steps 1–5, in green) and deSUMOylation (steps 6 and 7, in orange) are represented. Note that SUMO-processing (step 1), SUMO deconjugation (step 6) and SUMO chain-editing (step 7) activities are carried out by a family of enzymes termed SUMO-proteases. (Adapted from Ref. [164].)

1.8.2.2

Other Proteins Related to Ub

In yeast, Rub1 (in other eukaryotes also called NEDD8) displays a high homology to Ub, and is linked to all members of cullin (Cul)-family proteins through an enzymatic cascade analogous to ubiquitination [165].

Furthermore, other regulator proteins of Ub-related processes have evolved domains with Ub-like-three-dimensional structures mimicking certain aspects of monoubiquitination (referred in Section 1.8.1.1). Ub-binding and Ub-mimicking motifs have been uncovered in members of many distinct protein families, and link them to Ub-related mechanisms [166].

1.9
Aims of the Thesis

Despite a decade of work on the cellular processes underlying yeast adaptation to arsenic, and regulatory mechanisms of key transcription factor Yap8, the following fundamental questions on arsenic's mechanism of action were still open: (1) How do cells respond to arsenic challenge that perturb homeostasis?, (2) What are its cellular binding partners or targets?, (3) What are the post-translational modifications of Yap8, particularly upon arsenic stress?, (4) Is the stability/activity of the Yap8 protein also mediated by the SUMO modifier?

Aware of these unresolved fundamental questions and the existing extensive but incomplete data on arsenic's action at the molecular level, as well as intriguing aspects of the arsenic-mediated Yap8 stability, our aims were to:

- Foremost, undertake a DNA microarray approach in *S. cerevisiae* to compare expression profiles of normal cells and cells treated with arsenic in order to
- complement the diverse existing *S. cerevisiae* data, and provide a more consolidated view on the arsenic drug-targets in a defined cellular setting, within the long-term interest to
- identify signatures of molecular endpoints useful to address unresolved resistance problems to arsenic therapy in APL [167].

In addition, yeast two-hybrid system was a strategy for identification of the Ufd2 enzyme as a novel interactor of Yap8. Herein, we have focused on the immediate role of Ufd2 enzyme towards Yap8 stability, which was thought to be intimately associated with a transcriptional activation of *ACR* genes, therefore important for an adaptive stress response to arsenic. We have also performed biochemical and genetic assays using wild-type cells and mutants in the 'core' components upstream and downstream Ufd2 in order to enable the development of an interaction model, and to set the foundation for a comprehensive analysis of Yap8 regulation at the post-translational level. Finally, we also identified candidate lysines in Yap8 for

ubiquitination and SUMOylation, and discussed the effects of site-directed mutagenesis of these lysines on the protein stability and activity necessary for intracellular arsenic efflux.

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2

Perturbation of Ca^{2+} Homeostasis, and Activation of the Calcineurin/Crz1 Pathway by As

2.1
Preface

Calcium has been implicated in the regulation of a multitude of processes in biochemistry and biology. Cells must tightly regulate Ca^{2+} homeostasis to avoid pathological perturbations and cell death [1]. As described hereinafter, arsenic (As) disrupts the cellular Ca^{2+} homeostasis by inducing a transient increase in $[Ca^{2+}]_{cyt}$, in both the wild type and *yap1* mutant yeast strains, thereby activating a cascade that results in adaptation to arsenic stress and restores homeostasis. Calcineurin (CaN) is a highly conserved Ca^{2+}/calmodulin (CaM)-dependent phosphatase critical for Ca^{2+}-sensing and signal transduction to the transcriptional machinery ultimately leading to cell adaptive responses [2].

Our data obtained from yeast model system may help to understand the cellular and molecular mechanisms behind the action of As drug with effectiveness of treating acute promyelocytic leukaemia (APL) patients [3].

2.2
Journal Article

This Chapter consists of data published in the following journal article:

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2.2.1
Author's Contribution

I had a major participation in this study in planning and execution of experiments [Growth assays; *In vivo* monitoring of the Ca^{2+} pulse induced by arsenic stress; Fluorescence microscopy; β-galactosidase assays (cf. Methods Section of the article); and all yeast transformations and routine plasmid amplification purposes], data analysis, and paper writing.

2.3
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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 in therapeutics brings many benefits to human health, so it is therefore essential to discover the molecular mechanisms underlying arsenic stress responses in eukaryotic cells. Aiming to determine the contribution of Ca^{2+}-signaling pathways to arsenic stress responses, we took advantage of the use of *Saccharomyces cerevisiae* as a model organism. Here we show that Ca^{2+} enhances the 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+} signaling 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 exogenous Ca^{2+} sources, strongly supporting the notion that Ca^{2+} homeostasis is disrupted by arsenic stress. In response to an arsenite-induced increase of Ca^{2+} in the cytosol, Crz1 is dephosphorylated and 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 in the induction of the endogenous genes *PMR1, PMC1* and *GSC2*. Taken together, these data establish that activation of Ca^{2+} signaling pathways and the downstream

activation of the Crz1 transcription factor contribute to arsenic tolerance in the eukaryotic model organism *S. cerevisiae*.

INTRODUCTION

Arsenic (As) is a metalloid naturally occurring in the Earth's crust. Highly toxic arsenic ion species are released into the environment primarily through leaching from geological formations into aquifers, although anthropogenic sources also account for environmental contamination. Although it is used in 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 has 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, has been shown to be essential to regulate the induction of reactive oxygen species (ROS) homeostasis machinery in cells exposed to arsenic stress (Menezes *et al.*, 2004, 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-butyl hydroperoxide (tBOOH) 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 by either 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 & 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 endoplasmic reticulum (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 of a catalytic and a regulatory subunit, encoded by CNA1 and CNB1, respectively, the latter being essential for enzyme activity and induction by CaM (Cyert *et al.*, 1991; Cyert & Thorner, 1992; Cyert, 2003). CaN carries out multiple functions in yeast, in particular the regulation of the zinc-finger transcription factor Crz1. Like the mammalian orthologue 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 suggest that arsenic stress affects the expression of genes involved in Ca^{2+} signaling pathways, and these findings prompted us to investigate the role of the 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 the cell wall, Gsc2.

METHODS

Bacterial and yeast strains, plasmids and growth conditions. The Escherichia coli strain XL1-Blue {recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn 10 (Tetr)]} (Stratagene, Agilent Technologies) was used as the host for routine plasmid amplification purposes. Outgrowth was performed in super optimal broth with catabolite repression (SOC) medium at 37 °C. Plasmids were selected on LB plates supplemented with 100 µg ampicillin ml^{-1}.

A list of *S. cerevisiae* strains and oligonucleotide primers used in this work is presented in Tables 1 and S1, available with the online version of this paper, respectively. The microhomology PCR method (Güldener *et al.*, 1996) was used to generate the double mutant strains as follows: (1) *crz1cnb1*, deletion of the *CRZ1* coding region in the *cnb1* mutant; (2) *yap1crz1*, deletion of the *CRZ1* coding region in the *yap1* mutant; and (3) YAA3-*yap1*, deletion of the complete *YAP1* coding region in the YAA3 strain. The double mutant *yap1cbn1* was generated through the transformation of linearized plasmid pLF1 (encoding a YAP1 version in which the *HIS3* gene was inserted in the internal BamHI site of *YAP1*) in the *cnb1* strain. Gene disruptions were confirmed by PCR analysis of genomic DNA using upstream (A1) and downstream (A4) specific primers (Table S1). pEVP11-

Apoaequorin (Batiza *et al.*, 1996) was used to measure cytosolic free Ca^{2+} levels. Strains were grown in synthetic medium [SM; 0.67% (w/v) ammonium sulfate/yeast nitrogen base without amino acids, 2% (w/v) glucose, supplemented with 20 µg l-histidine-HCl monohydrate ml^{-1}, 60 µg leucine ml^{-1}, 20 µg uracil ml^{-1} and 20 µg l-methionine ml^{-1}]. Selective synthetic media were prepared omitting the amino acid corresponding to the auxotrophic marker. For solid media, agar was added to a final concentration of 2% (w/v). All spectrophotometric measurements of yeast biomass were performed using a polystyrene 45 mm path-length cuvette and the Bio-Rad SmartSpec 3000 spectrophotometer. Exponential growth phase cells were attained through the dilution of overnight cultures to an OD_{600} 0.1±0.01 in fresh media followed by incubation to OD_{600} 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 appropriate buffers and stored at −80 °C. Standard methods were used for genetic analysis, transformation and gene disruption procedures (Ausubel *et al.*, 1995).

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

Strain	Genotype	Source or reference
BY4742	*MATα his3 leu2 lys2 ura3*	EUROSCARF*
crz1	*MATα his3 leu2 lys2 ura3* *YNL027W*::*kanMX4*	EUROSCARF
cnb1	*MATα his3 leu2 lys2 ura3* *YKL190W*::*kanMX4*	EUROSCARF
crz1cnb1	*MATα his3 leu2 lys2 ura3* *YKL190W*::*kanMX4* *YNL027W*::*HIS3MX4*	This study
yap1	*MATα his3 leu2 lys2 ura3* *YML007W*::*kanMX4*	EUROSCARF
yap1crz1	*MATα his3 leu2 lys2 ura3* *YML007W*::*kanMX4* *YNL027W*::*HIS3MX4*	This study

Table 1. (*Continued*)

Strain	Genotype	Source or reference
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)**

* EUROpean *Saccharomyces Cerevisiae* ARchive for Functional analysis.

Growth assays. Phenotypic growth assays were carried out by spotting 5 µl of sequentially diluted cultures (approx. 5×10^3, 1×10^3, 5×10^2, 1×10^2, 5×10 and 1×10 cells) in SM containing up to 1.5 mM $NaAsO_2$ and/or 50 mM $CaCl_2$. Growth was recorded after 2 days of incubation at 30 °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 treated with 1.5 mM $NaAsO_2$. The cultures were incubated for 28 h at 30 °C with orbital agitation (200 r.p.m.) and the OD 600 was monitored at intervals of 2 h.

Microarray analysis. Total RNA was isolated as described elsewhere (Puig *et al.*, 2005) from exponentially growing BY4741 yeast cells that were either exposed or not to 2 mM sodium arsenate for 60 min; 50 µg total RNA was sent for labelling and hybridization to Affymetrix Yeast Genome S98 arrays. For further information about sample preparation, please see 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.

***In vivo* monitoring of the 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 plasmid pEVP11-Apoaequorin, kindly provided by Dr Patrick H. Masson. For the luminescence assays, stationary phase pre-cultures were diluted to OD_{600} 0.1±0.01 in fresh SM and grown to OD 600 1±0.1. To reconstitute functional aequorin, 50 µM native coelenterazine (Sigma; dissolved in methanol) was 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 washed three times with PBS (10 mM phosphate, pH 7.4, 138 mM NaCl, 2.7 mM KCl) to remove excess coelenterazine. The pellet was resuspended in SM and the cell suspension was transferred to a 96-well microplate. Before the induction of arsenic stress, the baseline luminescence was determined by 1 min of recording at 10 s intervals using a Perkin Elmer Victor 3 luminometer. $NaAsO_2$, to a final concentration of 1.5 mM, was injected into the samples and light emission was recorded for a further 9 min at 10 s intervals. The light emission is reported as relative luminescence units (RLU), expressed as counts per second (c.p.s.) per OD_{600} unit. 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 to the exponential growth phase, and were induced with 1.5 mM As(III). To specifically inhibit CaN function, the immunosuppressant cyclosporin A (CsA; Sigma) was used at 10 µg ml^{-1} final concentration 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 was immunoblotted as previously described (Menezes *et al.*, 2008). Crz1–GFP was detected using anti-GFP (Serrano *et al.*, 2011) and anti-rabbit (Santa Cruz) as primary and secondary antibodies, respectively. Pgk1 was used as the internal loading control. Protein detection was performed using the SuperSignal West Pico Chemoluminescent Substrate kit (Thermo Scientific).

Fluorescence microscopy. Yeast strains expressing GFP-tagged CRZ1 were grown to early exponential growth phase in SM and cells were induced with 2.5 mM $NaAsO_2$ for 15 min. DAPI (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, fixed with 3.7% (v/v) formaldehyde (Sigma-Aldrich) for 10 min at room temperature, and washed twice with PBS. After washing, cells were resuspended in 200 mM 1,4-diazadicyclo[2.2.2]octane [DABCO; dissolved in 75% (v/v) glycerol and 25% (v/v) PBS]. GFP signals were analysed in living cells using a Leica DMRXA fluorescence microscope equipped with a Roper Scientific MicroMax cooled CCD camera and MetaMorph software (Universal Imaging).

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 with either 1 mM $NaAsO_2$ or 20 mM $CaCl_2$ at 30 °C for 90 min. Cells were harvested by centrifugation, permeabilized with chloroform and assayed for enzyme activity using a BioTek Epoch microplate spectrophotometer. Miller units were calculated as described elsewhere (Miller, 1972). Experiments were performed using biological triplicates and the sd of the mean is shown.

Real-time PCR. RNA was extracted from early exponential phase cultures that were either untreated or exposed for 30 min to 1.5 mM $NaAsO_2$. DNA was removed by on-column DNase I digestion (RNase-Free DNase Set, Qiagen). Total RNA (1 µg) was reverse-transcribed with Transcriptor

Reverse Transcriptase (Roche Diagnostics). Quantitative PCRs (qPCRs) were performed in a LightCycler 480 Instrument (Roche), using LightCycler 480 SYBR 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 LightCycler 480 Software 1. Actin was used as a reference gene. All assays were done 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 determined the mRNA expression profile of wild-type cells upshifted to 2 mM Na_2HAsO_4-enriched medium for 60 min. Besides the upregulation of genes included in functional categories previously shown to be targeted by arsenic compounds, such as sulphur/methionine metabolism and redox homeostasis (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+} signaling pathways (Table S2). Despite several reports of genome-wide experiments, this is believed to be the first time that such a response has been detected, probably because none of the earlier studies addressed the mid-term response (60 min) induced by arsenate. Among these genes were *CRZ1*, encoding the master transcriptional regulator of Ca^{2+}-mediated signaling; *RCN1* and *CMP2*, encoding proteins related to CaN function; *CMD1*, *CMK2* and *RCK2*, encoding CaM and proteins directly regulated by CaM binding, respectively; and the calcium transporters encoded by *MID1* and *CCH1* (Table 2). Based on these results we hypothesized that Ca^{2+} signaling pathways could be involved in the response of yeast cells to arsenic stress.

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

Category	Gene	Function	Fold change
Ca^{2+} signaling	*CRZ1*	Transcription factor that activates transcription of genes involved in stress response	2.09
CaN complex	*RCN1*	Protein involved in CaN regulation during calcium signaling	2.78
	CMP2	CaN A; one isoform (the other is CNA1) of the catalytic subunit of CaN	1.90
Ca^{2+} binding	*CMD1*	CaM; Ca^{2+}-binding protein that regulates Ca^{2+}-independent and -dependent processes	1.37
CaM binding	*CMK2*	CaM-dependent protein kinase	−1.99
	RCK2	Protein kinase involved in the response to oxidative and osmotic stress	−1.40
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

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

To address the contribution of Ca^{2+} signaling 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 the Ca^{2+}-replete condition compared with the wild-type strain, indicating that the 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). As described previously (Menezes *et al.*, 2004), the *yap1* mutant strain displayed high growth sensitivity to 1 mM As(III), whereas the wild-type strain was resistant to 1 mM arsenite and moderately sensitive to 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 , middle and lower panels). Moreover, acquisition of tolerance mediated by Ca^{2+} was shown to be dose-dependent, as media supplementation with 100 mM $CaCl_2$ further enhanced the growth of these strains in the presence of arsenite. Our results therefore show that an increase in the availability of exogenous Ca^{2+} partially relieves arsenic toxicity and that Ca^{2+} may be implicated in the adaptive response to arsenic stress.

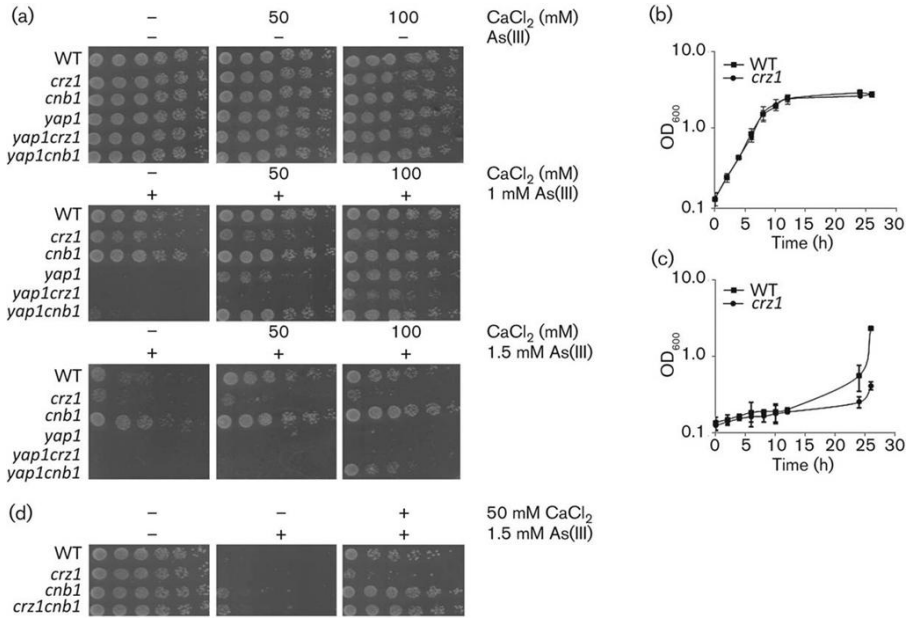

FIG. 1. Exogenous Ca^{2+} supply improves the tolerance of the wild-type and *yap1* strains to arsenite stress in a Crz1-dependent fashion. (a) Wild-type BY4742, *yap1* and the

FIG. 1. (*Continued*)

respective *crz1* and *cnb1* knockout strains were grown to early exponential phase and spotted onto SM supplemented with As(III) and/or $CaCl_2$. Growth was recorded after 48 h incubation at 30 °C. A representative experiment is shown. (b, c) Exponentially growing wild-type and *crz1* cells were diluted to OD_{600} 0.1±0.01, and induced or not induced with 1.5 mM As(III), and growth was monitored for 28 h at 30 °C. The growth curves show mean values±SD from at least three independent experiments. (d) Analysis of *CRZ1*/*CNB1*epistasis. Wild-type BY4742, *crz1*, *cnb1* and *crz1cnb1* knockout strains were grown to early exponential phase, and spot assays were performed as described above. WT, wild-type.

The zinc-finger transcription factor Crz1 is a central player in the Ca^{2+} signaling cascade. To determine whether Ca^{2+} contributes to arsenic tolerance through the activation of Ca^{2+} signaling pathways, we monitored, simultaneously, the growth of the respective *crz1* isogenic strains. First, we found that the *crz1* mutant was moderately sensitive to 1 and 1.5 mM As(III) (see Fig. 1a , lower and middle panels, and Fig. 1b, c), revealing therefore a link between the Crz1 pathway and the arsenic stress responses in yeast. Second, when *CRZ1* was disrupted in the *yap1* strain, the Ca^{2+}-mediated phenotype recovery of the double mutant was compromised in the presence of 1 mM arsenite plus 100 mM $CaCl_2$, when compared with the *yap1* single mutant, being completely abrogated in medium supplemented with only 50 mM $CaCl_2$. Finally, the growth recovery of *crz1* in the presence of Ca^{2+} was not as efficient as for the wild-type strain under all the stress conditions tested. Taken together, these results provide strong evidence of the importance of Crz1 and Ca^{2+} signaling pathways to full adaptive *S. cerevisiae* arsenic stress responses.

Under moderate arsenic stress conditions (1 mM $NaAsO_2$), Ca^{2+} induced some degree of tolerance even in the absence of Crz1 (Fig. 1a, middle panel), implying that Ca^{2+} may also mediate arsenic tolerance through the activation of Crz1-independent targets of the Ca^{2+} signallng cascade. The CaM kinase Cmk2, the related protein kinase Rck2, the glutamate decarboxylase Gad1 and the ER-residing proteins Hph1/Hph2 are potential candidates, due to their protective functions under oxidative and cell wall

stress (Cyert, 2001; Sánchez-Piris *et al.*, 2002; Heath *et al.*, 2004), which are known forms of cellular damage caused by arsenic stress (Menezes *et al.*, 2008; Thorsen *et al.*, 2009). Indeed, we found that *CMD1*, *CMK2* and *RCK2* were upregulated by arsenic in our transcriptomic analysis (Table 2).

Since the activation of Crz1 by Ca^{2+} is dependent on CaN function, we included in the phenotypic analysis the *cnb1* mutant, which is defective in the regulatory subunit of the CaN complex. As reported before, inactivation of Cnb1 renders the phosphatase complex non-functional (Cyert, 2003). Curiously, the *cnb1* mutant exhibited increased tolerance to high-dose arsenite stress compared with the wild-type strain, which was further increased when $CaCl_2$ was added to the medium (Fig. 1a , lower panel). The double mutant *yap1cnb1* displayed similar growth patterns under low-dose arsenite stress (Fig. S2). Although *yap1cnb1* was very sensitive to 1 mM arsenite, its growth was rescued through supplementation of the medium with $CaCl_2$. Under a high arsenite dose (1.5 mM), *yap1cnb1* growth recovery occurred only when media were supplemented with 100 mM $CaCl_2$ (Fig. 1a, middle and upper panels).

In order to understand the distinct behaviour of *crz1* and *cnb1* strains, we performed epistasis analysis. For this purpose, we constructed the double mutant *crz1cnb1* and compared the growth phenotypes of the three strains exposed to arsenic stress in the presence and absence of $CaCl_2$. The results in Fig. 1(d) clearly show that *CNB1* mutation is dominant over *CRZ1* mutation. This response is not specific to arsenic stress conditions, since *CNB1* is also dominant over *CRZ1* in the presence of high $CaCl_2$ concentrations (see Fig. S1). These results were not surprising, since CaN is upstream of Crz1 in the Ca^{2+}/CaM/CaN signaling pathway, regulating its translocation into the nucleus under conditions that trigger the mobilization of Ca^{2+} in the cytosol. A plausible explanation for this phenotypic paradox may lie in the fact that CaN regulates many substrates and therefore carries out multiple functions in yeast (Cyert, 2001). Indeed, it has already been reported that CaN mutants display defects that are not mimicked by the *crz1* mutant, which reinforces the participation of Crz1 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 by either activating proteins conferring toxicity or repressing those contributing to tolerance.

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

A transient increase in free cytosolic calcium is a mechanism used by eukaryotic cells to activate the Ca^{2+} signaling pathways and thus to regulate many cellular processes in response to specific environmental cues (Viladevall *et al.*, 2004; Araki *et al.*, 2009). Given that an excess of extracellular Ca^{2+} favours cell adaptation to arsenic stress and that Crz1 activity is required for 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 (approx. 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 cofactor coelenterazine as described in Methods. The baseline luminescence of both cultures, recorded for 10 min at 30 °C, did not increase over time (Fig. 2a, b, broken lines). The wild-type and *yap1* cells responded promptly to an arsenite shock, displaying a sharp rise followed by a rapid fall in luminescence, thus reflecting arsenite-induced fluctuations in free cytosolic Ca^{2+} (Fig. 2a, b, solid lines). The Ca^{2+} pulse amplitude exhibited by the arsenic-sensitive mutant *yap1* was slightly, but reproducibly, higher than that of the wild-type strain (compare Fig. 2a and b).

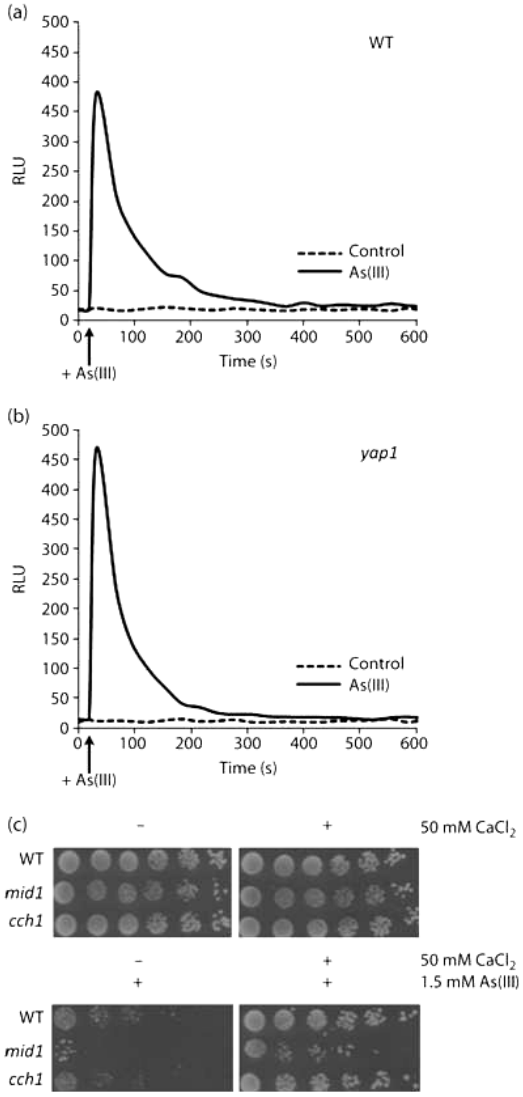

FIG. 2. Arsenite stress elicits a transient cytosolic Ca^{2+} pulse. Wild-type BY4742 (a) and *yap1* (b) cells expressing coelenterazine-reconstituted aequorin were injected or not injected with 1.5 mM As(III) directly in the luminometer plates and luminescence was recorded for 10 min. The arrows indicate the time of arsenite addition. Each determination was repeated at least three times with no significant variations. The dashed lines correspond to the baseline luminescence. RLU are expressed as c.p.s. per OD_{600} unit. (c) Mid1 is required for Ca^{2+}-mediated enhancement of arsenic tolerance. Wild-type BY4741 and the isogenic *mid1* and *cch1* strains were grown to early exponential phase and spotted onto SM supplemented with As(III) and/or $CaCl_2$. Growth was recorded after 48 h incubation at 30 °C. A representative experiment is shown.

These results indicate that arsenic compounds disturb Ca^{2+} homeostasis and further support the hypothesis that the activation of the Ca^{2+} signaling cascade may represent a mechanism employed by yeast cells to deal with arsenic stress.

Under stress conditions, an increase of cytosolic free Ca^{2+} can be generated by the mobilization of extracellular or vacuolar sources. The results of the phenotypic analysis reported here indicate that the

enhancement of extracellular Ca^{2+} availability promotes tolerance, suggesting that the arsenic-induced cytosolic Ca^{2+} burst might result from the mobilization of external sources. The facts that the mid1 mutant, which is defective in the plasma membrane high-affinity Ca^{2+} channel, is sensitive to arsenic compounds and that Ca^{2+} media supplementation did not efficiently improve the tolerance of this mutant to 1.5 mM As(III) (Fig. 2c), as happens with the wild-type strain, further support this hypothesis. Curiously, the *cch1* mutant exhibited growth patterns similar to those of the wild-type strain (Fig. 2c), suggesting that the role of Cch1 is dispensable for the arsenic-mediated activation of Ca^{2+} signaling. The discrepancy between the two mutants, whose genes encode proteins that cooperate to form a high-affinity Ca^{2+} influx system, has already been described and is attributed to the fact that Mid1 can also operate independently of Cch1 (Popa *et 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 CaN. One of the main functions of CaN is to dephosphorylate Crz1, thus triggering its nuclear accumulation when cytosolic Ca^{2+} levels rise. To provide additional evidence corroborating the importance of Ca^{2+} signaling and Crz1 activity to arsenic stress responses, we monitored Crz1–GFP phosphorylation status in the wild-type and *yap1* strains, and under conditions where CaN activity was specifically inhibited by CsA. As shown in Fig. 3(a), Crz1 was mainly phosphorylated under the control condition in the wild-type and *yap1* mutant strain. Treatment of cells with As(III) induced a partial shift of the protein to a faster-migrating form in the SDS-PAGE gel. This form corresponded to the dephosphorylated GFP–Crz1, since it completely disappeared when cells were treated with the CaN 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) induced 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 cytosolic Ca^{2+} release, leading to the induction of CaN, which in turn leads to Crz1 dephosphorylation and its consequent nuclear accumulation.

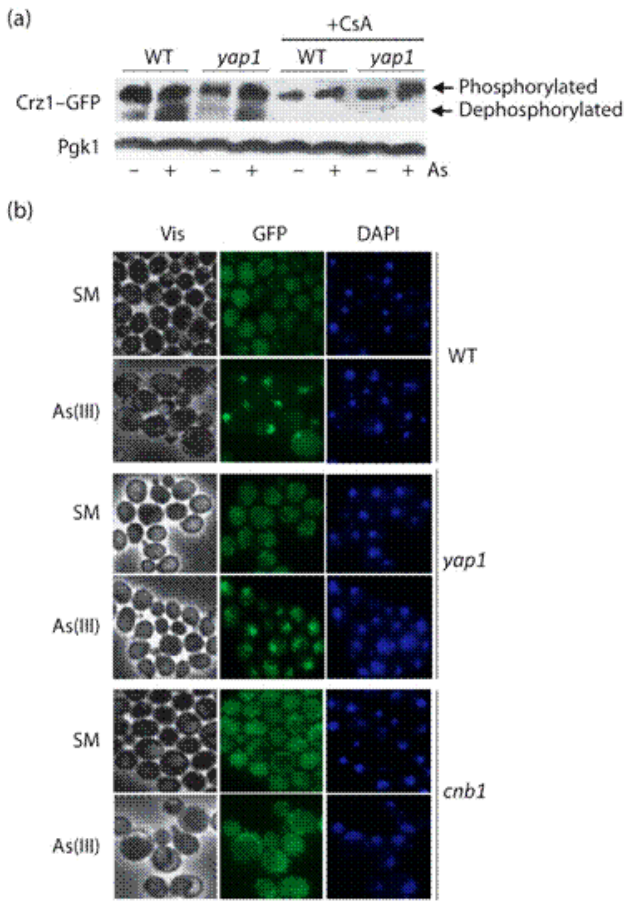

FIG. 3. Arsenite stress triggers Crz1 activation. (a) Wild-type and *yap1* cells expressing *CRZ1–GFP* were grown to early exponential phase, and at 30 min prior to induction with 1.5 mM As(III) were treated or not treated with CsA. Samples were collected at the indicated time points and proteins were 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.

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 known as CDRE 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 in which 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 10-fold increase compared with 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 the intact function of Crz1 and Cnb1. Exposure of wild-type cells to 1 mM arsenite led to an eightfold increase of β-galactosidase activity compared with the untreated condition (Fig. 4), which was a pattern of induction similar to that observed in the *yap1* strain (data not shown). Remarkably, some degree of lacZ activation was noted 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 CaN-dependent Crz1 activation by arsenite has the potential to induce the expression of endogenous CDRE-regulated genes.

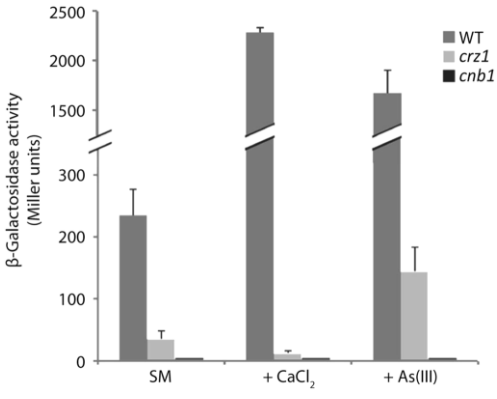

FIG. 4. Exposure to arsenite induces CDRE-driven expression. Wild-type, *crz1* and *cnb1* cells expressing the *CDRE–lacZ* reporter construct were upshifted to 1 mM As(III) or 20 mM $CaCl_2$ for 90 min at 30 °C. Cells were collected and permeabilized, and β-galactosidase activity was monitored. Measurements were performed in

FIG. 4 *(Continued)*

triplicate and the mean and SD are shown. WT, wild-type.

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+} signaling 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 that show that CDRE-regulated expression is partially dependent on Crz1 (Fig. 4), and are similar to those described in an earlier 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 either that 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) supports the former suggestion. Collectively, these results reinforce the hypothesis that arsenic stress induces a cytosolic Ca^{2+} pulse that activates the Ca^{2+} signaling pathways, which in turn culminates with the induction of at least *PMR1*, *PMC1* and *GSC2*.

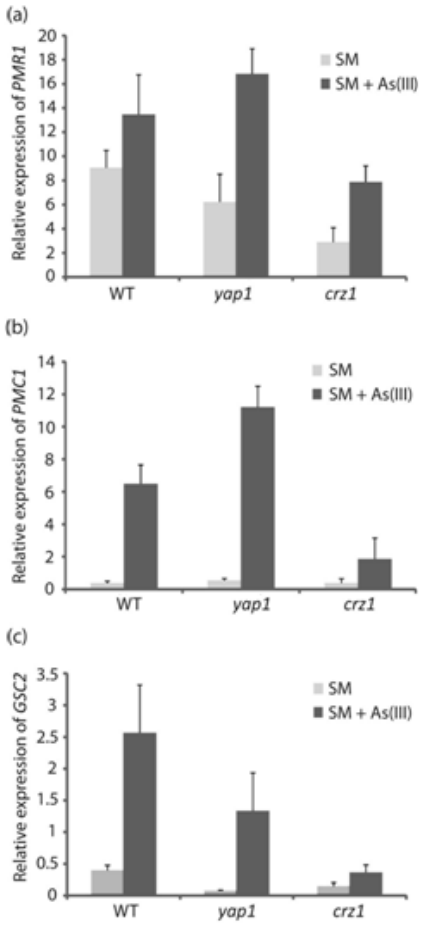

Fig. 5. *PMR1*, *PMC1* and *GSC2* are induced by arsenite stress. Early exponential phase wild-type, *yap1* and *crz1* cells were upshifted to 1.5 mM As(III) and harvested after 30 min incubation at 30 °C. The relative expression of *PMR1* (a), *PMC1*(b) and *GSC2* (c) was monitored by qPCR, using the relative quantification method with efficiency correction and *ACT1* as a reference gene. Measurements were performed in triplicate and the mean and SD are shown.

Concluding remarks

The deleterious effects and widespread distribution of the metalloid arsenic in the Earth's crust have 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 (ATO) 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 has been shown that ATO triggers ER stress responses and disturbs Ca^{2+} homeostasis (Binet *et al.*, 2010). Thus, to improve the effectiveness of arsenic-derived drugs in cancer therapy it is essential to recapitulate the cellular and molecular processes affected by the treatment.

In this work we took advantage of the eukaryotic model yeast *S. cerevisiae* to investigate how Ca^{2+} signaling pathways contribute to arsenic stress responses in eukaryotic cells. We report for what we believe is 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+} signaling cascade in this process. Upon arsenic stress, the burst of Ca^{2+} in the cytosol triggers CaN-dependent Crz1 dephosphorylation and nuclear accumulation, thereby leading to the induction of CDRE-driven expression of the endogenous *PMR1*, *PMC1* and *GSC2* genes.

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

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Supplementary Data

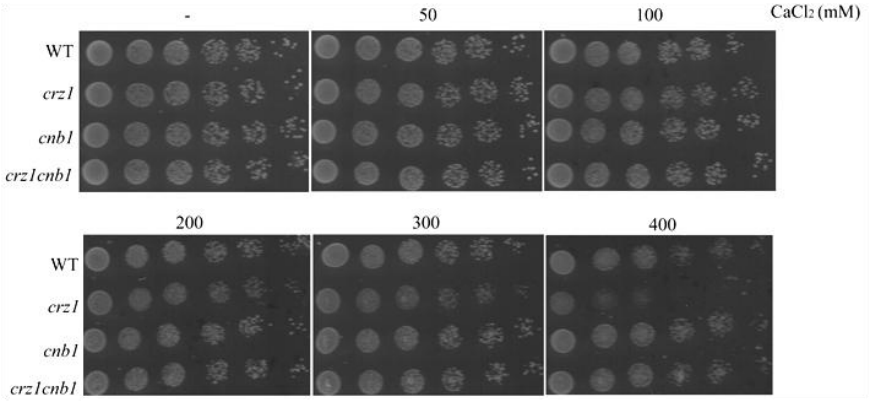

FIG. S1. Establishment of $CaCl_2$ non-toxic concentrations for the wild-type BY4742, *crz1*, *cnb1 and crz1cnb1* strains. The respective strains were grown to early exponential phase and were spotted onto synthetic medium supplemented with $CaCl_2$. Growth was recorded after 48 h incubation at 30 °C. A representative experiment is shown.

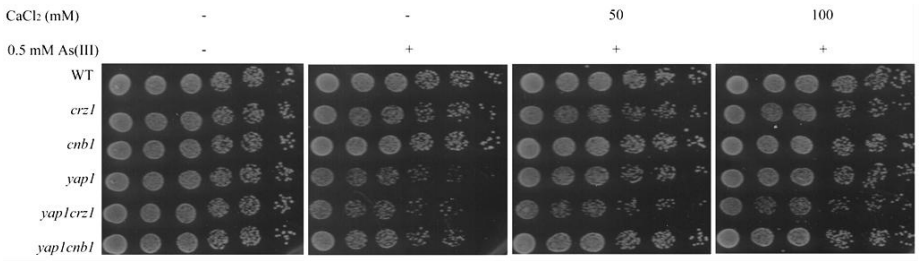

FIG. S2. Exogenous Ca^{2+} supply improves tolerance of the *yap1* strain to a low-dose arsenite stress in a Crz1-dependent fashion. Wild-type BY4742, *yap1* and the respective *crz1* and *cnb1* knockout strains were grown to early exponential phase and were spotted onto synthetic medium supplemented with 0.5 mM As(III) and/or $CaCl_2$. Growth was recorded after 48 h incubation at 30 °C. A representative experiment is shown.

Table S1. Oligonucleotides used in this work.

Primer	Sequence (5′–3′)
CRZ1-HIS-Fw*	TTTTTAGTCTCGGGAAGTTTCGTCAGACAGTACAAGGATC GTACGCTGCAGG
CRZ1-HIS-Rev*	GAAAAAAAAAATTCCTATTCAAAGCTTAAAAAAACAAAAATT AGGGAGACCGGCAGAT

Table S1. (*Continued*)

Primer	Sequence (5′–3′)
A1-CRZ1*	CAGGCTCAGATACAAGTACC
A4-CRZ1*	GGGCTGTCTAAGGAAATCTC
S1-YAP1*	CCACCCAAAACGTTTAAAGAAGGAAAAGTTCGTACGCTGCAGG TCGAC
S2-YAP1*	GAAAAAGTTCTTTCGGTTACCCAGTTTTCCATCGATGAATTCGA GCTCG
A1-YAP1*	GCTGATCTTACCGTGTTG
A4-YAP1*	TCTCAGCGTTGTGCCATC
PMR1-Fw†	CACCTTGGTTCCTGGTGATT
PMR1-Rev†	CCGGTTCATTTTCACCAGTT
PMC1-Fw†	GTGGCGCACCATTTTCTATT
PMC1-Rev†	TACTTCATCGGGGCAGATTC
GSC2-Fw†	CCCGTACTTTGGCACAGATT
GSC2-Rev†	GACCCTTTTGTGCTTTGGAA

*Gene disruption and mutant confirmation. †qPCR

Table S2. Calcium-related genes whose mRNA levels were up or downregulated in the wild-type strain upon arsenic treatment. The mean fold induction of triplicate samples is represented. An increase/decrease of 1.2-fold and a *P* value of <0.005 were used.

ORF	Gene	Function	Fold change
Calcium-mediated signaling:			
YLR260W	*LCB5*	Minor sphingoid long-chain base kinase	2.7
YNL027W	*CRZ1*	Transcription factor that activates transcription of genes involved in stress response	2.09
YOR171C	*LCB4*	Sphingoid long-chain base kinase	1.81
YDR294C	*DPL1*	Dihydrosphingosine phosphate lyase	1.68
YMR030W	*RSF1*	Protein required for respiratory growth	1.6

Table S2. (*Continued*)

ORF	Gene	Function	Fold change
Calcium transport/ calcium channel activity:			
YNL291C	*MID1*	N-glycosylated integral membrane protein of the ER and plasma membrane	−1.64
YGR217W	*CCH1*	Voltage-gated high-affinity calcium channel	−1.77
Cellular calcium homeostasis			
YBR036C	*CSG2*	ER membrane protein required for growth at high calcium concentrations	1.35
YEL031W	*SPF1*	P-type ATPase involved in ER function and Ca^{2+} homeostasis	−1.4
YBR127C	*VMA2*	Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar H^{+}-ATPase (V-ATPase)	−1.41
YGL020C	*GET1*	Subunit of the GET complex	−1.53
Calcium ion binding:			
YBR117C	*TKL2*	Transketolase, catalyses conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway	3.31
YAL048C	*GEM1*	Tail-anchored outer mitochondrial membrane GTPase which regulates mitochondrial morphology	2.81
YOR257W	*CDC31*	Calcium-binding component of the SPB half-bridge	2.68
YDR001C	*NTH1*	Neutral trehalase, degrades trehalose	2.36
YLR250W	*SSP120*	Protein of unknown function	2.22
YNL084C	*END3*	EH domain-containing protein involved in endocytosis	1.86
YGL085W	*LCL3*	Putative protein of unknown function	1.76

Table S2. (*Continued*)

ORF	Gene	Function	Fold change
YGR100W	*MDR1*	Cytoplasmic GTPase-activating protein; activates Ypt/Rab transport GTPases Ypt6p, Ypt31p and Sec4p	1.76
YBR001C	*NTH2*	Putative neutral trehalase, required for thermotolerance and resistance to other cellular stresses	1.67
YNL083W	*SAL1*	Probable transporter, member of the Ca^{2+}-binding subfamily of the mitochondrial carrier family	1.56
YGR058W	*PEF1*	Penta-EF-hand (PEF) protein required for polar bud growth and cell wall abscission	1.55
YDR373W	*FRQ1*	N-myristoylated calcium-binding protein that may have a role in intracellular signaling through its regulation of the phosphatidylinositol 4-kinase Pik1p	1.54
YBR109C	*CMD1*	Calmodulin; Ca++ binding protein that regulates Ca++ independent and dependent processes	1.37
YGR167W	*CLC1*	Clathrin light chain, subunit of the major coat protein involved in intracellular protein transport and endocytosis	1.34
YIR006C	*PAN1*	Part of actin cytoskeleton-regulatory complex Pan1p-Sla1p-End3p	1.3
YPL048W	*CAM1*	Nuclear protein required for transcription of MXR1	−1.31
YML072C	*TCB3*	Lipid-binding protein, localized to the bud via specific mRNA transport	−1.45
YPR074C	*TKL1*	Transketolase, similar to Tkl2p	−1.47
YJR131W	*MNS1*	Alpha-1,2-mannosidase involved in ER quality control	−1.52
YNL238W	*KEX2*	Calcium-dependent serine protease involved in the activation of proproteins of the secretory pathway	−1.64

Table S2. (*Continued*)

ORF	Gene	Function	Fold change
YIL140W	*AXL2*	Integral plasma membrane protein required for axial budding in haploid cells	−1.74
YPR021C	*AGC1*	Mitochondrial amino acid transporter	−2.11
YGR098C	*ESP1*	Separase, a caspase-like cysteine protease that promotes sister chromatid separation	−4.15
Calcium transport/ calcium channel activity:			
YNL291C	*MID1*	N-glycosylated integral membrane protein of the ER and plasma membrane	−1.64
Calcium-mediated signaling:			
YLR260W	*LCB5*	Minor sphingoid long-chain base kinase	2.7
YNL027W	*CRZ1*	Transcription factor that activates transcription of genes involved in stress response	2.09
YOR171C	*LCB4*	Sphingoid long-chain base kinase	1.81
YDR294C	*DPL1*	Dihydrosphingosine phosphate lyase	1.68
YMR030W	*RSF1*	Protein required for respiratory growth	1.6

3

Regulation of Transcription Factor Yap8, and the E4-Ubiquitin Ligase Ufd2

3.1
Preface

Cellular signals propagated by ubiquitin (Ub) involve enzymes associated to the activation, conjugation and ligation of Ub monomers as well as multi-ubiquitination and de-ubiquitination of substrate proteins [1]. Hereinafter, recent insights into the role of post-translational mechanisms affecting Yap8 stability and activity will be discussed. In the budding yeast, this transcription factor plays an important role in the adaptation to arsenic stress [2]; and we identified Ufd2, an enzyme of the ubiquitin-proteasome pathway (UPP), in regulating Yap8 activity under arsenic stress. Under physiological conditions, Yap8 is degraded via the UPP, however upon arsenic challenge, it bypasses degradation becoming highly stable in a process surprisingly dependent on Ufd2 function. While studies have been made to understand Ufd2 as a player in degradation [3], our study unveiled a link between this E4 Ub ligase and stabilization of transcription factor Yap8.

Extending the understanding of basic mechanisms of the UPP and key enzymes such as the E4s (Ub-protein ligases) may be valuable since the proteasome is considered an important target for drug development, in particular to combat malignancies [4].

3.2
Journal Article

Ferreira, R.T., Menezes R.A., Rodrigues-Pousada, C. E4-ubiquitin ligase Ufd2 stabilizes Yap8 and modulates arsenic stress responses independent of the U-box motif. *Biology Open*, **2015**: In Press.

Note: The article does not follow this thesis formatting, follow instead the rules of the Biology Open (BiO) Journal.

3.2.1
Author's Contribution

I had a major participation in this study: planning and execution of experiments [cf. Methods Section: Y2H analyses with the exception of the first screens performed by R.A.M. (data not shown); Co-immunoprecipitation assays; Cycloheximide chase and immunoblot analysis; β-galactosidase assays; Quantitative real-time PCR analyses; and Statistical analysis], data analysis, figures preparation, and paper writing.

3.3
References for Chapter's Preface

1. Hoppe, T., *Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all.* Trends Biochem Sci, 2005. **30**(4): p. 183-7.
2. Menezes, R.A., et al., *Yap8p activation in Saccharomyces cerevisiae under arsenic conditions.* FEBS Lett, 2004. **566**(1-3): p. 141-6.
3. Bohm, S., et al., *Cellular functions of Ufd2 and Ufd3 in proteasomal protein degradation depend on Cdc48 binding.* Mol Cell Biol, 2011. **31**(7): p. 1528-39.
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E4-Ubiquitin ligase Ufd2 stabilizes Yap8 and modulates arsenic stress responses independent of the U-box motif

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ABSTRACT

Adaptation of *Saccharomyces cerevisiae* cells to arsenic stress is mediated through the activation of arsenic detoxification machinery by the Yap8 transcription factor. Yap8 is targeted by the ubiquitin proteasome system for degradation under physiological conditions, yet it escapes proteolysis in arsenic-injured cells by a mechanism that remains to be elucidated. Here, we show that Ufd2, an E4-Ubiquitin (Ub) ligase, is upregulated by arsenic compounds both at mRNA and protein levels. Under these conditions, Ufd2 interacts with Yap8 mediating its stabilization, thereby controlling expression of *ACR3* and capacity of cells to adapt to arsenic injury. We also show that Ufd2 U-box domain, which is associated to the ubiquitination activity of specific ubiquitin ligases, is dispensable for Yap8 stability and has no role in cell tolerance to arsenic stress. Thus, our data disclose a novel Ufd2 role beyond degradation. This finding is further supported by genetic analyses showing that proteins belonging to Ufd2 proteolytic pathways, namely Ubc4, Rad23 and Dsk2, mediate Yap8 degradation.

KEY WORDS

Ufd2, U-box domain, AP-1 like transcription factor, Yap8, Arsenic

INTRODUCTION

Arsenic (As) is a toxic element widely spread in nature due to natural and anthropogenic sources (Mandal and Suzuki, 2002). Several studies have revealed that As mediates toxicity via inducing the production of reactive oxygen species (ROS), inhibiting DNA repair, altering DNA methylation and increasing cell apoptosis (Flora, 2011; Jomova *et al.*, 2011). Paradoxically, due to its anticancer effect, arsenic trioxide (As_2O_3) has been proposed as a front-line agent for treatment of acute promyelocytic leukemia (APL) (Mathews *et al.*, 2011; Iland and Seymour, 2013).

In the yeast *Saccharomyces cerevisiae*, protective response to arsenic stress involves multiple cellular mechanisms. They include the activation of antioxidant defense machinery, the calcium-signaling pathways, the repression of the high affinity iron uptake system, the adjustment of sulfur metabolism to enhance GSH biosynthesis associated to intra- and extra-cellular chelation of arsenic, the regulation of cell cycle progression as well as enhancement of proteasomal degradation of misfolded/damaged proteins (Menezes *et al.*, 2008; Migdal *et al.*, 2008; Ferreira *et al.*, 2012; Jacobson *et al.*, 2012; Thorsen *et al.*, 2012; Batista-Nascimento *et al.*, 2013). The most important adaptive mechanism triggered in cells exposed to As(V) and As(III) requires the activity of Yap8 transcription factor. It modulates As detoxification by the activation of *ACR2* and *ACR3* genes encoding an arsenate reductase, which catalyses the conversion of As(V) into As(III), and an arsenite efflux pump, respectively (Wysocki *et al.*, 1997, 2004; Bobrowicz and Ulaszewski, 1998; Haugen *et al.*, 2004; Rodrigues-Pousada *et al.*, 2004). Yap8 is the most divergent member of the yeast AP-1 like family of transcription factors displaying restricted DNA-binding specificities in comparison with the other family members (Ilina *et al.*, 2008; Amaral *et al.*, 2013). Its activity is tightly controlled at different levels. Previously, we have reported that Yap8 shifts between the cytoplasm and the nucleus under non-stressed conditions, while arsenic compounds trigger its retention in the nucleus (Menezes *et al.*, 2004). A further mechanism of Yap8 regulation relies on the post-translational control of its protein levels by the ubiquitin-

proteasome pathway (UPP). It was shown that Yap8 is ubiquitinated and degraded by the proteasome under physiological conditions, and upon arsenic injury, it escapes degradation by a mechanism that is not yet elucidated (Di and Tamas, 2007).

The cycle of ubiquitin (Ub) attachment to the lysine residues of target proteins commonly involves the catalytic activities of E1-Ub-activating and E2-Ub-conjugating enzymes, and E3-Ub-ligases (Finley, 2009; Hochstrasser, 2009; Ciechanover and Stanhill, 2014). Nevertheless, efficient multiubiquitination of specific substrates also requires the activity of E4 enzymes, which work in association with E1s, E2s and E3s to catalyse Ub chain assembly necessary for recognition and degradation by the 26S proteasome (Hoppe, 2005). Among the few yeast E4 enzymes, the ubiquitin fusion degradation enzyme Ufd2 is the best characterized and the first identified member (Koegl *et al.*, 1999). It belongs to a family of proteins containing a domain with 70 amino acids at their C-terminus, termed the U-box, conserved among eukaryotes (Hatakeyama and Nakayama, 2003). This domain is associated to the elongation of Ub chains being structurally related to the RING finger domain found in certain E3-Ub ligases (Aravind and Koonin, 2000; Tu *et al.*, 2007). The U-box is generally considered as the essential functional unit of E4s, however, it was reported that the U-box of human UFD2a is not required for the proteasomal turnover of p73 (Hosoda *et al.*, 2005).

The mechanisms by which Yap8 circumvents proteolysis under As stress are still elusive constituting a matter of investigation in the present study. Although Ufd2 was shown to act as an E4 enzyme active in degradation, our study leads to novel insights on Ufd2 function in yeast. *UFD2* deletion in the yeast genome reveals that Yap8 is destabilized under As conditions, its transcriptional activity is decreased, and cellular tolerance to As compounds is compromised. Interestingly, Ufd2 function in Yap8 regulation seems to be independent of the U-box domain. Our results describe the involvement of Ufd2 in a new function beyond proteolysis.

RESULTS

Yap8 levels are tightly controlled by arsenic

Arsenic stress responses in *S. cerevisiae* require the AP-1 like transcription factor Yap8, which drives the expression of genes involved in As detoxification processes (Menezes *et al.*, 2004). Aiming at elucidating further the mechanisms underlying Yap8 regulation, we analysed by immunoblotting the levels and the stability of HA-tagged Yap8 in wild type (WT) cells, either in the presence or the absence of arsenite (Fig. 1). Yap8-HA levels were shown to be low in cells incubated under control conditions, and increased in response to 90 min treatment with As(III) (Fig. 1A, lanes 1 and 2). Furthermore, inhibition of proteasome activity with MG132 led to an increase of Yap8-HA levels under non-inducing conditions (Fig. 1A, lanes 1 and 3), but not in the presence of As(III), as indicated by cell co-treatment with MG132 and As(III) (Fig. 1A, lanes 2 and 4). These data indicate that Yap8 is not degraded by proteasome under arsenic stress conditions. In DMSO-treated cells, Yap8 levels are comparable to those observed in the control condition (Fig. 1A, lanes 1 and 5). We have then determined Yap8 stability in the presence and absence of As by using the protein synthesis inhibitor cycloheximide (CHX). For that, cells were first pre-treated with 1.5 mM arsenite for 30 min followed by CHX treatment up to 240 min. It is clear that Yap8 levels were enhanced during this period (Fig. 1B, left panel) in contrast to what does occur if As was completely removed from the medium before CHX treatment (Fig. 1B, right panel). Measurement of Yap8 half-life in both conditions revealed that it is strongly reduced in the absence of As (>240 min vs 94 min).

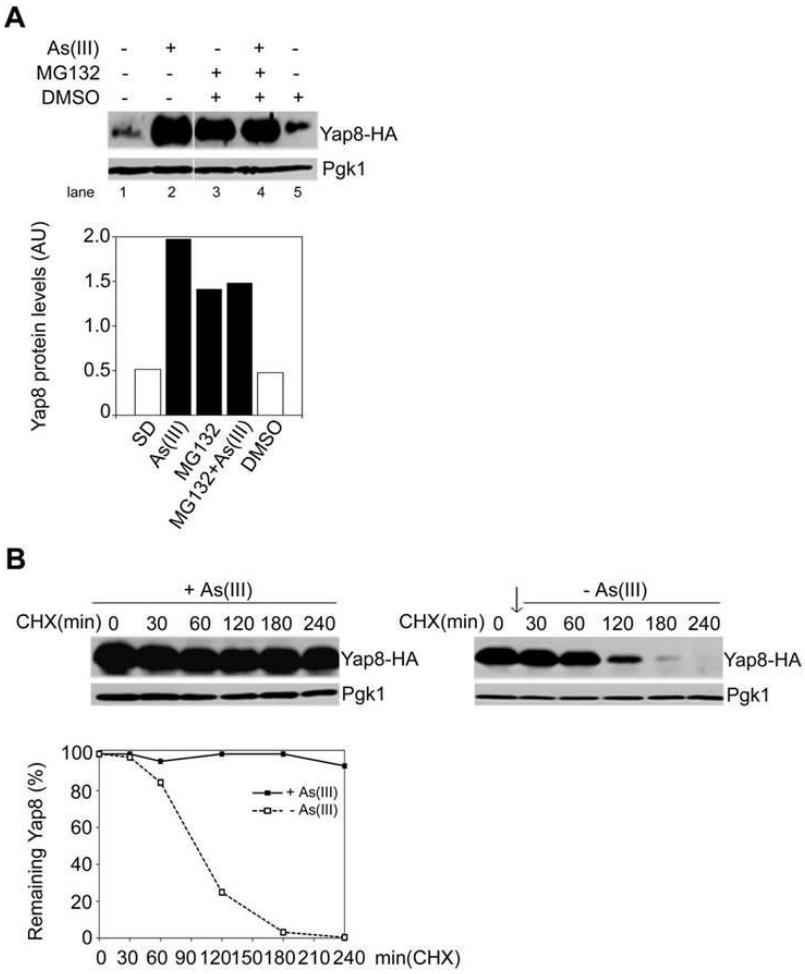

Fig. 1. Arsenic triggers Yap8 stabilization circumventing proteasomal degradation. (A) Wild type cells exposed to arsenite exhibit enhanced Yap8 levels. BY4742 wild type (WT) cells expressing Yap8-HA were left untreated (SD) or exposed to 1.5 mM As(III) alone or in combination with 100 µM MG132 and DMSO, for 90 min. The graph represents relative Yap8 levels normalized against Pgk1 levels (arbitrary units, AU). (B) As(III) triggers Yap8 stabilization. Cells were first exposed to 1.5 mM As(III) for 30 min, cycloheximide (CHX) was then added to a final concentration of 0.1 mg/ml up to 240 min and protein extracts were subjected to immunoblotting using a anti-HA antibody. Pgk1 was used as loading control. The graph represents the percentage of remaining Yap8 after CHX addition. Left panel: As(III) was maintained in the medium; estimated Yap8 half-life >240 min. Right panel: As(III) was removed from the medium before CHX addition; estimated Yap8 half-life is 94 min. The arrow indicates the removal of As(III). Representative experiments are shown.

These findings are in agreement with those previously reported using different As conditions (Di and Tamas, 2007), and are consistent with the notion that Yap8 is a substrate for proteasomal degradation under physiological conditions whereas it is stabilized by arsenic.

The E4-Ufd2 enzyme interacts with Yap8 under arsenic stress conditions

To get further insights into the molecular basis of Yap8 stabilization that circumvents degradation upon arsenic stress, we have screened a yeast two-hybrid cDNA library fused to the Gal4 activation domain ($Gal4^{AD}$), using Gal4 DNA binding domain-Yap8 ($Gal4^{DBD}$Yap8) fusion as a bait protein. Yap8 is strongly activated by arsenic, therefore, to increase the likelihood of identifying new Yap8-interaction partners, the cDNA library was generated from cells induced with a sub-lethal dose of pentavalent inorganic arsenic As(V). Performing the screening in the presence of 0.5 mM As(V) allowed us to identify new Yap8-interaction partners (data not shown), among them the ubiquitin fusion degradation enzyme Ufd2. In order to assess the specific interaction between Yap8 and Ufd2, cells co-expressing $Gal4^{DBD}$Yap8 and $Gal4^{AD}$Ufd2 along with the respective controls, were treated or not with 2 mM As(V) for 60 min, and interaction was followed through induction of lacZ reporter gene in quantitative β-galactosidase assays (Fig. 2A). A high β-galactosidase activity was detected in $Gal4^{DBD}$Yap8/$Gal4^{AD}$Ufd2-expressing cells only under conditions where Yap8 is activated, i.e. in the presence of arsenic. The β-galactosidase signal observed in control cells expressing $Gal4^{DBD}$Yap8/$Gal4^{AD}$ and challenged with arsenic is due to the Yap8 transactivation potential (Menezes *et al.*, 2004). Notwithstanding, $Gal4^{DBD}$Yap8/$Gal4^{AD}$Ufd2 interaction has yielded β-galactosidase activity values significantly higher than those determined for $Gal4^{DBD}$Yap8/$Gal4^{AD}$. As a positive control we have used cells expressing the well-known interacting proteins, p53 and SV40 T-antigen, fused to the $Gal4^{DBD}$ and $Gal4^{AD}$, respectively ($Gal4^{DBD}$p53/$Gal4^{AD}$T) (Li and Fields, 1993). Similarly to what we observed for non-stressed cells co-expressing

Gal4^{DBD}Yap8/Gal4^{AD}Ufd2, we did not detect β-galactosidase activity for Gal4^{DBD}/Gal4^{AD}Ufd2 and Gal4^{DBD}LamC/Gal4^{AD}T control cells.

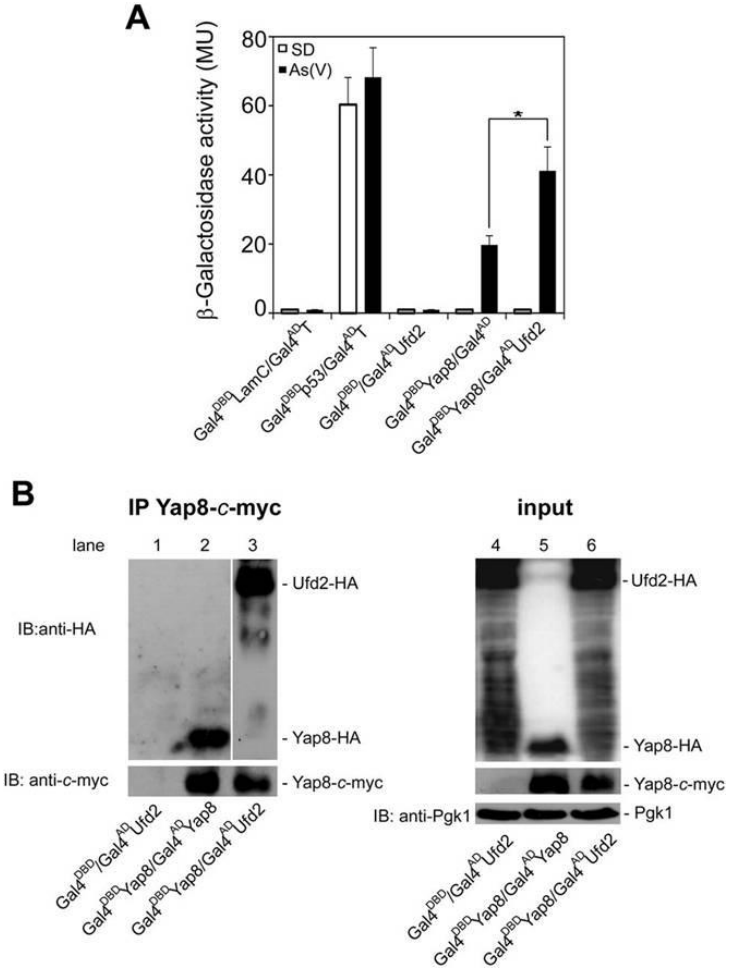

Fig. 2. Yap8 interacts with Ufd2 upon arsenic stress. (A) Yeast two-hybrid assays reveal Ufd2 as a Yap8-interaction partner. The Y187 strain was co-transformed with plasmids encoding *GAL4^{DBD}LamC/GAL4^{AD}T*, *GAL4^{DBD}p53/GAL4^{AD}T*, *GAL4^{DBD}/GAL4^{AD}UFD2*, *GAL4^{DBD}YAP8/GAL4^{AD}* or *GAL4^{DBD}YAP8/GAL4^{AD}UFD2* and β-galactosidase activity was measured in cells challenged or not with 2 mM As(V) (MU, Miller Units). Values represent the mean±standard deviation (s.d.) of three biological replicates and statistical differences denoted as **P*<0.05. (B) Ufd2 co-immunoprecipitates together with Yap8. Y187 cells co-transformed with *GAL4^{DBD}/GAL4^{AD}UFD2* (lanes 1 and 4), *GAL4^{DBD}YAP8/GAL4^{AD}YAP8* (lanes 2 and 5) or *GAL4^{DBD}YAP8/GAL4^{AD}UFD2* (lanes 3 and 6) were exposed to 2 mM

Fig. 2 (*Continued*)
As(V) for 60 min and Gal4^{DBD}Yap8, bearing a *c*-myc epitope, was immunoprecipitated with anti-*c*-myc antibody. Immunoblotting was performed using anti-HA, anti-*c*-myc and anti-Pgk1 antibodies. A representative experiment is shown. IP, immunoprecipitation; IB, immunoblotting.

The specificity of Yap8/Ufd2 interaction was further corroborated by co-immunoprecipitation using Gal4^{DBD}Yap8 and Gal4^{AD}Ufd2 constructs, which also comprise the *c*-myc and HA epitopes, respectively, and similar conditions to that of the two-hybrid analysis (Fig. 2B). The fusion Yap8-*c*-myc was efficiently immunoprecipitated using the anti-*c*-myc antibody (Fig. 2B, lanes 2 and 3). Co-immunoprecipitation signals were only detected in cells co-expressing Yap8-*c*-myc together with Ufd2-HA (Fig. 2B, lane 3) revealing the specific interaction between Yap8 and Ufd2. The formation of Yap8 homodimeric complexes (R.A.M., unpublished observations; Di and Tamas, 2007) was used as positive control (Fig. 2B, lane 2). As revealed by immunoblotting analysis of whole cell extracts, Yap8 and Ufd2 were properly expressed in all conditions tested (Fig. 2B, lanes 4-6). To avoid artefacts that could interfere with Yap8 and Ufd2 interaction, including the presence of Gal4 tags and overexpression of fusion proteins, reciprocal co-immunoprecipitation assays were performed using BY4742 cells carrying *YAP8-c-myc* and *UFD2-HA* in the centromeric (*CEN-ARS*) vectors and under the control of native promoters. Yap8-*c*-myc was efficiently co-immunoprecipitated together with Ufd2-HA in cells challenged with As(III) for 90 min but not in cells left untreated (supplementary material Fig. S1).

Overall, these data reveal Yap8 as an interaction partner of Ufd2 in cells exposed to arsenic stress conditions.

Ufd2 modulates arsenic stress responses

The results above suggest that Ufd2 may play a role in cells exposed to arsenic stress. To characterize the mechanisms by which Ufd2 is implicated in arsenic stress responses, we first evaluated its requirement to arsenic tolerance. We have therefore carried out phenotypic growth assays as well as growth curves of the WT and isogenic *ufd2* mutant strains, either in the

absence or presence of both As(V) or As(III). *UFD2* gene is not essential for yeast cell viability (Bohm *et al.*, 2011), however its deletion leads to a slight growth impairment in the control condition (Fig. 3A), as previously reported (Yoshikawa *et al.*, 2011; Marek and Korona, 2013). Remarkably, *ufd2* displayed sensitivity to both As(V) and As(III) stresses (Fig. 3A), which is restored after the reintroduction of an episomal copy of *UFD2* in the mutant strain (supplementary material Fig. S2). In agreement with the notion that As(III) is more toxic than As(V), we also noted that *UFD2* deletion severely impairs cell growth in the presence of As(III). These results bring to light a novel role for Ufd2 in yeast arsenic adaptation.

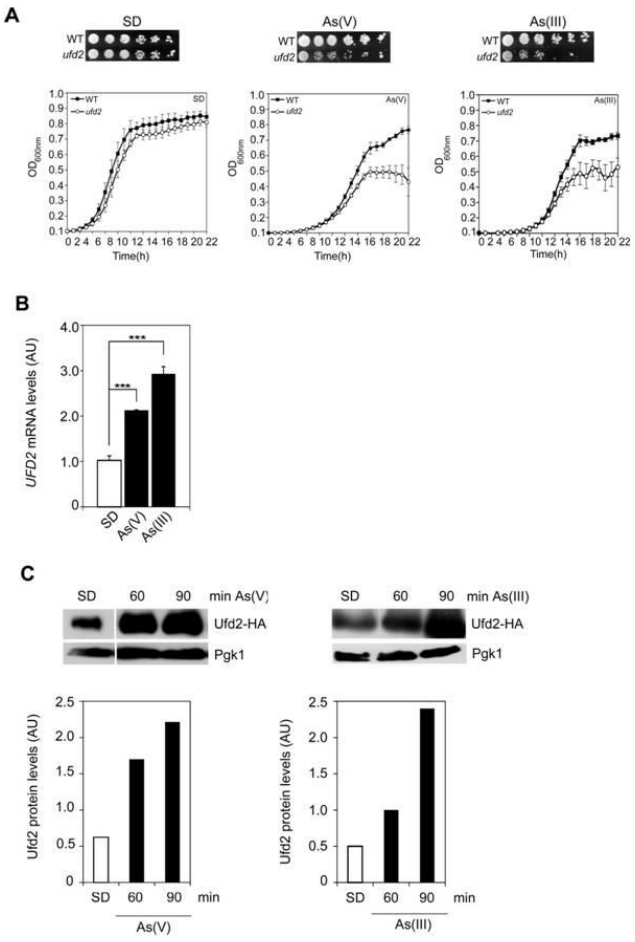

Fig. 3. Ufd2 mediates arsenic tolerance. (A) *ufd2* cells are sensitive to arsenic stress. Exponential phase BY4742 wild type (WT) and the *ufd2* mutant were serially diluted and spotted onto SC media supplemented or not with 1.5 mM As(III) or 2 mM As(V) or 1.5 mM As(III). SD, control. Growth was recorded after 2 days incubation at 30°C.A representative experiment is shown. Cell growth was also monitored by means of growth curves. Exponential phase BY4742 WT and *ufd2* mutant cells were

Fig. 3 (*Continued)*
exposed or not to 2 mM As(V) or 1.5 mM As(III) for 22 h and OD_{600} was monitored in intervals of 1 h. The curves represent the mean±s.d. of three biological replicates. (B) *UFD2* is induced in cells injured with arsenic. BY4742 cells were challenged or not with 1.5 mM As(III) or 2 mM As(V) or 1.5 mM As(III) and *UFD2* mRNA levels were determined by qRT-PCR (AU, Arbitrary Units). Values represent the mean±s.d. of three biological replicates and statistical differences denoted as ***P<0.001. (C) Ufd2 protein levels increase during arsenic stress. BY4742 cells expressing Ufd2-HA were treated with 2 mM As(V) or 1.5 mM As(III) and harvested at the indicated time-points. SD, control. Immunoblottings were performed using anti-HA and anti-Pgk1 antibodies. Pgk1 was used as loading control. The graphs represent relative Ufd2 levels (AU, Arbitrary Units). Representative experiments are shown.

We have further examined how As stress stimuli affect Ufd2. First, it was analysed arsenic-mediated changes in *UFD2* gene expression by qRT-PCR. As shown in Fig. 3B, *UFD2* mRNA levels increased 2-fold and 3-fold after 90 min exposure to As(V) and As(III), respectively. Consistent with *UFD2* mRNA analyses, Ufd2 protein levels were also enhanced upon As(V) or As(III) stress (Fig. 3C).

Altogether, these results show the activation of Ufd2 by arsenic suggesting that it is a novel regulator of metalloid stress response. Since Ufd2 function seems to be more critical in cells exposed to As(III), this condition was chosen for further studies.

Ufd2 regulates Yap8 stabilization

Ufd2 was shown to be involved in proteasomal degradation pathways (Liu *et al.*, 2010). On the other hand, our data suggest that interaction of Ufd2 with Yap8 protects yeast cells against the toxicity of As compounds. To clarify the functional relationship between Ufd2 and Yap8, we have monitored the kinetics of Yap8 fused to HA epitope (Yap8-HA) induced by As(III) in WT and *ufd2* strains. As shown in Fig. 4A, *ufd2* displayed lower Yap8 levels when compared to WT cells indicating that Yap8 stability may be compromised in the absence of Ufd2.

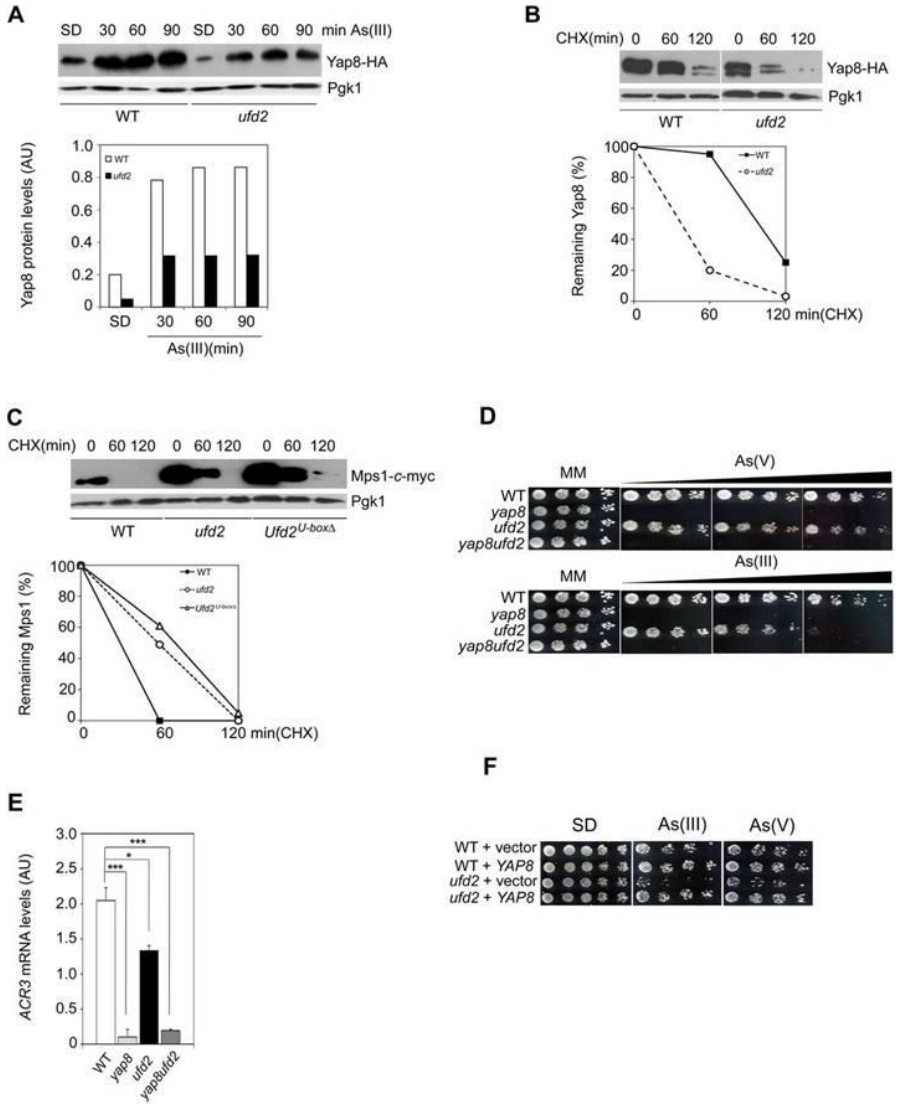

Fig. 4. Ufd2 mediates Yap8 stabilization. (A) Yap8 levels are reduced in *ufd2* mutant cells compared to the wild type strain. BY4742 wild type (WT) and *ufd2* mutant strains expressing Yap8-HA were incubated with 1.5 mM As(III), harvested at the indicated time-points and subjected to immunoblotting using anti-HA and anti-Pgk1 antibodies. The graph represents relative Yap8 levels (AU, Arbitrary Units). (B) Yap8 is destabilized in the *ufd2* mutant. The same strains were first exposed to 1.5 mM As(III) for 90 min, washed and subsequently treated with 0.1 mg/ml cycloheximide (CHX) up to 120 min prior to immunoblotting with the antibodies indicated above. The graph represents the percentage of remaining Yap8 protein after CHX addition. Estimated Yap8 half-life is 98 min in the WT

Fig. 4 (*Continued*)
strain and 37 min in the *ufd2* mutant. (C) Mps1 stability is increased in *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ mutant cells in comparison to WT strain. BY4741 WT, *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ mutant strains carrying the *GAL1*^{promoter}*MPS1-c-myc* construct were induced with galactose before being challenged with glucose and 0.1 mg/ml CHX. Cells were harvested at the indicated time-points and subjected to immunoblotting using anti-*c*-myc and anti-Pgk1 antibodies. The graph represents the percentage of remaining Mps1 protein after CHX addition. A representative experiment is shown. (D) Epistasis analyses of *YAP8* and *UFD2*. Exponential phase BY4742 WT, *yap8*, *ufd2* and *yap8ufd2* cells were serially diluted and spotted onto MM media supplemented or not with increasing concentrations of As(V) (up to 2 mM; upper panel) or As(III) (up to 1.5 mM; lower panel). Growth was recorded after 2 days incubation at 30°C. A representative experiment is shown. (E) *ACR3* expression is similar in the double *yap8ufd2* and single *yap8* mutants. The same strains referred in D were challenged with 1.5 mM As(III) for 90 min and *ACR3* mRNA levels were determined by qRT-PCR (AU, Arbitrary Units). Values represent the mean±s.d. of three biological replicates and statistical differences denoted as **P*<0.05 or ****P*<0.001. (F) *YAP8* overexpression recovers *ufd2* growth in cells exposed to arsenic stress. Exponential phase BY4742 WT and the *ufd2* mutant strain overexpressing *YAP8* or the vector alone were serially diluted and spotted onto SD media supplemented with 1.5 As(III) or 2 mM As(V). Growth was recorded after 2 days incubation at 30°C. A representative experiment is shown.

We next examined Yap8 stability in WT and *ufd2* using CHX. Given that both Ufd2 and Yap8 are highly expressed in cells exposed to 1.5 mM arsenite for 90 min (Figs 3C and 4A), this condition was used to induce Yap8 expression in the cycloheximide chase experiments. After arsenic removal, cells were resuspended in fresh media containing CHX and incubated up to 120 min. Remarkably, Yap8 turnover rate was significantly increased in the *ufd2* mutant (half-life 37 min) as compared to the WT (half-life 98 min) (Fig. 4B). Moreover, we also observed a reduced Yap8 stability in *ufd2* cells exposed to 2 mM arsenate for 60 min prior to CHX treatment (supplementary material Fig. S3). Altogether, these results indicate that Ufd2 regulates Yap8 stabilization. To support the specificity of Ufd2-dependent Yap8 stabilization, it was monitored the Mps1 kinase turnover, a well known substrate of Ufd2 degradation pathway (Liu *et al.*, 2011). We therefore performed CHX chase assays to assess the stability of *c*-myc tagged Mps1 under the regulation of

GAL1-inducible promoter (supplementary material Table S2) in WT, *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ strains. The latter corresponds to a mutant in which the U-box domain, essential for Ufd2-mediated Mps1 proteolysis, was deleted (Liu *et al.*, 2011). As expected, Mps1 was readily degraded in WT cells and the absence of Udf2 as well as of Udf2 U-box domain clearly decreases its degradation rates, consistent with Mps1 being targeted to proteasome (Fig. 4C).

Our data suggest that impaired Yap8 levels observed in *ufd2* cells may be responsible for their reduced tolerance to arsenite. Aiming at strengthening this hypothesis we have performed epistasis analyses to compare cellular growth and expression of the Yap8 target gene *ACR3* in WT, single *yap8* and *ufd2* mutants as well as in the double *yap8ufd2* mutant. In agreement with previous findings, *yap8* is very sensitive to arsenic stress due to a severe downregulation of *ACR3* expression as revealed by qRT-PCR (Fig. 4D,E) (Menezes *et al.*, 2004; Wysocki *et al.*, 2004). The growth patterns and *ACR3* expression profile of the double *yap8ufd2* mutant are almost identical to those observed for *yap8* (Fig. 4D,E) implying that Ufd2 seems to contribute to arsenic tolerance through the regulation of Yap8 stability and transcriptional activity (Fig. 4A,B,E). In contrast, the *ufd2* mutant is more resistant to arsenic than *yap8* being only sensitive to high doses of As(III) and As(V) (Figs 3A and 4D). In line with the notion that *ACR3* expression levels determine the extent of arsenite tolerance, *ACR3* mRNA levels were significantly higher in *ufd2* cells than in the *yap8* strain (Fig. 4E). Additionally, *YAP8* overexpression in the *ufd2* mutant restores arsenic tolerance of this strain (Fig. 4F) indicating that enhancement of *YAP8* expression compensates for lower protein stability in the absence of Ufd2.

Collectively, these results point out Ufd2-mediated stabilization as a further regulatory mechanism contributing to the tight control of Yap8 activity.

Ufd2 U-box motif is not required for Yap8 stability and arsenic tolerance

Several reports indicate that Ufd2 U-box motif is essential for its ubiquitination activity (Aravind and Koonin, 2000; Tu *et al.*, 2007). We have

therefore investigated whether this activity was also required for Yap8 stabilization and cell tolerance to arsenic stress. BY4741 isogenic strains were used for these experiments since the *Ufd2*$^{U\text{-}box\Delta}$ mutant is available exclusively in this yeast background (Liu *et al.*, 2011). First, it was examined Yap8-HA kinetics in WT, *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ strains induced for 90 min with As(III). Corroborating the data from the BY4742 strain, Yap8 levels were found to be reduced in the *ufd2* mutant (Fig. 5A). Remarkably, the *Ufd2*$^{U\text{-}box\Delta}$ mutant displayed similar Yap8 levels as compared to the WT strain indicating that Ufd2 U-box domain is not involved in the regulation of Yap8 protein levels under arsenic stress. Next, Yap8 stability was monitored in the same strains. Cells were induced for 90 min with As(III) after which arsenic was removed and cells were treated with CHX for further 90 min. Supporting previous results obtained in the BY4742 strain (Fig. 4B), Yap8 stability is compromised in the *ufd2* mutant (Fig. 5B, half-life 25 min). Notwithstanding, *Ufd2*$^{U\text{-}box\Delta}$ mutant cells displayed similar Yap8 half-life compared to WT cells (67 and 63 min, respectively) confirming that Ufd2 U-box motif is dispensable for Yap8 stabilization during arsenic stress.

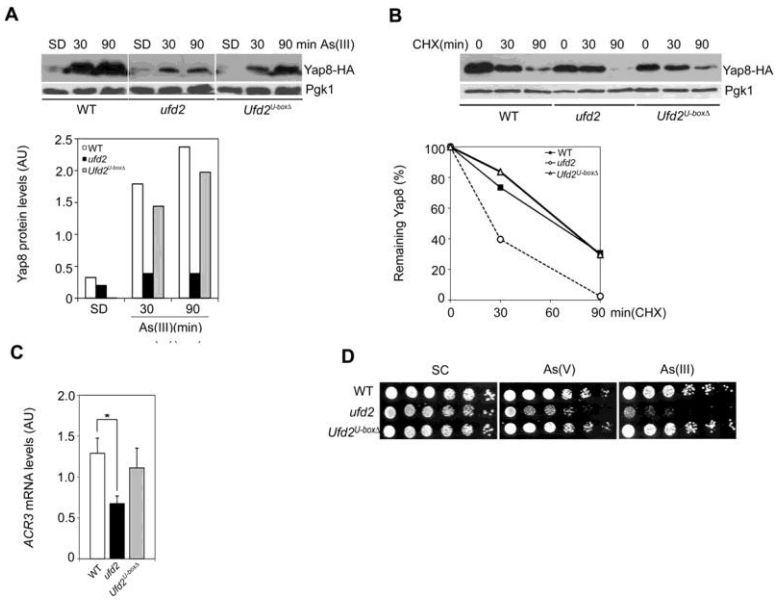

Fig. 5. Ufd2 U-box motif is not required for Yap8 stabilization. (A) Yap8 levels are unaffected in the *Ufd2*$^{U\text{-}box\Delta}$ mutant strain compared to the wild type strain. BY4741 wild

Fig. 5 (*Continued*)
type (WT), *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ strains expressing Yap8-HA were incubated with 1.5 mM As(III), harvested at the indicated time-points and subjected to immunoblotting using anti-HA and anti-Pgk1 antibodies. The graph represents relative Yap8 levels (AU, Arbitrary Units). A representative experiment is shown; SD, control. (B) Yap8 stability is similar in WT and *Ufd2*$^{U\text{-}box\Delta}$ mutant strains. The same strains were first exposed to 1.5 mM As(III) for 90 min, washed and subsequently treated with 0.1 mg/ml cycloheximide (CHX) up to 90 min prior to immunoblotting, as indicated above. The graph represents the percentage of remaining Yap8 protein after CHX addition. Estimated Yap8 half-life is 63 min in the WT strain, 25 min in *ufd2* and 67 min in *Ufd2*$^{U\text{-}box\Delta}$. (C) *ACR3* mRNA levels are unaltered in the *Ufd2*$^{U\text{-}box\Delta}$. The same strains were challenged with 1.5 mM As(III) for 90 min and *ACR3* mRNA levels were determined by qRT-PCR (AU, Arbitrary Units). Values represent the mean±s.d. of three biological replicates and statistical differences denoted as **P*<0.05. (D) The *Ufd2*$^{U\text{-}box\Delta}$ mutant is tolerant to arsenic stress. Exponential phase BY4741 WT, *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ cells were serially diluted and spotted onto SC media supplemented or not with 2 mM As(V) or 1.5 mM As(III). Growth was recorded after 2 days incubation at 30°C. A representative experiment is shown.

Having established that Ufd2 mediates Yap8 stabilization independent of the U-box motif, these strains were used to cement the premise that Yap8 stabilization correlates with the enhancement of Yap8 activity and tolerance to arsenite stress. We have then analysed *ACR3* expression in WT, *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ cells by qRT-PCR and found that the *Ufd2*$^{U\text{-}box\Delta}$ mutant and the WT strain exhibit similar *ACR3* mRNA steady state levels, which are higher than the *ufd2* mutant (Figs 4E and 5C). Consequently, cells lacking the U-box domain were tolerant to 1.5 mM As(III) and 2 mM As(V), as it was the WT strain, while growth of *ufd2* was impaired under these conditions (Figs 3A, 4D and 5D). In agreement with our previous results showing that Yap8 is not regulated at the transcriptional level (Menezes *et al.*, 2004), *YAP8* mRNA levels were similar in WT, *ufd2* and *Ufd2*$^{U\text{-}box\Delta}$ cells (data not shown). These results indicate that Ufd2 does not regulate *YAP8* expression, yet it is involved in post-translational mechanisms modulating Yap8 levels.

Altogether, these data firmly establish that the U-box domain is not essential for Ufd2 regulation of Yap8 levels and further support the

hypothesis that arsenic-sensitive phenotype of the *ufd2* mutant is mediated via diminished Yap8 activity.

Ufd2 stabilizes Yap8 independent of the ubiquitin-proteasome pathway

As to determine whether Ufd2 role on Yap8 stabilization is connected to UPP we next investigated the requirement of the UPP components Ubc4, Rad23 and Dsk2 to Yap8 stability under arsenic stress conditions. The E2 enzyme Ubc4 is upstream of Ufd2 in the ubiquitination process of proteins targeted to proteasome (Jentsch *et al.*, 1990; Sommer and Seufert, 1992) whereas Rad23 and Dsk2 are Ufd2-downstream players bridging ubiquitinated proteins to the proteasome (Chen and Madura, 2002; Funakoshi *et al.*, 2002; Kim et al., 2004). Importantly, the concerted action of Ufd2, Rad23 and Dsk2 in the degradation of Mps1 kinase was already described (Liu *et al.*, 2011). In contrast to the reduced Yap8 stability observed in the ufd2 strain, Yap8 half-life was found to be higher than the WT strain in the *ubc4*, *rad23* and *dsk2* mutants challenged with arsenite (Fig. 6A-C). Furthermore, arsenite-mediated upregulation of *ACR3* is not significantly affected in these mutants (Fig. 6D) indicating that Ubc4, Rad23 and Dsk2 are dispensable for Ufd2-mediated Yap8 stabilization and transcriptional activity. These results are also consistent with the observation that Yap8 is stabilized in mutants with defective proteasomal activity (Di and Tamas, 2007).

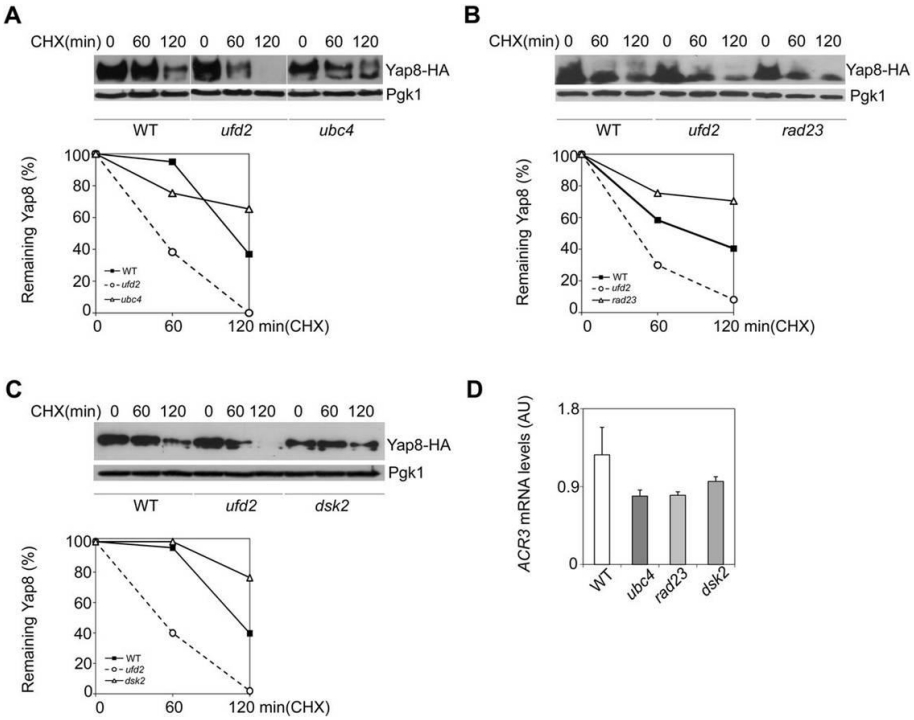

Fig. 6. Ubiquitin proteasome pathway (UPP) enzymes Ubc4, Rad23 and Dsk2 do not interfere with Yap8 stability in arsenic-exposed cells. BY4742 wild type (WT), *ubc4* (A), *rad23* (B) and *dsk2* (C) mutant strains expressing Yap8-HA were first exposed to 1.5 mM As(III) for 90 min, washed and subsequently treated with 0.1 mg/ml cycloheximide (CHX) up to 120 min prior to immunoblotting using anti-HA and anti-Pgk1 antibodies. The graphs represent the percentage of remaining Yap8 protein after CHX addition. Representative experiments are shown. (D) *ACR3* mRNA levels remain unaltered in *ubc4*, *rad23* and *dsk2* mutant cells. The same strains were challenged with 1.5 mM As(III) for 90 min and *ACR3* mRNA levels were determined by qRT-PCR (AU, Arbitrary Units). Values represent the mean±s.d. of three biological replicates. No significant statistical differences were observed.

Overall, these data disclose a novel Ufd2 role beyond degradation independent of Ubc4, Rad23 and Dsk2.

DISCUSSION

The b-ZIP transcription factor Yap8 plays a key role in arsenic stress responses as it regulates expression of the arsenic detoxification genes *ACR2* and *ACR3*. It was previously shown that Yap8 activity is controlled at different levels, including its degradation by the UPP under physiological conditions and stabilization upon arsenic stress (Fig. 1) (Menezes *et al.*, 2004; Di and Tamas, 2007). Nevertheless, the mechanisms by which Yap8 circumvents degradation under arsenic stress have not yet been deciphered. Here, we move a step forward in the characterization of these mechanisms by identifying the E4-Ub fusion degradation enzyme Ufd2 as a Yap8-interaction partner (Fig. 2) and a mediator of arsenic tolerance in *S. cerevisiae*. This conclusion is supported by data indicating that *ufd2* cells are less tolerant to either arsenite or arsenate than the parental WT strain (Figs 3A, 4D and 5D) and that *UFD2* expression is highly induced in cells challenged with these compounds (Fig. 3B,C).

The mechanism by which Ufd2/Yap8 interaction governs yeast adaptation to arsenic stress was unveiled by the demonstration that *ufd2* cells exhibit decreased Yap8 levels as a consequence of impaired protein stability (Figs 4A,B and 5A,B). Hence, the reduction of Yap8 levels in the *ufd2* mutant compromises *ACR3* expression thereby affecting yeast tolerance to arsenic stress (Figs 3A; 4D,E and 5C,D). The molecular pathway underlying Ufd2 role in cellular protection against arsenic injury was strengthened by epistasis analyses revealing that *yap8* and the double *yap8ufd2* mutant cells challenged with arsenic compounds display almost identical growth patterns and *ACR3* expression profiles (Fig. 4D,E). These data, reinforced by the demonstration that Ufd2 and Yap8 interact *in vivo*, clearly connect Ufd2-mediated arsenic tolerance to Yap8 stability and transcriptional activity (Fig. 4A,B,E). The fact that *YAP8* overexpression restores arsenic tolerance of *ufd2* cells (Fig. 4F) may indicate that Ufd2 operates an additional regulatory mechanism contributing to the tight control of Yap8 activity.

The recapitulation of Ufd2-dependent degradation of Mps1, an essential protein kinase required for spindle pole body (SPB) duplication (Liu *et al.*, 2011), was performed in our experimental conditions to corroborate the specificity of Ufd2-mediated Yap8 stabilization (Fig. 4C). Thus, maintenance of Yap8 levels by the E4-Ub ligase Ufd2, a well-known component of the proteolytic machinery, represents the most striking finding of this work and the first report of a yeast Ub-ligase stabilizing a transcription factor. Interestingly, it was shown that the Ufd2 human orthologue, UFD2a, also attenuates degradation of the ΔNp63α regulator by increasing its half-life and transcriptional activity (Chatterjee *et al.*, 2008). The current knowledge of Ub ligases is far more complex and diverse. For example, the E3 MDM2 mediates p53 regulation via the UPP (Moll and Petrenko, 2003), but it can also bind to the p53 relative protein, p73, stabilizing its levels (Ongkeko et al., 1999).

Yeast Ufd2 contains a U-box domain, which is present in proteins from yeast to humans, and is associated to the enzymatic activity of E4s necessary for their proteolytic function (Tu *et al.*, 2007). To show that Ufd2 stabilization role upon Yap8 is independent of its proteolytic function, various assays using the *Ufd2*$^{U\text{-}boxΔ}$ mutant were performed. The data resumed in Fig. 5 show that the U-box motif is required neither for Yap8 stability nor its activity. In line with the hypothesis that Ufd2 mediates arsenic tolerance through the regulation of Yap8 levels, the U-box domain is dispensable for cell adaptation to arsenic injury. This novel Ufd2 function was further reinforced by genetic analyses showing that Ubc4, Rad23 and Dsk2, components of proteolytic pathways, are not required for Yap8 stabilization (Fig. 6). Taking together, these results indicate that Ufd2, usually associated to protein degradation pathways, may be a bi-functional protein exerting also a role beyond proteolysis regulation.

To conclude, we have shown here a novel function of the Ub-fusion degradation enzyme Ufd2 in the regulation of Yap8 stability and consequently arsenic tolerance. However, further efforts are required for a deep understanding of the molecular events regulating Yap8 stability by Ufd2.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The yeast strains and plasmids used in this study are listed in supplementary material Tables S1 and S2, respectively. To generate the *yap8ufd2* double mutant, the *UFD2* gene was disrupted in the *yap8* mutant strain using the oligonucleotides 1 and 2 (supplementary material Table S3) and the microhomology PCR method (Gueldener *et al.*, 2002). *UFD2* deletions were confirmed by PCR using genomic DNA and *UFD2* specific oligonucleotides (supplementary material Table S3, oligonucleotides 3 and 4). HA-tagged UFD2 was generated by homologous recombination into the pRS416 vector (Agilent Technologies, Santa Clara, CA, USA) previously linearized with SmaI (Fermentas™ Thermo Fisher Scientific Inc., Rockford, IL, USA), using the In-Fusion® HD Cloning Plus CE kit (Clontech Laboratories, Inc., Mountain View, CA, USA) and oligonucleotides indicated in supplementary material Table S3 (oligonucleotides 10 to 13) to generate pRS416-*UFD2-HA*. *UFD2-HA* was then sub-cloned into YCplac111 vector (Agilent Technologies) as a *Sma*I fragment. *GAL4*^{AD}*UFD2* fusion was obtained by PCR amplification of *UFD2* gene lacking the ATG codon (oligonucleotides 8 and 9, supplementary material Table S3) and subsequent cloning into the pGADT7-Rec vector (Clontech Laboratories, Inc.), previously linearized with *Sma*I. Sequence integrity of *UFD2* constructions was confirmed by sequencing with the oligonucleotides listed in supplementary material Table S3 (oligonucleotides 5 to 7). PCR reactions were performed using the Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, US) and a Trio-ThermoBlock (BioMetra, Goettingen, Germany).

Yeast strains were maintained in YPD solid medium [1% yeast extract, 2% bactopeptone, 2% glucose and 2% of agar (Difco™ Thermo Scientific Inc.)]. They were grown in synthetic complete (SC) medium [0.67% ammonium sulfate-yeast nitrogen base without amino acids (Difco™ Thermo Scientific Inc.), 2% glucose, supplemented with essential amino acids and bases], synthetic defined (SD) medium [0.67% ammonium sulfate-yeast

nitrogen base without amino acids, 2% glucose, supplemented with required amino acids and bases] or minimal medium (MM) [0.67% ammonium sulfate-yeast nitrogen base without amino acids, 2% glucose, supplemented only with amino acids and bases corresponding to the respective auxotrophic markers] at 30°C, with orbital agitation (200 rpm). Absorbance at 600 nm (OD600) was measured using a SmartSpec™ 3000 (Bio-Rad Laboratories, Hercules, CA, USA). When indicated, 1.5 mM sodium arsenite [As(III), $NaAsO_2$], 2 mM sodium arsenate [As(V), $Na_2HAsO_4.7H_2O$], 100 µM MG132 or 0.1 mg/ml cycloheximide (CHX), all purchased from Sigma-Aldrich (St. Louis, MO, USA), were added to cultures. Phenotypic growth assays were carried out by spotting 5 µl of sequentially diluted (~5×103 to 10 cells) early exponential phase cells (OD_{600} 0.5±0.05) onto the surface of the SC, SD or MM medium containing As(III) or As(V). Growth was recorded after 2 days at 30°C. For the growth curves, early exponential phase cultures were diluted to 0.1±0.01, and were exposed or not to As(III) for 24 h at 30°C, with agitation, in a 96-well microplate. OD_{600} was monitored in intervals of 1 h using the Epoch™ BioTek spectrophotometer (Winooski, VT, US). *MPS1-c-myc* encoding cells were pre-grown to early exponential phase (OD_{600} 0.5±0.05) in YEP-Raffinose (2% peptone, 1% yeast extract, 2% D-glucose, 1% raffinose) liquid media, resuspended in YEP-Galactose (2% peptone, 1% yeast extract, 2% galactose) liquid media and incubated for 180 min at 30°C with orbital agitation (200 rpm).

The bacterial Escherichia coli strain XL1-Blue (Agilent Technologies) was used as a host for routine cloning purposes. Ampicillin (Sigma-Aldrich) to a final concentration of 100 µg/ml was used to select recombinant cells.

Yeast two-hybrid (Y2H) analyses

The $Gal4^{DBD}$Yap8 fusion was used as a bait protein to screen a yeast cDNA library constructed in the pGADT7-Rec vector (Clontech Laboratories, Inc.). Library construction and screening, by mating, were performed according to Matchmaker™ Gold Yeast Two-hybrid System (Clontech Laboratories, Inc.). Diploid cells displaying histidine and adenine prototrophies and β-galactosidase activity were selected for further studies. The plasmids were

isolated, amplified in *E. coli* and re-tested under the same conditions. The resulting prey plasmids were sequenced and the respective DNA sequences identified using the BLAST algorithm.

Co-immunoprecipitation assays

Co-immunoprecipitation assays were performed as previously described (Soutourina *et al.*, 2006), with minor modifications. Briefly, Y187 cells co-expressing $Gal4^{DBD}$Yap8/$Gal4^{AD}$Ufd2 were grown until early exponential phase (OD_{600} 0.5±0.05) and challenged with 2 mM As(V) for 60 min. Protein extracts were generated in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, 10% glycerol) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) to a final concentration of 1 mM, by cell disruption with a FastPrep®-24 instrument (MP Biomedicals, France). *c*-myc-tagged proteins were immunoprecipitated by the incubation of cell lysates with anti-*c*-myc mouse monoclonal antibody (9E10; Roche; Cat. No. 11 667 149 001), prebound to Dynabeads Pan Mouse IgG (Invitrogen™ Thermo Fisher Scientific Inc.) in a rotating wheel, overnight at 4°C. Immunoprecipitates were washed three times with phosphate-buffered saline (PBS) and were eluted from beads by heating the samples for 10 min at 65°C using Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue dye). Immunoprecipitated proteins, along with the whole cell extracts, were loaded on a 12% polyacrylamide gel and were analysed by immunoblotting, as described below, using anti-*c*-myc, anti-HA and anti-Pgk1 antibodies. Reciprocal co-immunoprecipitation assays were performed using BY4742 cells, co-expressing Yap8-*c*-myc/Ufd2-HA, challenged with 1.5 mM As(III) for 90 min. HA-tagged proteins were immunoprecipitated by the incubation of cell lysates with anti-HA mouse monoclonal antibody (12CA5; Roche; Cat. No. 11 583 816 001).

Cycloheximide chase and immunoblot analysis

Immunoblottings were performed using early exponential phase cells (OD^{600} 0.5±0.05) challenged with 1.5 mM As(III) or 2 mM As(V), and harvested at

the indicated time-points. For Yap8-HA stability assays, cells were pre-exposed either to 1.5 mM As(III) or to 2 mM As(V), and washed three times with phosphate-buffered saline (PBS) to remove arsenic compounds. Cells were then resuspended in fresh media supplemented with cycloheximide (CHX) to a final concentration of 0.1 mg/ml, and samples were collected at the indicated time-points. Also, co-treatments with As(III) and CHX were carried out. Total proteins were extracted by the TCA lysis method and protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories) being then 70–100 µg resolved by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), using a Trans-Blot Semy Dry transfer system (Bio-Rad Laboratories). Immunoblottings were performed following standard procedures (Ferreira *et al.*, 2012) and using the following antibodies: anti-HA-Peroxidase high affinity rat monoclonal antibody (3F10; Roche; Cat. No. 12 013 819 001), anti-*c*-myc mouse monoclonal antibody (9E10; Roche; Cat. No. 11 667 149 001), anti-Pgk1 (Invitrogen; Cat. No. 459250), and goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-2314). Peroxidase signals were detected using the Super Signal® West Pico and West Femto Maximum Sensitivity Substrates (Thermo Fisher Scientific). Quantification of protein signals was carried out using the ImageJ software (NIH, Bethesda, MD). Pgk1 was used as loading control in all assays. In CHX chase assays, the percentage of proteins present after CHX addition is shown. Protein half-life was estimated as previously described (Boban *et al.*, 2014).

β-galactosidase assays

β-galactosidase measurements were performed as previously described (Menezes *et al.*, 2004). Briefly, early exponential phase cells subjected or not to Na_2HAsO_4 were harvested after 60 min and enzyme activity was assayed by following the degradation of the colorimetric substrate ONPG (o-nitrophenyl-β-D-galactopyraniside) (Sigma-Aldrich) at A_{420} using a microplate spectrophotometer (Epoch™ BioTek). Values were normalized against the number of cells of each culture. Miller units were calculated as previously

described (Pimentel *et al.*, 2014). The results are the average of at least three biological replicates (n=3).

Quantitative real-time PCR analyses

RNA was isolated from early exponential phase cultures (OD_{600} 0.5±0.05) that were either non-exposed or exposed to 1.5 mM $NaAsO_2$, and harvested at the indicated time-points. RNA samples were treated with the TURBO DNA-free™ kit (Ambion, Cambridge, UK) according to the manufacturer's instructions, and purified by on-column DNAse I digestion using the RNase-Free DNase Set (Qiagen, Hilden, Germany). Total RNA (1 µg) was reverse transcribed with Transcriptor Reverse Transcriptase (Roche). qRT-PCR reactions were performed in the Light Cycler 480 Real-Time PCR System using Light Cycler Fast Start DNA Master SYBR Green I (Roche). Relative standard curves were constructed for each gene, using triplicate serial dilutions of cDNA. The relative expression of the genes was calculated by the relative quantification method with efficiency correction, using the LightCycler Software 4.1 (Roche). Actin (*ACT1*) was used as a reference gene. All assays were made using biological triplicates. The oligonucleotides used are listed in supplementary material Table S3 (oligonucleotides 14 to 23).

Statistical analysis

The results reported in this study are the averages of at least three independent experiments and are represented as the means±s.d. Differences amongst treatments were detected by the parametric Student's t-test using the XLSTAT statistical software 2015.1. Statistical differences between treatments are denoted as **P*<0.05 and ****P*<0.001.

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FOOTNOTES

Competing interests

The authors declare no competing or financial interests.

Author contributions

R.T.F., R.A.M. and C.R.P. conceived and designed the experiments, analysed the data and wrote the manuscript. R.T.F. and R.A.M performed the experiments.

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Supplementary Material

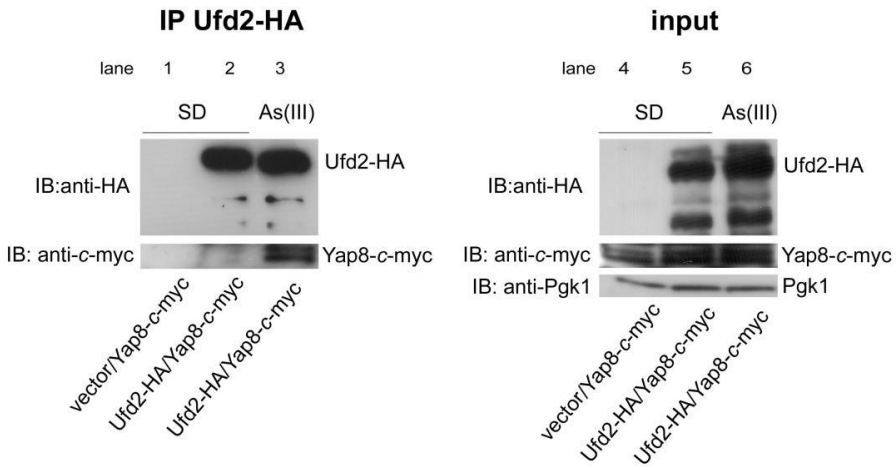

Fig. S1. Reciprocal co-immunoprecipitation assays showing arsenic-mediated interaction between Yap8 and Ufd2. BY4742 cells co-transformed with vector/*YAP8-c*-myc (lanes 1 and 4) or *UFD2*-HA/*YAP8-c*-myc (lanes 2, 3, 5 and 6) were exposed or not to 1.5 mM As(III) for 90 min and Ufd2-HA was immunoprecipitated with anti-HA antibody. Immunoblotting was performed using anti-HA, anti-*c*-myc and anti-Pgk1 antibodies. A representative experiment is shown. IP – immunoprecipitation; IB – immunoblotting; SD – Synthetic Defined medium, control condition.

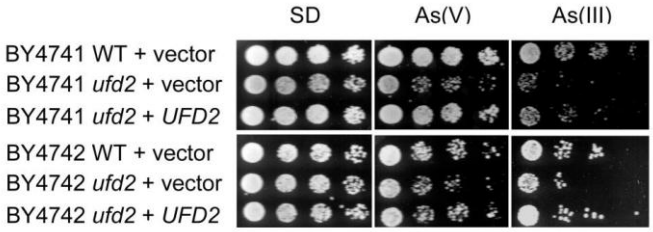

Fig. S2. As(V)- and As(III)-sensitivity phenotypes of *ufd2* are restored by expressing an episomal copy of *UFD2*. Exponential phase BY4741 and BY4742 wild type (WT) and *ufd2* cells expressing an episomal copy of *UFD2* or the respective control vector were serially diluted and spotted onto SD media supplemented or not with 2 mM As(V) or 1.5 mM As(III). Growth was recorded after 2 days incubation at 30ºC. A representative experiment is shown.

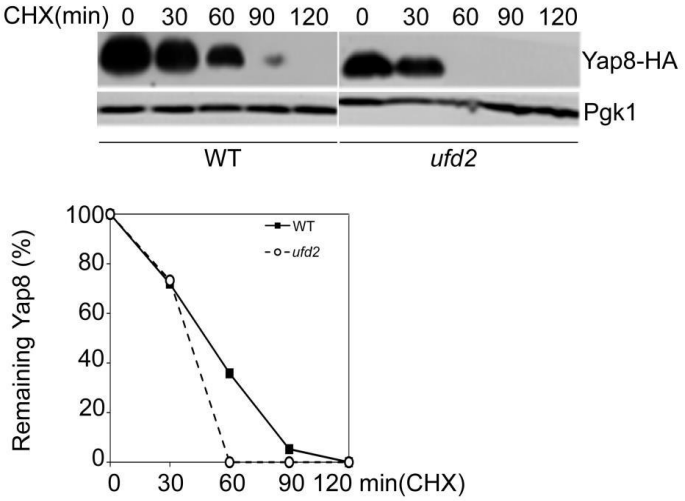

Fig. S3. Ufd2 mediates Yap8 stabilization under As(V) stress conditions. BY4742 wild type (WT) and *ufd2* mutant strains expressing Yap8-HA were pre-treated with 2 mM As(V) for 60 min, washed and subsequently treated with 0.1 mg/mL cycloheximide (CHX) up to 120 min prior to immunoblotting using anti-HA and anti-Pgk1 antibodies. The graph represents the percentage of remaining Yap8 protein after CHX addition. A representative experiment is shown.

Table S1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference or source
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0* ura3Δ0	EUROSCARF*
ufd2	*MATα his3Δ1 leu2Δ0* lys2Δ0 *ura3Δ0 YDL190c::kanMX4*	EUROSCARF
yap8	*MATα his3Δ1 leu2Δ0* lys2Δ0 *ura3Δ0 YPR199c::kanMX4*	EUROSCARF
yap8ufd2	*MATα his3Δ1 leu2Δ0* lys2Δ0 *ura3Δ0 YPR199c::kanMX4 YDL190c::HIS3MX4*	This study
ubc4	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBR082c::kanMX4*	ATCC**
rad23	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YEL037c::kanMX4*	EUROSCARF
dsk2	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YMR276w::kanMX4*	EUROSCARF
Y187	*MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3 112 gal4Δ met-gal80Δ URA3::GAL1UAS-GAL1TATA-lacZ MEL1*	Clontech Laboratories, Inc.
BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0* ura3Δ0	EUROSCARF
ufd2	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDL190C::kanMX4*	EUROSCARF
Ufd2$^{U\text{-}box\Delta}$	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDL190C2568-2886 bp::HIS3*	Chang Liu *et al.* (2011)

*EUROpean *Saccharomyces Cerevisiae* ARchive for Functional analysis
** American Type Culture Collection

Table S2. Plasmids used in this study.

Name	Features	Reference or source
pRS416	*CEN*, *URA3*	Agilent Technologies
pRS416-*YAP8-HA*		Our unpublished work
pRS416-*YAP8-c-myc*		Amaral *et al*. (2013)
pRS416-*UFD2-HA*		This study
pGADT7-Rec	2μ, LEU2, HA	Clontech
pGADT7-*T-antigen*		Clontech
pGADT7-*UFD2*		This study
pGBKT7	2μ, *TRP1*, *c-myc*	Clontech
pGBKT7-*LamC*		Clontech
pGBKT7-p53		Clontech
pGBKT7-*YAP8*		Our unpublished work
YCplac111	*CEN, LEU2*	Agilent Technologies
YCplac111-*UFD2-HA*		This study
YEplac181	2μ, LEU2	Agilent Technologies
YEplac181-*YAP8-HA*		Our unpublished work
pGal-*MPS1-c-myc*	*Integrative, URA3, GAL1promoter*	Chang Liu *et al*. (2011)

Table S3. Oligonucleotides used in this study.

#	Name	Sequence (5' – 3' UTR)
1	UFD2-HIS-Fw [D]	GGGAAAAGTTAACTTTGAAAGTAGAACCCTCATTCCATAGATCGTACGCTGCAGG
2	UFD2-HIS-Rv [D]	TTGATTAGGGTCAATTTTGCAATTTATTCTATCACTTATTTTAGGGAGACCGGCAGAT
3	A1-UFD2 [D]	GGCTTGCTGGTACAATATGG
4	A4-UFD2 [D]	CCAGAGCTTTGAGAAGAG
5	UFD2 400-Fw *	GAAAGGTAAAGTTGAC
6	UFD2 1300-Fw *	CTTAAACTCAAGGAC
7	UFD2 2200-Fw *	GGTAAATTAGTGCAG
8	UFD2-pGADT7-Fw [C]	ACCGCCATAGAAGATATTTTAC
9	UFD2-pGADT7-Rv [C]	TCACTCGCTTGCTTTATG

Table S3. *(Continued)*

#	Name	Sequence (5' – 3' UTR)
10	pRS416-SmaI-UFD2-P1000-Fw [C]	GAATTCCTGCAGCCCAATATTCTGTTA TTG
11	HA-UFD2-Rv [C]	AGCGTAATCTGGAACATCGTATGGGTA CATTCACTTATTCATTCA
12	HA-UFD2-Term500-Fw [C]	ATGTACCCATACGATGTTCCAGATTAC GCTTAGAATAAATTGCAA
13	UFD2-Term500-pRS416-SmaI-Rv [C]	ACTAGTGGATCCCCCAGTTGGCTGAA TTGA
14	UFD2-Fw ●	GACTTTCCTGTTGGATGAAG
15	UFD2-Rv ●	CTCTTCCTCTCTTGTTGGTG
16	ACR2-Fw ●	AGGCAACTCAAGGCCTAAT
17	ACR2-Rv ●	GAACATGCCAAGCGTTTGTA
18	ACR3-Fw ●	AAGAGGGTCTGGGGAAGAAA
19	ACR3-Rv ●	GCAATTGCCAGGGATAGTTC
20	YAP8-Fw ●	AACCGCCCACATGTAACACT
21	YAP8-Rv ●	TCCAACACACACTGAGAGCAG
22	ACT1-Fw ●	CTATTGGTAACGAAAGATTCA
23	ACT1-Rv ●	CCTTACGGACATCGACATCA

Fw – forward; Rv – reverse

[D] Gene disruption and mutant confirmation, *Sequencing**, C** Cloning, ●qRT-PCR

4

Role of Ubiquitin- and SUMO-Covalent Modification in the Regulation of the Yeast Transcription Factor Yap8

4.1

Preface

Similarly to non-proteolytic functions of Ub, SUMO has been shown to regulate protein localization, activity and to interfere with protein-protein interactions [1]. On the other hand, the molecular mechanisms underlying Yap8 post-translational regulation by Ubc4 [2], and Ufd2 [3] are still elusive. To provide further insights into Yap8 regulation, in this study it was addressed the prediction of putative Yap8 Lys targeted for Ub- and SUMO-attachment. These predictions constituted the starting-point to investigate the role of selected Lys residues in the post-translational control of Yap8 transcription factor. The replacement of single Lys62 or Lys198 by Arg resulted in the loss of Yap8 stability, down-regulation of *ACR3* mRNA levels, and decreased cell tolerance to arsenic. As revealed by the *in silico* analysis of Yap8 sequence, Lys62 and Lys198 are predicted sites of both ubiquitination and SUMOylation. However, our data suggest that these sites are not involved in the control of Yap8 by the UPS, at least under stress conditions; this is in agreement with previous demonstration that Yap8 escapes proteolysis in response to arsenic stress [3]. It remains to be elucidated whether the regulatory role of K62 and K198 is associated to the mono-ubiquitination and/or SUMOylation, thereby affording new clues on the molecular mechanisms modulating arsenic adaptation. Importantly, arsenic

induces SUMO-dependent pathways in human cells which in turn affects substrates in different ways, and particularly in leukemic cells [4].

4.2
Manuscript in Preparation

Ferreira, R.T., Menezes R.A., Rodrigues-Pousada, C. *Post-translational control of Yap8 transcription factor by ubiquitination and SUMOylation during arsenic stress response in Saccharomyces cerevisiae*.

Note: The manuscript does not completely follow this thesis formatting.

4.2.1
Author's Contribution

I had a major participation in this study: planning and execution of all experiments [cf. Methods Section: Site-directed Mutagenesis; Phenotypic Analysis; Immunoblot and CHX Chase Assays; Real-Time PCR; and all yeast transformations and routine plasmid amplification purposes], data analysis (with the exception of the *in silico* predictions), figures preparation, and manuscript preparation.

4.3
References for Chapter's Preface

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2. Di, Y. & Tamas, M.J. Regulation of the arsenic-responsive transcription factor Yap8p involves the ubiquitin-proteasome pathway. *J Cell Sci* 120, 256-264 (2007).
3. Ferreira, R.T., R.A., M. & Rodrigues-Pousada, C. E4-Ubiquitin ligase Ufd2 stabilizes Yap8 and modulates arsenic stress responses independent of the U-box motif. *Biology Open (BiO)* (2015).

4. Zhu, J. *et al.* A sumoylation site in PML/RARA is essential for leukemic transformation. *Cancer Cell* 7, 143-153 (2005).

Article in Preparation

Post-translational control of Yap8 transcription factor by ubiquitination and SUMOylation during arsenic stress response in *Saccharomyces cerevisiae*

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Abstract

Arsenic (As) stress response in *S. cerevisiae* is mainly controlled by the specific transcriptional regulator Yap8, that is involved in the activation of the As cellular detoxification machinery. Yap8 regulation occurs at the level of its sub-cellular localization, as well it is carried out by the Ubiquitin (Ub) system. Under non-stressed conditions, Yap8 shuttles between the cytoplasm, and the nucleus being rapidly retained in this cellular compartment upon As stress. Also, under uninduced conditions Yap8 is degraded by the proteasome mediated by the Ub (E2) enzyme Ubc4; whereas in presence of arsenic, the Ufd2(E4) binds to and stabilizes Yap8, thus revealing the importance of post-translational modifications (PTMs) in the regulation of Yap8. Here, we demonstrate that all the six predicted lysines (Lys, K) on Yap8 for Ub- and/or small ubiquitin-like modifier (SUMO)- attachment are important for the protein stability, and particularly single mutations of putative

high-scored SUMOylation sites (K62 and K198) decreased the protein half-life and transcriptional activity, as well as they compromise the cell tolerance to As(III). K62 and K198- also predicted as sites for Ub, suggest that a possible interplay between the Ub and SUMO systems may modulate Yap8 regulation. Finally, we observe that Yap8 stability is impaired in the temperature-sensitive *ulp1*^{ts} mutant, devoid of SUMO-protease activity at the restrictive temperature, suggesting for the first time a link between Yap8 regulation and (de)SUMOylation. Overall, Yap8 regulation by Ub and SUMO appears to constitute a step among the stability and activity processes of this transcription factor in yeast cells treated with As.

Introduction

In the budding yeast *Saccharomyces cerevisiae*, an attractive biological model to study environmental stress responses [5], resistance to arsenic is mainly achieved through the activation of the basic region-leucine zipper (bZIP) transcriptional regulator Yap8. It regulates transcriptional activation of the *ACR2* and *ACR3* genes, which encode arsenic detoxification proteins [6]. PTMs of Yap8 constitute a key step among the stability and activity of Yap8, either associated with its proteasomal degradation mediated by the E2-Ub enzyme Ubc4 under non-stressed conditions [2], or its stabilization and enhanced activity through the binding of E4-Ub enzyme Ufd2 under arsenic treatment [3]. Although the importance of Ufd2 in Yap8 stabilization has been established, the underlined Yap8 PTMs are still poorly understood. The Ub proteolytic system is one of the major protein-modification systems required for the selective degradation of cellular proteins [7]. Ubiquitination is a multi-step process which involves the components E1, E2, E3 and E4 catalytic components, resulting in the formation of an isopeptidic bond between the C terminus of ubiquitin and the ε-amino group of a lysine in the targeted protein for degradation by the 26 S proteasome [8, 9]. Notably, Yap8 represents a novel substrate of yUfd2 - among the very few previously identified in *S. cerevisiae* [8, 10, 11] – however, whose modification leads to

protein stabilization upon arsenic stress instead of multi-ubiquitination, and consequently degradation.

Besides ubiquitin, eukaryotic cells also express a group of ubiquitin-like proteins, such as SUMO that is also conjugated to the lysine residues of targeted substrates by an isopeptidic bond similar to that observed for the ubiquitin system [12]. SUMO regulates many aspects of cellular physiology to maintain cell homeostasis, both under normal and during stress conditions [13]. In contrast to ubiquitination, the covalent attachment of Ub-like proteins, SUMOylation- has critical roles in diverse cellular processes including genomic stability, cell cycle progression, intracellular trafficking, and transcription [14]. In most cases, SUMO conjugation affects localization, stability and/or activity of specific transcription factors, therefore emerging as an important post-translational regulation of transcriptional function [1]. Up to date, some studies have revealed that SUMO serves to repress transcription, while others have discussed that SUMO can also have profound positive effects on transcription [13]. As a dynamic and reversible process, substrates can be rapidly SUMOylated and deSUMOylated by specific proteases in the cell, termed Ub-like protein-specific proteases (Ulps). *S. cerevisiae* cells express a single SUMO paralogue, called Smt3, and two SUMO proteases- Ulp1 and Ulp2, that cleave Smt3 from distinct sets of substrates. Ulp1 is responsible for both removing SUMO/Smt3 from specific target proteins, and processing precursor SUMO into its conjugation-competent form (which turns Ulp1 essential for viability); whereas Ulp2 only appears associated to the disassembly of polySUMO chains [15, 16]. Moreover, Ulp1 localizes to the nuclear envelope and is encoded by an essential gene [17].

Aiming at unraveling the molecular mechanisms underlying Yap8 regulation by PTMs, we investigated the role of lysine residues in the modulation of Yap8 activity. Although at preliminary stages, our study provides indirect evidences that fine-tuning regulation of Yap8 may be associated to SUMOylation occurring at lysines that can also serve as ubiquitin modification sites. These amino modifications might be responsible

for a switch between Yap8 degradation in cells grown under normal conditions and Yap8 stabilization known to occur under As treatment.

Materials and Methods

Plasmids, Strains and Growth Conditions Used. Genotypes and vectors are listed in Table S1. Yeast strains were grown at 30ºC in YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose and 2% agar, all from Difco). For plasmid selection, synthetic dropout medium (SD) (0.67% yeast nitrogen base without amino acids, 2% glucose and 2% bacto-agar for solid medium), supplemented with required amino acids and bases was used. Transformations of *S. cerevisiae* strains were done using the standard lithium acetate protocol [18]. Transformations into the temperature-sensitive *ulp1*^{ts} strain were performed at 25ºC as described previously [19]. The *E. coli* strain was grown at 37ºC in LB broth (1% NaCl, 0.5% yeast extract and 1% bacto-tryptone, all from Difco). Selection of recombinant clones was performed after grown in the presence of 100 µg/mL ampicilin (Sigma-Aldrich).

Prediction of PTM sites. Putative SUMOylation and ubiquitination sites were predicted using the UbPred software (www.ubpred.org) [20] and the Abgent SUMOplot™ Analysis (http://www.abgent.com/sumoplot) program. In UbPred, lysine residues with a score of >0.69 were considered ubiquitinated with medium confidence. Low level threshold with cut-off values ranging from 0.62-0.69 were selected. In the SUMO analysis, lysine residues with a score of >0.8 were considered SUMOylated with high probability. Low level threshold with cut-off values ranging from 0.27-0.59 were selected.

Site-directed Mutagenesis. From pRS416-*YAP8-HA* and pRS416-*YAP8* templates, six single mutants at Lysine putative SUMOylation/ubiquitination sites were generated by PCR reactions using the primer pairs designed for introducing mutations in the cDNA nucleotide sequence of *YAP8* (Table S2). by using existing convenient restriction sites. The replaced fragments and the restriction site junctions were sequenced, as earlier described [21].

Phenotypic Analysis. Yeast cells were plated onto SD solid medium supplemented with $NaAsO_2$ [(arsenite; As(III)] (Sigma-Aldrich). Exponential phase grown cells were diluted in phosphate buffered saline (PBS) in order to spot cells. Growth at 30ºC was recorded after two days. Transformation with the empty vector was used as control. Cells were spotted onto SD plates lacking uracil.

Cycloheximide Chase and Immunoblot Assays. Cycloheximide (CHX) chase assays were performed as already reported, as well as determination of protein half-lives [3]. The Abs used for western blotting were: anti-HA-Peroxidase high affinity rat monoclonal (3F10), anti-Pgk1 (Invitrogen™ Thermo Fisher Scientific Inc, Illinois, USA) and goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology, Dallas, Texas, USA). The band intensity of the different immunoblot signals was estimated by Image J Software (NIH, Bethesda, MD). Pgk1 was used as loading control.

Real-Time PCR. Cells were grown until early exponential phase, cultures were left untreated or treated with 1.5 mM As(III). Cells were harvested after 30 and 90 minutes and RNA was isolated. qRT-PCR was performed as described earlier. Primers used in this assay are listed in Table S2.

Statistical Analysis. The qPCR results reported in this study are the averages of at least three independent experiments and are represented as the means±s.d. For the comparison between the samples, the parametric Student's *t*-test using the XLSTAT statistical software 2015.1 was applied. The statistical significant difference was considered when $^{**}P<0.01$ and $^{***}P<0.001$. All experiments were done in triplicate and the data are mean +/− SD of three independent experiments.

RESULTS

Identification of putative Ub and SUMO modification sites in Yap8. As one of the most important reversible protein PTMs, ubiquitination has been reported to be involved in diverse biological processes *S. cerevisiae* [22], being closely implicated in Yap8 post-translational regulation [3, 4]. Identification of ubiquitination sites in targets proteins is a critical step to

understand further the regulatory mechanisms UPP. The UbPred (www.ubpred.org) bioinformatics tool was used to predict ubiquitination sites from Yap8 amino acid sequence (Fig. 1A, left panel). With the assistance of UbPred, eight putative Yap8 acceptor Lys were identified at positions 46, 47, 62, 63, 90, 96, 126 and 198, some of which lie in the b-ZIP domain (K46, K47, K62; Fig. 1B). The Lys 46, 47, 90 and 96 were identified as medium confidence sites for ubiquitination, whereas the remaining residues K62, K63, K126 and K198 were identified as low confidence sites. The high impact of SUMOylation in the stability and activity of transcription factors in yeast and other organisms [1] led us to hypothesize that Yap8 could also be regulated through SUMOylation. Thus, we used the Abgent SUMOplot™ Analysis Program (http://www.abgent.com/tools) to predict and score potential SUMOylation sites within the Yap8 (Fig. 1A, right panel). The software identified eight putative SUMOylation sites at positions K8, K37, K46, K62, K96, K198, K284 and K288. Strikingly, the high-scored residues K62 and K198 were also predicted as putative Ub modification sites (Fig. 1B).

Figure 1. Mapping of the Ub- and SUMO- acceptor sites on Yap8. (*A*) The UbPred and Abgent SUMOplot™ Analysis Programs were used to predict and score ubiquitination and

Figure 1. (*Continued*)

SUMOylation sites on Yap8 *in silico*. (*B*) Inspection of Yap8 amino acid sequence. Amino acids marked with green represent putative ubiquitination sites whereas predicted SUMOylation sites on Yap8 are marked with red. Amino acids marked with blue were predicted to be both SUMO- and Ub- attachment sites on Yap8 with higher scores for SUMO or Ub, as indicated in subscript. The predicted Ub sites with medium confidence (K46, K47, K90, K96, and K198), and the highest scored SUMO sites (K62 and K198) are marked by an asterisk. Note that Yap8-K46, Yap8-K47 and Yap8-K62 localize to basic region of Yap8, known to be crucial for its DNA-binding specificity.

Lysine-to-Arginine substitutions of specific residues affect Yap8 stability. In order to determine whether any of the *lysine residues* was involved in the stability of Yap8, we introduced point mutations converting Lys (K) residues to Arg (R) residues. The four predicted lysine residues with medium confidence for ubiquitination (K46, K47, K90, K96 and K198) and the two putative high-scored lysines targeted by SUMO (K62 and K198) were chosen for the analyses. Following transformation of HA-tagged Yap8 wild-type or mutants in *yap8* mutant cells, cell lysates were subjected to immunoblotting and the turnover rates of Yap8 proteins were monitored by means of cycloheximide (CHX)-chase experiments (Fig. 2). Cells were first pre-incubated in the presence of As(III) to enhance Yap8 levels, which are very low under physiological conditions [4, 20]. Then, As(III) was removed from the growth medium, and CHX was added for up to 120 min to inhibit de novo protein synthesis. Yap8 protein half-lives were (Fig. 2). HA-tagged wild-type Yap8 (Yap-wt) exhibited a half-life of c.a. 104 min (Fig. 2). Faster degradation rates were determined for all Yap8 mutants, being the mutations K46R, K62R and K198R that mostly affected Yap8 stability (estimated half-lives are 58 min, 50 min and 43 min, respectively) (Fig. 2). These findings suggest that Yap8 Lys residues are essential for Yap8 stability, thus representing putative targets of PTMs, yet the nature of these modifications is currently unknown. Same results were obtained in cells expressing Yap8-wt-HA and Yap8-K62R-HA co-treated with CHX and As(III), the latter revealing a shorter half-life, although the Yap8 levels were enhanced in both

strains during the CHX treatment (data not shown), in agreement with that we have recently shown [3].

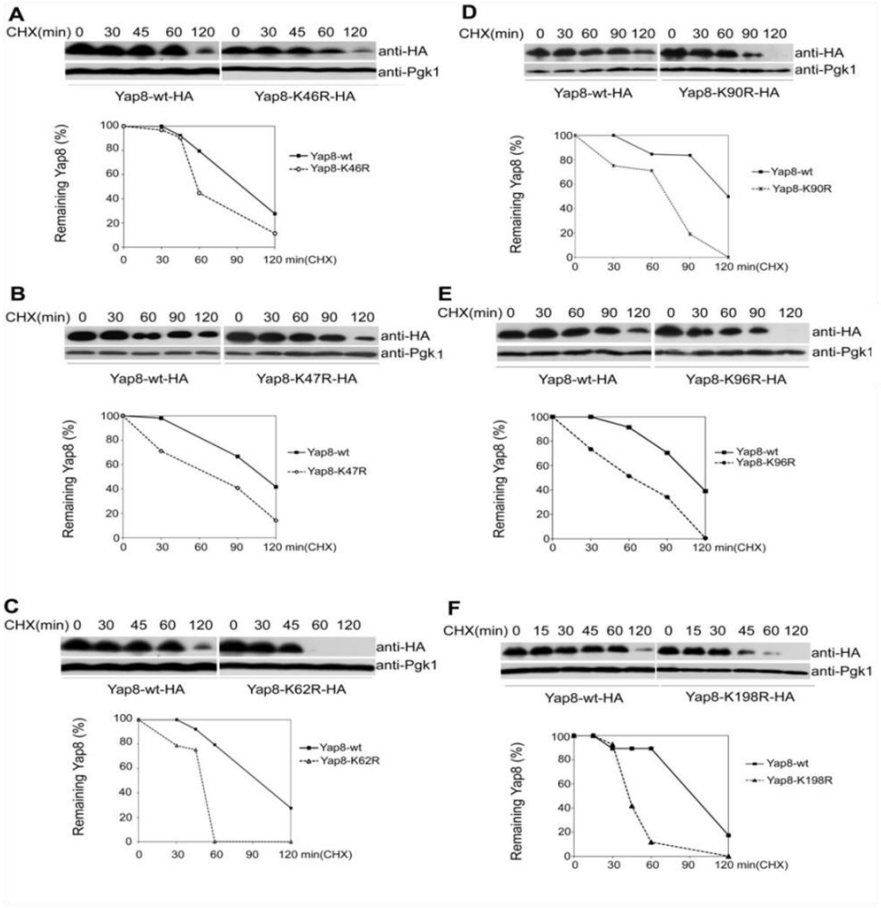

Figure 2. Mutations of putative Ub and SUMO modification sites in the Yap8 affect protein stability. Putative ubiquitination- or SUMOylation-deficient Yap8 mutants show decreased half-lives. BY4742 *yap8* mutant cells transformed with the HA-tagged Yap8-wild-type (Yap8-wt-HA) or the Yap8 mutant versions (Yap8-K46R-HA, Yap8-K47R-HA, Yap8-K62R-HA, Yap8-K90R-HA, Yap8-K96R-HA and Yap8-K198R-HA) were treated with 1.5 mM As(III) for 30 min, then washed to remove As(III) from the medium, and subsequently treated with cycloheximide (CHX) 0.1 mg/ml up to 120 min in a fresh growth medium. Western blots for Pgk1 were performed as internal loading controls. The graphs represent the percentage of remaining Yap8-wt or Yap8 mutants after CHX addition. The initial levels of the proteins were normalized to 100%. Representative experiments are shown. Yap8 half-lives: (*A*) 94 min (Yap8-wt) and 58 min (Yap8-K46R); (*B*) 110 min (Yap8-wt) and

Figure 2. (*Continued*)
70 min (Yap8-K47R); (*C*) 94 min (Yap8-wt) and 50 min (Yap8-K62R); (*D*) 120 min (Yap8-wt) and 72 min (Yap8-K90R); (*E*) 110 min (Yap8-wt) and 62 min (Yap8-K96R); and (*F*) 93 min (Yap8-wt) and 43 min (Yap8-K198R).

Lysine-to-Arginine substitutions of specific residues repress Yap8 activity, and cell tolerance to arsenic stress. To shed light on the role of putative Yap8 Lys residues targeted for PTMs, we investigated the requirement of Lys residues indicated above for arsenic stress responses. For that, *yap8* cells were transformed with a CEN plasmid carrying or not wt *YAP8* or the respective mutants. The WT strain transformed with the vector was used as a control. As shown in Fig. 3 A, *YAP8* expression in the *yap8* strain allowed the recovery of cell tolerance to As(III). Remarkably, only the Yap8 mutants K46R, K47R, K62R and K198R were found to be sensitive to As(III). To address the importance of these residues on Yap8 transcriptional activity, *ACR3* mRNA levels were quantified in cells expressing the Yap8 mutated versions K46R, K62R and K198R by RT-PCR. As shown in Fig. 3B, *ACR3* mRNA levels were severely decreased in cells expressing Yap8-K62R and Yap8-K198R. Mutation of Lys198 (Yap8-K198R) was sufficient to abrogate *ACR3* expression, suggesting that it exerts an essential role in Yap8 activity.

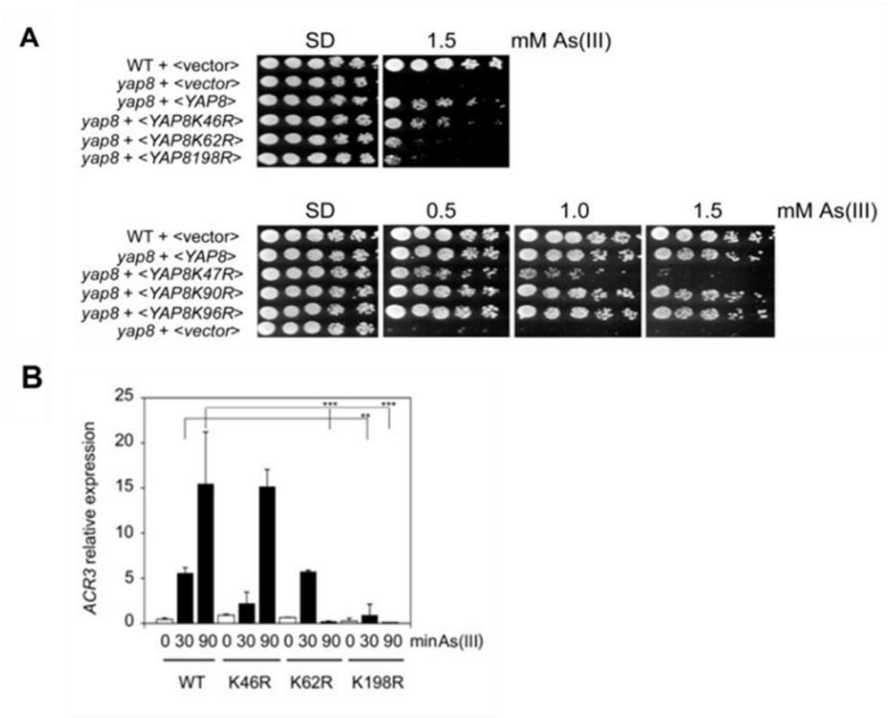

Figure 3. Yap8 Lys62 and Lys198- putative sites for Ub and SUMO, seem to be related to arsenic cell tolerance. Phenotypic sensitivity to As(III) and *ACR3* down-regulation in cells expressing Lys62- and Lys198-mutated Yap8. (*A*) Exponential-phase BY4742 cells transformed with the plasmid pRS416 [either empty, or carrying wild-type *YAP8* or *YAP8* mutants (*YAP8-K46R*, *YAP8-K62R*, *YAP8-K198R*, *YAP8-K47R*, *YAP8-K90R*, and *YAP8-K96R*)] were serially diluted, and spotted onto SD medium, supplemented with As(III) up to 1.5 mM; SD, control. Growth was recorded after 2 days at 30ºC. Representative experiments are shown. (*B*) Arsenic-mediated induction of *ACR3* mRNA levels is diminished or abolished in cells expressing Lys62- and Lys198- mutated Yap8, in contrast to *yap8* cells carrying the *YAP8-wt* (WT) or *YAP8-K46R* (K46R) constructs. *yap8* mutant cells carrying the native *YAP8-wt*, or mutations *YAP8-K46R*, *YAP8-K62R* and *YAP8-K198R* were exposed to 1.5 mM As(III) up to 90 min. Cells were harvested at the indicated times, RNA was extracted and analyzed for the kinetic study of *ACR3* expression, by RT-PCR. *ACR3* mRNA levels were normalized to reference *ACT1*. Values represent the means±s.d. of three biological replicates and statistical differences denoted as ***P*<0.01 and ****P*<0.001.

Impairment of deSUMOylation decreases Yap8 stability. Yap8 K62 and K198 are putative high-scored SUMOylation sites. Because the SUMO

pathway is controlled and is reversible by means of specific isopeptidase Ulp1 [22], we monitored Yap8 stability under arsenic conditions in the *ulp1*^{ts} mutant strain, exhibiting deficient Ulp1 isopeptidase activity, thus accumulating SUMOylated proteins. Yap8 stability was assessed by means of a cycloheximide (CHX) chase. The results in Fig. 4A indicate that impairment of deSUMOylation (or SUMO deconjugation) increases Yap8 turnover; estimated Yap8 half-life is 50 min in the WT strain vs. 18 min in the *ulp1*^{ts} mutant. Furthermore, the phenotype of *ulp1*^{ts} mutant cells shows that these cells are substantially more sensitive to increasing [As(III)] than their wild-type (WT) counterpart (Fig. 4B). These findings indicate that deSUMOylation process is associated to cell protection under arsenic treatment. Our results also suggest that Yap8 regulation is likely to involve SUMO, possibly at Lys62 and Lys198, as a mechanism to control Yap8 protein levels, and transcriptional activity. Further work is required to cement the hypothesis of Yap8 as a substrate for SUMO modification.

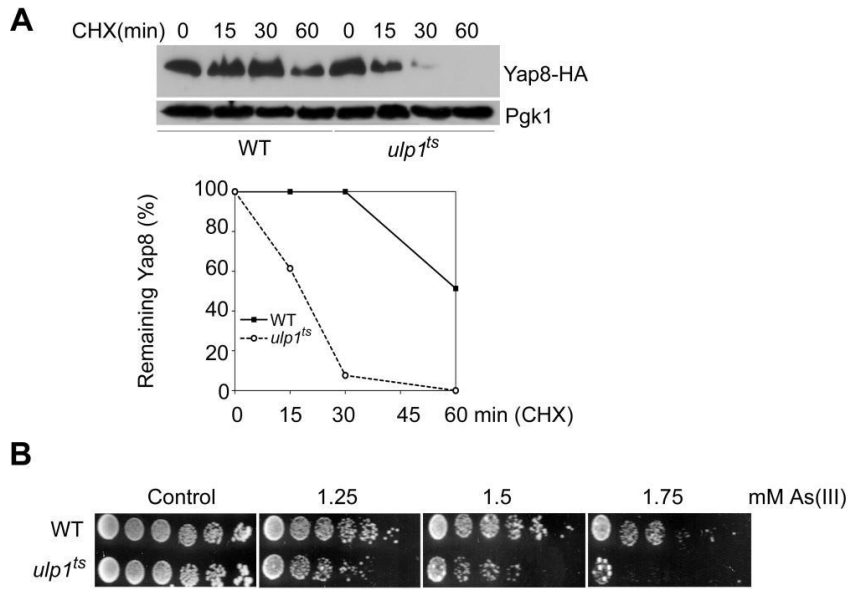

Figure 4. SUMO deconjugation might positively regulate Yap8 stability, thus mediating yeast tolerance to arsenic. (*A*) Dysfunctional Ulp1 enzyme results in decreased Yap8 protein stability *in vivo*. Cycloheximide (CHX) chase was carried out with a minor change in

Figure 4. (*Continued*)
previous treatment with 1.5 mM As(III) that was done for 90 min. Yap8 half-lives: 50 min (WT) and 18 min (*ulp1*^{ts}). (*B*) Spotting assay using the strains in *A*. Yeast cells were spotted in 10-fold dilution on plates containing As(III) concentrations up to 1.75 mM, or not (Control). Growth was recorded after 2 days at 30ºC. Representative experiments are shown.

DISCUSSION

In *S. cerevisiae*, Yap8 is the key transcription factor orchestrating transcriptional activation of the *ACR2* and *ACR3* genes, encoding an arsenate reductase and an arsenite efflux pump, respectively [6]. It was previously shown that Yap8 activity is predominantly modulated by cytoplasmic-nuclear shuttling [21], though the regulation of Yap8 levels by UPP has also been reported [2]. Very recently, the results emerging from an yeast two-hybrid (Y2H) screening led us to better understand how Yap8 becomes stabilized under arsenic conditions [3]; through Y2H assays, we have identified the Ub ligase (E4) Ufd2 (an enzyme of the multiubiquitination pathway) that stabilizes Yap8. The findings revealed that PTMs exert an important role in the regulation of Yap8 activity.

Moreover, an increasing body of evidences has indicated that SUMOylation has a key role in the modulation of protein activity, particularly transcription factors, affecting their stability, sub-cellular localization and etc [22]. Aiming at deciphering the molecular mechanisms underlying Yap8 regulation by PTMs, we have identified multiple Lys residues putatively targeted for ubiquitination and/or SUMOylation (Fig. 1). Genetic analyses revealed that the amino acid residues K62 and K198, representing putative sites for both modifications, are essential for a proper Yap8 regulation. Lys-to-Arg substitution of these residues caused a reduction of Yap8 stability (Fig. 2) and activity (Fig. 3), ultimately leading to a severe sensitivity phenotype of these strains (Fig. 3). It remains to elucidate: (1) whether these residues also serve as ubiquitination sites controlling Yap8 degradation under physiological conditions, and (2) whether Yap8 undergoes stress-inducible SUMO modification at K62 and/or K198. *In vivo* and *in vitro*

ubiquitination and SUMOylation assays should be made to clarify these issues.

Furthermore, Yap8 is a nuclear and cytoplasmatic protein under physiological conditions, whereas it becomes nuclear in response to arsenic [21]. Because earlier studies pointed to functions of SUMO in target-specific nuclear import, we also wonder whether Yap8 SUMOylation could represent a mechanism controlling Yap8 sub-cellular distribution.

In addition, the biological significance of SUMO deconjugation in the arsenic stress response was revealed (to some extent) by the demonstration that a mutant strain encoding the temperature-sensitive allele of *ULP1* (*ulp1*^{ts}) is sensitive to As(III) (Fig. 4). In *S. cerevisiae*, *ULP1* encodes the major SUMO-protease associated to disassembly of polySUMO chains from substrates, and therefore has a central role in the dynamics of SUMOylation processes [16]. Curiously, Yap8 stability was shown to be impaired in the *ulp1*^{ts} mutant (Fig. 4), thus providing a link between Yap8 regulation, and SUMO conjugation/deconjugation.

Taken together, our results point out a role of Lys62 and Lys198 in Yap8 post-translational regulation. Although the two Lys have been predicted with high score as putative SUMOylation consensus motifs, it is imperative to verified if they are effective SUMO acceptor sites. On the other hand, since these residues were predicted as putative sites for both the Ub- and SUMO-modifiers, it is reasonable to hypothesize that they could be at the center of an eventual interplay between the two PTMs. Indeed, ubiquitination and SUMOylation were reported to often communicate [23]; SUMO and Ub are both linked to proteins through Lys residues, and several SUMO target proteins were also found to be modified by Ub. It will be of interest to investigate if Yap8 can be modified by SUMOylation and ubiquitination at the same Lys residues, as described to other substrates in mammals [24, 25].

To summarize, although we have not directly addressed the question of Yap8 SUMOylation in As-stressed cells, it seems likely that a crosstalk between Ub and SUMO systems might be regulating this transcription factor at the post-translational level. Importantly, it is already known that arsenic trioxide (ATO) treatment is linked to the degradation of the promyelocytic

leukaemia protein (PML) via a SUMO-triggered RNF4/Ubiquitin-mediated pathway [26, 27]. Recent studies also gave novel insights into the SUMO deconjugation process required for arsenic-triggered ubiquitination of PML, and also SUMOylaltion of PML in response to arsenic(III) [26-28].

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COMPETING INTERESTS. The authors declare no competing interests.

AUTHOR CONTRIBUTIONS. Conceived and designed the experiments: R.T.F., R.A.M. and C.R.P.; performed the experiments: R.T.F.; analyzed the data: R.T.F., R.A.M. and C.R.P.; wrote the manuscript: R.T.F., R.A.M. and C.R.P.

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Supporting Tables

Table S1. Strains and plasmids used in the present study.

Strain	Genotype	Reference or source
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0* ura3Δ0	EUROSCARF*
ufd2	*MATα his3Δ1 leu2Δ0* lys2Δ0 *ura3Δ0 YDL190c::kanMX4*	EUROSCARF
yap8	*MATα his3Δ1 leu2Δ0* lys2Δ0 *ura3Δ0 YPR199c::kanMX4*	EUROSCARF
Yap8-K46R(-HA)	*YAP8* mutant in pRS416	This study
Yap8-K47R(-HA)	*YAP8* mutant in pRS416	This study
Yap8-K62R(-HA)	*YAP8* mutant in pRS416	This study
Yap8-K90R(-HA)	*YAP8* mutant in pRS416	This study
Yap8-K96R(-HA)	*YAP8* mutant in pRS416	This study
Yap8-K198R(-HA)	*YAP8* mutant in pRS416	This study
W303-1A	MAT a; ura3-1; trp1-1; leu2-3_112; his3-11; ade2-1; can1-100	Shahpasandzadeh *et al*. (2014)
ulp1^{ts}	*MAT**a**his3-Δ200 leu2-3,112::LEU2::ulp1-333 ura3-52 lys2-801 trp1-1 ulp1Δ1::his3::URA3*	Shahpasandzadeh *et al*. (2014)

*EUROpean *Saccharomyces Cerevisiae* ARchive for Functional analysis

Table S2. Oligomers used in the present study.

Oligomers	sequence 5' to 3'
***YAP8* mutagenesis**	
Y8L46R.fw	GAAGAACTAGAGCGGAAAGAA
Y8L46R.rv	GTTAGCTGAGCTTCTTTCCGC
Y8L47R.fw	GAAGAACTA GAG AAGCGGGAAGCTCAGCTAAC
Y8L47R.rv	GTTAGCTGAGCTTCCCGCTTCTCTAGTTCTTC
Y8L62R.fw	CAAATTCACATATTACGGAAG
Y8L62R.rv	GTTCATTTTCCTTCCGTAATAT
Y8L90R.fw	GAACGGAACATTAGTCGGGCCTGTTG
Y8L90R.rv	GGTTTTTCTTCACAACAGGCCCGACTA
Y8L96R.fw	AAGGCCTGTTGTGAAGAACGGCCGCC
Y8L96R.rv	GTTACATGTGGGCGGCCGTTCTTCACA
Y8L198R.fw	GTGGGACTGAAATACGGGAGC
Y8L198R.rv	GAAATTATTTGGCTCCCGTATT
q-RT-PCR	
qRTACR3-Fw	AAGAGGGTCTGGGGAAGAAA
qRTACR3-Rv	GCAATTGCCAGGGATAGTTC
ACT1_Fw	CTA TTG GTA ACG AAA GAT TCA
ACT1_Rv	CCT TAC GGA CAT CGA CAT CA

5

Discussion

5.1
Final Discussion

5.1.1
CaN/Crz1 Pathway Activation in Arsenic Stress Response

Although numerous studies have addressed the cellular and molecular changes during the stress response to arsenic (a therapeutic drug, and also a group1 human carcinogen [1]), we wanted to see novel signaling mechanisms underlying the stress response of budding yeast *S. cerevisiae* against arsenic. In order to start out with the identification of the novel gene categories associated with arsenic treat, we precisely performed a microarray DNA analysis of *S. cerevisiae* cells exposed to arsenic. For a better understanding of it was discussed in Chapter 2, Figure 5.1 presents a model of observed changes in calcium-related proteins, and activation of the calcineurin/Crz1 pathway by arsenic [2]. The cellular and molecular effects of arsenic stress are: (i) cytoplasmic Ca^{2+}-binding to Calmodulin, (ii) Calmodulin-binding to the CnA-CnB complex, Calcineurin (CsA immunosuppressant drug inhibits Calcineurin [3]), (iii) dephosphorylation of Crz1 by Calcineurin leading to Crz1 nuclear import, and (iv) expression of Crz1-dependent genes (*PMR1*, *PMC1*, and *GSC2*) (Crz1 contains zinc finger motifs, and binds specifically to the CDRE).

In our research, we have also observed that calcineurin inhibition protects yeast cells from arsenic toxicity [2]. Potentially, this observation can be related to the involvement of calcineurin-dependent degradation of Yap1 [4] (Figure 5.1); the calcineurin-mediated Yap1 degradation was reported as an adaptive response that assures a G_2 delay in response to stress that causes the activation of the calcium signaling pathways. Considering that Yap1 can be a substrate of calcineurin [4], and Yap1 contributes towards cellular adaptation upon oxidant challenge caused by arsenic [5], we need to understand how yeast cells achieve a balance between the two responses elicited by calcineurin that can be protective or toxic. Therefore, we need to investigate the molecular mechanisms underlying Yap1 regulation, and Yap1 as a potential substrate of calcineurin under arsenic conditions. Also, the hypothesis of As(III)-dependent stabilization of Yap1 (presented in Figure 5.1) must be tested.

On the other hand, we observed that the double *yap1cnb1* mutant is tolerant to arsenic drug [2], therefore we hypothesize that calcineurin inhibition might have other effects beyond Yap1 that can escape degradation. CaN/Crz1-independent pathways might also be activated under arsenic treatment (Figure 5.1). For example, the Ca^{2+}/calmodulin-dependent protein kinases (CaMK) are ubiquitously expressed multifunctional protein kinases that function through Ca^{2+}-signaling to regulate many different processes in the cell [6].

There is ongoing investigation on the arsenite effects on calcineurin signaling. The key transcriptional regulator of this pathway- Crz1 is the yeast ortholog of the human Nuclear Factor of Activated T cells (NFATc) [7]. A study advanced that micromolar concentrations of arsenite inhibit calcineurin phosphatase activity, and decrease NFATc nuclear translocation, indicating that arsenite affects calcineurin transduction route, and that calcineurin may serve as a potential link between ROS exposure and impaired tumor suppression [8]. Also, a study published by the Toxicol Lett. Journal [9] indicates that As_2O_3 at clinically and environmentally relevant concentrations (of 100 pM to 1 µM) perturbs calcium homeostasis. Within this concentration range, As_2O_3 had cell type specific cytotoxic effects. The study has shown

that the As_2O_3-induced cell death could be triggered or mediated by $[Ca^{2+}]_i$ signals, suggesting that As_2O_3 at low concentrations is able to interfere with specific physiological processes in diverse cell models.

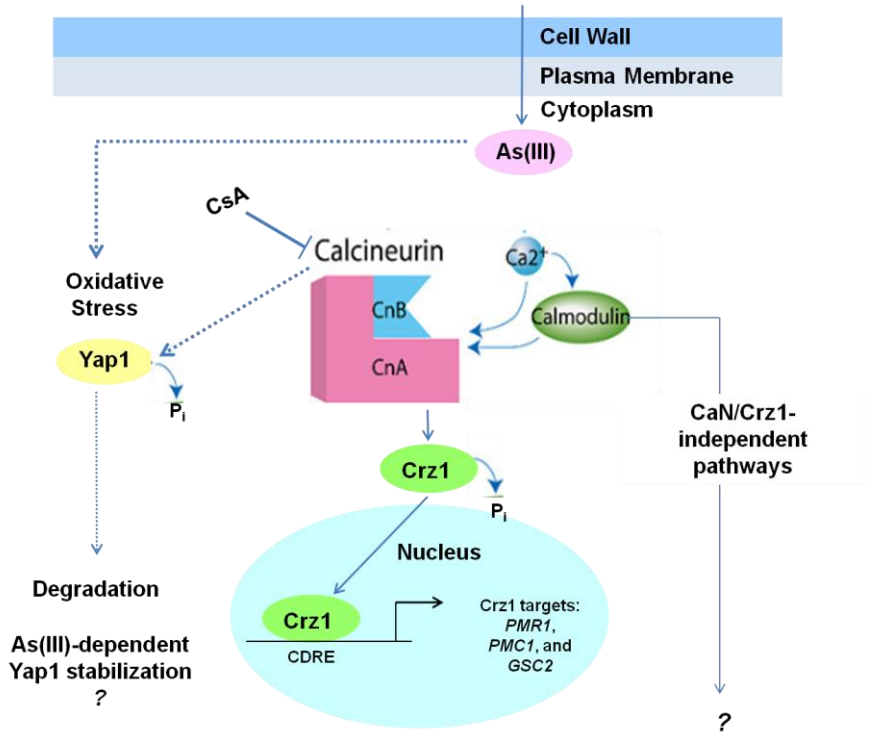

Figure 5.1 Model for activation of the calcineurin/Crz1 pathway by As(III). See text for details.

5.1.2

Post-translational Control of Yap8 by Ufd2

Earliest research on regulation of Yap8 transcription factor indicated that Yap8 primary control lies in a redox-regulated Crm1-dependent nuclear export [10]. Later on, it was reported that Yap8 is targeted by the Ubiquitin-Proteasome System (UPS) for degradation only under non-stress conditions [11]. In the Chapter 3, we provided novel insights into the biological role of the E4-Ub ligase Ufd2 during arsenic stress, which appeared as a new interactor of Yap8 [12]; the results unraveled the importance of Ufd2 in Yap8

stabilization, and *ACR2*/*ACR3* expression driven by the Yap8 transcription factor in the nucleus of *S. cerevisiae* cells (see Figure 5.2 below).

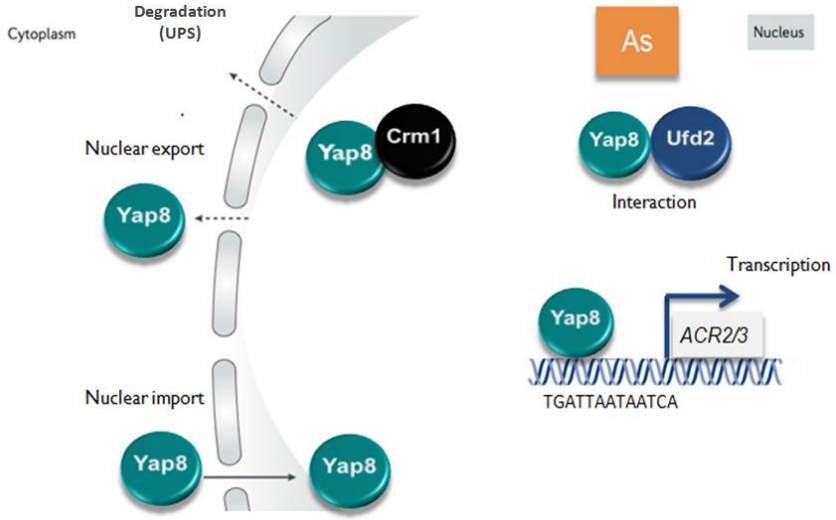

Figure 5.2 Model for mechanisms of activation, and attenuation of AP-1-like Yap8. See text for details.

Still, our understanding of the molecular mechanisms underlying Yap8 stabilization by Ufd2 remains limited. As such, identification of the interaction domains in both proteins, and eventually the Yap8 amino acid residues involved, would greatly help to elucidate this issue.

Finally, the current study also suggests that ubiquitination factors E4 have far more functions than the previously established multiubiquitination activity of the ubiquitin-proteasome system. In fact, it was already seen that this novel fundamental Ufd2 function is not restricted to organisms much simpler like yeast (whereby human UFD2a acts to attenuate cisplatin-mediated degradation of isoform ΔNp63α [13]), thus reflecting the high degree of conservation of basic cellular processes between yeast and higher eukaryotes [14].

5.1.3
Deciphering Yap8 Regulation via Ubiquitination, and SUMOylation

Chapter 4 focuses on lysines in Yap8 protein sequence putatively in the closest proximity to Ub and SUMO, representing a starting point resource for Yap8 SUMOylation studies. Nevertheless, only a detailed characterization of knockout strains defective in the SUMO process, *in vitro* SUMOylation assays, as well as mass spectrometry data may indicate which residues serve as target sites for SUMO-attachment to the transcription factor Yap8.

5.2
Future Research

The cellular Ca^{2+}-signaling around the CaN/Crz1 'core' complex presented in this thesis represents a concise overview of Ca^{2+} homeostasis perturbation as a side effect of arsenic, and how homeostasis can subsequently be restored. Nevertheless, a large-scale extension of this signaling network will be crucial for a refined picture of Ca^{2+} signal transduction pathways activated by arsenic. A comprehensive functional analysis of the Ca^{2+}-signaling networks and their components mediated by arsenic, in yeast, holds the potential to identify further, in human cells, key proteins within these pathways suitable for As pharmacological targeting in synergy with CaN/Crz1.

The observation of Ufd2-Yap8 interaction under arsenic conditions is associated with a new fundamental function of the yeast ubiquitin ligase (E4) Ufd2 in stabilizing the Yap8 transcription factor (as aforementioned, it was also observed a similar function for the human UFD2 towards p63 transcription factor). This new concept poses numerous novel challenges around studies of the ubiquitin-proteasome system, and its individual components. In addition, further experimental approaches are required to elucidate and refine our current model for mechanisms of Yap8 post-translational regulation, as well as it remains to be observed whether Ufd2-Yap8 interaction localizes to the yeast cell nucleus. Furthermore, it will be

relevant to explore alternative targets that could also contribute for Yap8 stabilization and transcriptional activity. Most importantly, it remains to be observed if the new function of Ufd2 in response to arsenic can also be observed in mammalian cells, particularly in APL cells during treatment stages.

Concerning our preliminary data on Yap8 mutants with single lysine-to-alanine substitutions that are more prone to degradation, the question of Yap8 SUMOylation remains open, and must be tested. We know that ATO drug potentiates SUMO modifications on substrates in human cell lines. On the light of this scenario, future research in yeast will be conducted on challenging existing paradigms of arsenic drug with focus on SUMO, and other Ub-like modifiers that can control the key Yap8 transcription factor in yeast response to arsenic toxicity.

A major limitation to studying the Yap8 regulator has been the absence of orthologs for this protein. However, Rodrigues-Pousada laboratory was also engaged in characterizing the Yap8 protein-DNA interactions [15], and it was possible to construct a model of the complex Yap8bZIP-DNA based on Pap1-DNA crystal structure due to high similarity of the respective basic regions of Yap8 and Pap1 [15]. (Pap1 is also a bZIP containing protein of *Schizosaccharomyces pombe* that has homology and similar DNA binding specificity to the mammalian c-Jun protein [16].)

5.3
Final Conclusions

This thesis enables a novel view in yeast of the calcineurin-Crz1 and ubiquitin ligase Ufd2, as the cellular targets of arsenic compounds. A DNA microarray approach measuring the gene expression of yeast cells treated with arsenate resulted in the quantitative identification of significantly up-regulated, and interconnected CaN-Crz1 ‘core’ complex that links arsenic to key components of the calcium signaling pathways. Moreover, the analysis of cytosolic free [Ca^{2+}] revealed that arsenic perturbs calcium homeostasis, and a new link by which arsenic stress is converted to signaling for the Ca^{2+}-

activated calcineurin and Crz1 response to restore homeostasis and cell integrity. Furthermore, a two-hybrid analysis uncovered that the prototype U-box protein Ufd2 interacts with Yap8 upon arsenic stress, and stabilizes the protein, changing our view of the yeast Ufd2 as an ubiquitin ligase (E4) associated with proteolysis, and contributing to a novel concept in research of the ubiquitin-proteasome system.

This thesis underlines the importance of the understanding of how yeast cells adapt to arsenic for the elucidation of arsenic action in clinical, cellular, and molecular contexts.

> "*Almost everything we know about the fundamental properties of living cells – how they grow and divide, how they express their genetic informations, and how they use and store energy – has come from the study of model organisms*."- Fields and Johnston (After Ref. [17])

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6
Epilogue

I hold a degree in biochemistry from the Lisbon Faculty of Sciences, although I had done my diploma thesis in macromolecular crystallography at ITQB from NOVA University (2006-2007). At an earlier stage of my career, I had to make the decision to return to ITQB and applying for a Ph.D experience, in 2010. Indeed, that was something that made me nervous for several reasons, but I not regret it. I leave ITQB with fresh knowledge, concepts and new skills, allowing me to display competence naturally when I take my career to the next level.

Thought:

"Imagination disposes of everything; it creates beauty, justice, and happiness, which are everything in this world." -Blaise Pascal

Appendix

Table 1. Human diseases related to functions of yeast genes. (After Ref.[1], adapted.)

Human disease-gene effect/defect	Yeast gene
Immunodeficiency	*AAH1*
Hypertension	*ACS1*
Autism features	*ADE13*
Acute alcohol intoxication	*ALD2*
*Chediak-Higashi syndrome (CHS) (also murine beige protein); decreased pigmentation; immunodeficiency	*BPH1*
*Menke's disease (MNK); neurodegeneration	*CCC2*
*Wilson disease (WND); toxic accumulation of Cu in liver and brain	*CCC2*
*Migraine (CACNL1-A4); familial hemiplegic migraine; episodic ataxia	*CCH1*
Metabolic acidosis	*ERG10*
Fumaric aciduria; encephalopathy	*FUM1*
*Werner syndrome (WT1); nephroblastoma	*FZF1*
*Fanconi syndrome (CLCN5); Nephrolithiasis	*GEF1*
Glycogen storage disease Skeletal muscle insufficiency	*GPH1*
*Hyperglycerolemia; poor growth; mental retardation	*GUT1*
Coproporphyria; psychatric symptoms	*HEM12*
Protoporphyria	*HEM15*
Hepatic porphyria	*HEM2*
*Neurofibromatosis (NF1)	*IRA2*
Maple syrup urine disease; Lactic acidosis	*LPD1*
Homocystinuria; psychosomatic symptoms	*MET12*
*Lissencephaly (LIS1); malfunction of platelet-activating factor acetylhydrolase	*MET30*
*HNPCC; hereditary nonpolyposis colon cancer	*MLH1/MSH2*
Neuropathy	*PEX1*
Neuropathy	*PEX5*
Neuropathy	*PEX6*
*Adrenoleukodystrophy (ALD); neurodegeneration	*PXA1*
Cockayne syndrome (CS-B)	*RAD26*
Cockayne syndrome (CS-A)	*RAD28*
A-Thalessimia (ATRX); mental/psychomotoric retardation	*RAD54*
Hypermethioninanemia; mental retardation	*SAM1/2*
*Bloom's syndrome (BLM); growth defect; predisposition to cancer Werner's syndrome (WRN); premature aging	*SGS1*
*Lowe syndrome (OCRL); cataracts	*SJH1*
*Amylotrophic lateral sclerosis	*SOD1*
Venous thrombosis	*SPT14*
*Moncytic leukemia (MOZ);erythrophagocytosis	*TAS1/SAS3*
*Ataxia telangiectasia (ATM)	*TEL1*
Hemolytic anemia	*URA6/ADK1*
*Cystic fibrosis (CFTR); impaired clearance	*YCF1*
*Myotubular myopathy (MTM1); muscle-specific disease	*YJR110w*
*Myotonic dystrophy (DM); neurodegeneration	*YNL161w*
*Niemann-Pick disease (NPC1); fatal neurovisceral disease	*YPL006w*
Hemolytic anemia	*ZWF1*

Asterisks indicate that the human gene was identified by positional cloning.

Table 2 Examples for biopharmaceuticals produced in *S. cerevisiae*. (After Ref.[1].)

Product group	Product	Commercial name and function
Prokaryotic products	Tetanus toxin fragment C	
	Streptokinase	
Surface antigens of viruses	Hepatitis B antigen	Ambirix (combination vaccine containing rHBsAg)
		HBVAXPRO (rHBsAg)
		Twinrix (adult pediatric forms in EU; combination vaccine containing rHBsAg)
		Infanrix-Hexa (combination vaccine containing rHBsAg)
		Hexavac (combination vaccine containing rHBsAg)
		Primavax (combination vaccine containing rHBsAg)
		Comvax (combination vaccine containing rHBsAg)
		Recombivax (rHBsAg)
	Vaccines against foot and mouse disease, influenza, polio, polyoma	Gardasil (quadrivalent human papillomavirus (HPV) recombinant vaccine; contains major caspid proteins from four HPV types)
Animal products	Hirudin	r Hirudin as anticoagulant
	Porcine interferon	
	Interleukin	
	Trypsin inhibitor	
Human hormones	Insulin	Insulin determir, long-acting rh Insulin analogue
		rh Insulin formulated as short-, intermediate-, or long-acting product
		Insulin aspart, short-acting rh Insulin analogue
	Parathyroid hormone	
	Growth hormone	Valtropin (somatropin, rh, GH)
	Chorionic gonadotropin	
	Glucagon	Glucagen (rh glucagon)

Table 2. (*Continued*)

Product group	Product	Commercial name and function
Human growth factors	IGF1	Insulin-like growth factor
	NGF	Nerve growth factor
	EGF	Epidermal growth factor
	CSF	Colony stimulating factor
	GM-CSF	Granulocyte-monocyte stimulating factor
	PDGF	Regranex (rh PDGF)
	TNF	Tumor necrosis factor
Human blood proteins	Hemoglobin	
	Factors VIII and XIII	
	Alpha-1-antitrypsin	
	Antithrombin III	
	Serum albumin	Recombumin
Enzymes and further compounds	Urate oxidase	Fasturtec (Elitex in US) (rasburicase; r urate oxidase)
	Transferirn	
	Hydrocortisone	
	Ergosterone	
	Polypeptides	

r, recombinant; rh, recombinant human

Reference for Appendix

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