Mechanisms of toxic metal tolerance in yeast

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Abstract

Toxic metals are an integral part of our environment and all organisms possess systems to evade toxicity and acquire tolerance. Studies in yeast have revealed a number of important tolerance systems encompassing metal uptake and export pathways, metal binding and sequestration systems as well as the regulatory mechanisms that the cell utilizes to control these systems. The study of the physiological, molecular, and genetic details of the function of these systems has significantly contributed to our understanding of toxic metal tolerance acquisition. This review will focus on tolerance mechanisms to toxic metals including cadmium, arsenic, antimony, mercury, and selenium in the model eukaryote *Saccharomyces cerevisiae* (bakers' yeast) and other fungi.

1 Introduction

All living organisms are exposed to metals through natural geological as well as anthropogenic sources. Many metals serve as essential nutrients, while others are either toxic or harmful in excessive quantities. Deposition of nonessential toxic metals in the environment has dramatically increased during the last century. Cadmium, arsenic, mercury, and lead are extensively distributed in nature and can reach relatively high concentrations in some locations. These metals are highly toxic and pose a considerable threat to the environment and to human health. Metal intoxication often occurs through occupational exposure or through ingestion of contaminated food and water. In fact, organisms have been exposed to toxic metals since the origin of life and have therefore developed various tolerance mechanisms early during evolution. Currently, metal pollution leads to the spread of plasmids containing resistance genes among prokaryota.

Metal tolerance mechanisms in bacteria are relatively well-described where plasmids containing specific operons account for this phenomenon (Silver 1998, 2003; Nies 1999; Rosen 2002). Similarly, there has been a tremendous advance in the understanding of nutrient metal homeostasis and detoxification in many organisms. These mechanisms are extensively reviewed in other chapters in this volume and will not be considered here. However, the mechanisms of tolerance to various nonessential metals in eukaryotic organisms have remained poorly explored. The increasing use of toxic metals in medical therapy, *e.g.*, the use of arsenic for the treatment of certain forms of cancer and of diseases caused by protozoan parasites,

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as well as the need to develop systems for phytoremediation of contaminated sites, has spurred research in this field and led to a significant progress in understanding metal responses and tolerance acquisition mechanisms in eukaryotic organisms (Tamás and Wysocki 2001; Alkorta et al. 2004; Desoize 2004; Macek et al. 2004). In particular, the use of the yeast *Saccharomyces cerevisiae* (bakers' yeast) as a eukaryotic model organism has proved very useful to unravel the molecular mechanism of many cellular metal tolerance systems. Here, we review the current knowledge about tolerance mechanisms to cadmium, arsenic, antimony, mercury, and selenium in *S. cerevisiae*.

1.1 Metal abundance, distribution, and usage

Arsenic is a semimetal or metalloid and as such, it has intermediate properties between those of metals and nonmetals. Arsenic compounds, in the form of sulphides and oxides as well as in the form of calcium, sodium, and potassium salts, are naturally occurring and ubiquitous in the environment. Arsenic contamination of drinking water is a serious problem worldwide: Bangladesh, West Bengal, Vietnam, and Taiwan are the most affected areas where global epidemic of arsenic poisoning is observed (Frisbie et al. 2002; Nordstrom 2002). The sources of arsenic in underground water supplies in these areas are geologically deposited sediments. Elevated concentrations of arsenic in soil and surface water are also associated with the use of arsenic compounds as pesticides, fungicides, insecticides, and wood preservatives (Mukhopadhyay and Rosen 2002). Nonferrous ore smelting, semiconductor, and glass manufacturing as well as power generation by the burning of arsenic-contaminated coal further contributes to arsenic pollution (Hei and Filipic 2004).

Arsenic has a long and well-documented history of usage in medicine since ancient times (Waxman and Anderson 2001; Ravandi 2004). In the 18th century, potassium arsenite in the form of Fowler's solution was used to treat a number of ailments. The use of arsenic in treating leukaemia was first described in the 19th century and its efficacy was confirmed in the 1930s (Evens et al. 2004). At the beginning of the 20th century, Paul Ehrlich and his co-worker Sahachiro Hata developed the arsenic-containing 'compound 606' (Salvarsan) and introduced this drug for the treatment of syphilis and trypanosomiasis (Sorgel 2004). This was the first example of modern chemotherapy; in fact, Paul Ehrlich coined the term 'chemotherapy'. However, arsenic was often overdosed producing severe side effects. With the introduction of penicillin and other less toxic drugs, arsenic was no longer in use. Recently, arsenic trioxide was re-introduced in the treatment of acute promyelocytic leukaemia (APL) and multiple myeloma (Zhu et al. 2002; Ravandi 2004). Arsenic trioxide exerts its effect by inducing differentiation of leukaemia cells (by promoting degradation of the leukemogenic PML-RARα fusion protein) and/or by inducing apoptosis (Hu et al. 2005).

The metalloid antimony is related to arsenic. Antimony is not abundant but is found in more than 100 mineral species. Antimony, in the form of sulphide called stibnite, has been known since Biblical times as a medicine and as a cos-

metic. Today, antimony compounds are used for instance in the making of flame-proofing formulations and glass paints. In addition, pentavalent antimony is an active component of Pentostam and Glucantime, the first line drugs in the treatment of leishmaniasis (Sundar and Rai 2002; Berman 2003).

Cadmium is a highly toxic metal at very low concentrations and is found in increasing abundance in the environment due to industrial activities. Cadmium occurs naturally in zinc, lead, copper, and other ores, which can serve as sources to ground and surface waters, especially when in contact with soft, acidic waters. Nowadays, cadmium is released in air, water, and soil, mainly due to mining, smelting, battery, and paint manufacturing and car exhaust (Goyer and Clarkson 2001).

Although, mercury is a very rare element in the earth's crust, it was used already in ancient Egypt from 3500 BC. Mercury is primarily obtained from the mineral cinnabar (HgS). Mercury easily forms alloys with other metals called amalgams, which are used in gold extraction from ores, dental fillings, and batteries. Due to its high toxicity, mercury chloride ($HgCl_2$) was once used as pesticide, antiseptic, and wood preservative (Goyer and Clarkson 2001).

In contrast to the metals described above, selenium [Se(0)] is an essential trace element in all organisms and non-toxic at low levels. In nature, selenium is found in a few rare minerals such as eucairite (CuAgSe), crooksite (CuThSe), and clausthalite (PbSe) as well as in many sulphide ores. In living organisms, it is a component of the amino acid selenocysteine, which is present in several enzymes with oxidoreductase activity (Stadtman 1996). On the other hand, selenate [Se(VI)], selenite [Se(IV)], and selenide [Se(II)] are highly reactive and may cause increased production of reactive oxygen species (Turner et al. 1998).

2 Effects of nonessential metals on biological systems

The toxicity of a compound depends on the dose as well as on the contamination pathway, *i.e.*, whether it is absorbed via skin, ingested via the intestinal tract, or inhaled via the lungs. In the case of metals, other parameters influencing toxicity include the oxidation state and the speciation of the metal. Most metals affect various organ systems but at the lowest dose where effects occur, each metal tends to affect first a specific organ or tissue. A common property of nearly all toxic metals, including Hg(II), As(III), Cd(II), and Pb(II) is their high reactivity with sulphhydryl groups. Other toxic metals such as Cr(VI), Se(III), and Se(VI) are less sulphhydryl reactive though they are generally reduced in the cell by glutathione. Many metals have been shown to cause oxidative stress, lipid peroxidation, DNA strand breaks, and alteration of the cellular glutathione pool. In addition, the toxic and carcinogenic effects of metals may be induced by targeting cellular regulatory proteins or signalling proteins involved in proliferation, cell cycle regulation, apoptosis, DNA repair and differentiation (reviewed in: Stohs and Bagchi 1995; Ercal et al. 2001; Goyer and Clarkson 2001; Chen and Shi 2002; Harris and Shi 2003).

2.1 Effects on human health

Arsenic has for long been known to be toxic and carcinogenic. Epidemiological studies have indicated that arsenic can cause cancers of the skin, bladder, liver, and lung. In addition, arsenic exposure has been associated with diabetes and hypertension (Smith et al. 1992; Evens et al. 2004; Shi et al. 2004). Arsenic exists in various oxidation states exhibiting different biological properties and degrees of toxicity. Arsenate [As(V)] is a phosphate analogue and it inhibits oxidative phosphorylation, short-circuiting the cell's main energy-generation system (Goyer and Clarkson 2001). Arsenite [As(III)] is more potently toxic and exposure leads to increased production of free radicals in mammals, especially hydroxyl radicals (Liu et al. 2001). Enhanced oxidative stress is proposed to be responsible for arsenic-induced toxicity and carcinogenicity. However, the mechanisms of free radical production and the details of arsenic-induced cytotoxicity remain largely unknown (Ercal et al. 2001; Evens et al. 2004; Hei and Filipic 2004; Shi et al. 2004)

Antimony belongs to the same periodic group as arsenic and has the same oxidation states. In contrast to arsenic, the genotoxic and carcinogenic properties of antimony has received little attention and most information has been obtained from industrial experiences. Chronic exposure to antimony may affect the skin and lungs and also produce alterations in cardiac function. Pentavalent antimony [Sb(V)] inhibits glucose catabolism and ATP-formation while trivalent antimonite [Sb(III)] may impair protein function by interacting with sulphhydryl groups. Whether antimony can induce oxidative stress is unclear (Gebel 1997; Goyer and Clarkson 2001).

Cadmium contaminating food is absorbed through the intestinal tract and transferred to the liver where it is chelated to glutathione and transferred to metallothionein. This complex is reabsorbed into the kidney where it may stay up to 20 years; its accumulation over time and progressive release when chelating capacities are exceeded may cause toxicity. In Japan, the accidental exposure of inhabitants of the Jinzu river basin eating cadmium-contaminated rice provoked a severe disease called Itai Itai disease (Itai! means Ouch!), which is particularly painful to kidneys and bones. Cadmium is also considered genotoxic and carcinogenic for lung, kidney, and prostate (Waalkes 2003).

The major forms of mercury are methylmercury (MeHg) and Hg(0) (vapour). Inside the cell, Hg(0) may be converted to Hg(II), which is thought to produce H_2O_2 leading to oxidative stress. Mercury has also been described to perturb calcium homeostasis and to inhibit oxidative phosphorylation. Finally, mercury accumulates in liver, kidney, and brain and is poorly excreted (Ercal et al. 2001).

Selenium salts are toxic at high concentrations and selenium sulphide is considered carcinogenic. However, selenium is an essential trace element; small amounts of selenium are required to synthesize the amino acid selenocysteine present in glutathione peroxidases and thioredoxin reductases in mammals (Stadtman 1996). Dietary selenium has also been shown to prevent chemicals from inducing tumours in mammals (Rayman 2000). Selenium inhibits the intracellular JNK/SAPK signalling and $p38^{MAPK}$ cascades (Park et al. 2000) as well as some transcription factors (Handel et al. 1995; Kim and Stadtman 1997). Some of these inhibitory ef-

fects occur through a thiol redox mechanism (Park et al. 2000), but it is not known whether this mechanism is responsible for the anti-carcinogenic properties of this element.

2.2 Toxic metals cause oxidative stress

Oxidative stress originates from toxic levels of oxygen-derived reactive species. Reactive oxygen species (ROS) are mainly singlet oxygen ($O^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^{\bullet}$). Hydrogen peroxide is also included as ROS although this molecule is reactive only in some specific conditions (*e.g.* in the presence of reduced iron or copper; see Section 2.3.3). Hydroxyl radical is considered the most toxic ROS since it can attack and damage all macromolecules of the cell leading to protein oxidation, lipid peroxidation and DNA damage (reviewed in: Halliwell and Gutteridge 1984; Toledano et al. 2003).

2.2.1 Genetic data

Cadmium has been described to cause oxidative stress (Brennan and Schiestl 1996), lipid peroxidation (Howlett and Avery 1997), and mutagenesis (Jin et al. 2003). In line with this, yeast genes encoding oxidative stress defence functions (*SOD1*, *SOD2*, *TRR1*, *GLR1*, *TRX1*, *TRX2*, *GPX3*; see also Table 1 for abbreviations of genes/proteins and their function) are necessary for cadmium tolerance (Brennan and Schiestl 1996; Vido et al. 2001; Avery et al. 2004). Yap1, the main transcriptional activator controlling the oxidative stress response is central for cadmium tolerance (Wemmie et al. 1994b) and also for tolerance to Hg(II) (Westwater et al. 2002), Se(III) (Pinson et al. 2000), As(III) (Menezes et al. 2004; Wysocki et al. 2004), and Sb(III) (Wysocki et al. 2004). In contrast to these data, a recent genome-wide analysis found that strains lacking genes encoding oxidative stress defence functions were not particularly As(III) sensitive (Haugen et al. 2004). On the other hand, the same study confirmed the importance of Yap1-mediated induction of oxidative stress defence genes by As(III) (Haugen et al. 2004).

2.2.2 Lipid peroxidation

Yeast cells exposed to Cd(II) have an elevated level of lipid peroxidation and cadmium-induced lipid peroxidation is influenced by the degree of plasma membrane fatty acid unsaturation (Howlett and Avery 1997). A correlation between membrane peroxidation, membrane permeabilisation, and cadmium sensitivity has been reported, suggesting that lipid peroxidation is contributing to cadmium toxicity (Howlett and Avery 1997). Phospholipid hydroperoxidases are considered the principal cellular enzymes capable of repairing membrane lipid peroxides. *S. cerevisiae* expresses at least three different enzymes (Gpx1, Gpx2, and Gpx3) with phospholipid hydroperoxidase activity (Avery and Avery 2001). Among them,

Table 1. List of genes described in this review, the function of the corresponding gene products and their role in metal tolerance.

Gene	Function and role in metal tolerance
Sulphur assimilation and methyl cycle	
SUL1, SUL2	Sulphate transport
MET3	ATP sulphurylase
MET14	APS kinase
MET16	PAPS reductase
MET5, MET10	Sulphite reductase
MET25	O-acetylhomoserine sulphydrylase
MET6	Homocysteine methyl-transferase
SAM1, SAM2	S-adenosylmethionine synthase
SAH1	S-adenosylhomocysteinase
Glutathione biosynthesis	
CYS4	Cystathionine β-synthase
CYS3	Cystathionine γ-lyase
GSH1	γ-glutamyl-cysteine synthase
GSH2	Glutathione synthetase
GTT2	Glutathione S-transferase
Oxidative stress defence and antioxidants	
GLR1	Glutathione reductase
GPX1, GPX2, GPX3	Glutathione peroxidase
GRX1, GRX2, GRX5	Glutaredoxin
TRX1, TRX2	Thioredoxin
TRR1	Thioredoxin reductase
TSA1	Thiol peroxidase
SOD1, SOD2	Superoxide dismutase
CUP1, CRS5	Metallothionein
Signalling proteins and transcriptional regulators	
HOG1	Mitogen-activated protein kinase (MAPK); mediates As(III) and Sb(III) tolerance through multiple mechanisms
YAP1	AP-1-like bZIP transcription factor; controls expression of genes encoding oxidative and metal stress defence functions as well as functions in redox metabolism; activated by oxidants and metals; mutant is sensitive to oxidants and metals
YAP8/ACR1/ARR1	AP-1-like bZIP transcription factor; controls expression of ACR2 and ACR3; activated by As(III); mutant is As(V) and As(III) sensitive
ORP1/GPX3	Required for Yap1 activation by peroxide
YBP1	Required for Yap1 activation by peroxide
SKN7	Activator of the heat shock factor family; involved in numerous cellular processes including oxidative stress; role in metal tolerance unclear
MET4	bZIP transcriptional activator of MET genes; required for Cd(II) and As(III) tolerance
MET28, MET31, MET32, CBF1	DNA-binding factors that are required for tethering Met4 to the promoter DNA of target genes
MSN2, MSN4	Zinc finger family of transcriptional activators; activators of the 'general stress response'
RAD9	DNA damage checkpoint protein

Gene	Function and role in metal tolerance
Metal transporters	
PHO84, PHO87, PHO88	Phosphate transport; entry of As(V)
FPS1	Aquaglyceroporin; entry of As(III) and Sb(III)
ZRT1	High affinity zinc uptake; Cd(II) entry
SMF1	Manganese, copper, and iron uptake; Cd(II) entry
FET4	Low affinity iron uptake; Cd(II) entry
ACR3/ARR3	As(III) and perhaps Sb(III) export
SSU1	Sulphite efflux; Cd(II) efflux
PCA1	P-type ATPase involved in copper and iron homeostasis; Cd(II) efflux
YCF1	ABC transporter; sequesters glutathione conjugated substrates into the vacuole; As(III), Sb(III), Cd(II), Hg(II), Pb(II) detoxification
BPT1	ABC transporter in vacuolar membrane, Cd(II) detoxification
ZRC1, COT1	Vacuolar zinc uptake; Cd(II) detoxification
ATR1, FLR1	Multidrug transport
Other functions	
ACR2/ARR2	Arsenate reductase
PDC1, PDC6	Pyruvate decarboxylase; role in sulphur sparing programme
CRM1/XPO1	Nuclear export receptor (exportin)
PSE1/KAP121	Nuclear import receptor (importin)
RCK2	MAPK-activated protein kinase; mutant is As(III) and Cd(II) sensitive
SIC1	CDK-inhibitor protein; mutant is As(III) sensitive
MET30	F subunit of the SCF^{Met30} ubiquitin ligase
REV3	DNA polymerase (zeta subunit)
URE2	Regulator of nitrogen utilization; shows glutathione peroxidase activity

Gpx3 was shown to play a major role in cadmium resistance through its phospholipid hydroperoxidase activity (Avery et al. 2004). Taken together, these data strongly suggest that an important toxic effect of cadmium is membrane lipid peroxidation. Arsenic has also been shown to induce lipid peroxidation in animal models (Hei and Filipic 2004; Shi et al. 2004). Whether this is also the case in yeast is not known.

2.2.3 Mutagenic effect

Cadmium has strong mutagenic effects even at low concentrations; it induces recombination events (Brennan and Schiestl 1996), base substitution mutations and frame-shift mutations at a high rate (Jin et al. 2003). Cadmium causes hypermutability by inhibiting the mutation avoidance system rather than by direct DNA damage; it strongly inhibits the DNA mismatch repair system (Jin et al. 2003) by blocking the ATPase activity of the MSH2-MSH6 complex (Banerjee and Flores-Rozas 2005). Thus, cadmium strongly increases the number of mutations arising from endogenous processes (Jin et al. 2003). Arsenic exposure can also lead to

various types of DNA damage including chromosomal aberrations and sister chromatid exchange. It has been proposed that arsenic interferes with DNA repair systems (Shi et al. 2004). However, yeast strains lacking genes encoding DNA repair functions were not found particularly sensitive to arsenite (Haugen et al. 2004). For the other toxic metals described here, it is not known whether their potential mutagenic properties are due to similar mechanisms (*i.e.* inhibition of DNA repair systems) or to metal-mediated DNA damage. However, the yeast DNA repair mutants *rad9Δ* and *rev3Δ* are hypersensitive to selenite (Pinson et al. 2000).

2.3 Possible molecular mechanisms leading to oxidative stress

Cd(II), Hg(II), and Pb(II) are redox-inactive metals and cannot undergo simple oxidation reactions. The molecular mechanisms leading to oxidative stress are still largely unknown and are probably indirect. From literature, three possible and non-exclusive scenarios can be proposed: (i) binding and inhibition of specific enzymes, (ii) depletion of free glutathione pools, and (iii) Fenton reactions.

2.3.1 Binding and inhibition of specific enzymes

Nonessential toxic metals are generally thought to bind proteins through thiol groups of cysteine residues (Stohs and Bagchi 1995), which may lead to inhibition of essential enzymes. Methyl mercury (MeHg) strongly inhibits the yeast L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) which catalyses the synthesis of glutamine-6-phosphate (Naganuma et al. 2000). Overexpression of this enzyme allows cells to resist MeHg, suggesting that GFAT is a MeHg target. Whether MeHg inhibits this enzyme by binding to a thiol group is unknown.

Cadmium is also described to bind thiol groups in proteins. An *in vitro* search for Cd(II) binding site on phytochelatin synthase (Maier et al. 2003) and thioredoxin (Rollin-Genetet et al. 2004) identified, as expected, thiol-containing peptides. In addition, the frequent implication of Asp and Glu residues in Cd(II) binding-sites suggests an important participation of carboxylate groups (Maier et al. 2003; Rollin-Genetet et al. 2004). *In vitro* analysis showed that human thiol transferases (glutathione reductase, thioredoxin reductase, thioredoxin) are inhibited by Cd(II) (Chrestensen et al. 2000). It has been proposed that Cd(II) binds the two essential cysteine residues of thiol transferase active sites. As these enzymes are involved in oxidative stress defence, their inhibition would lead to increased oxidative stress in the cell. Although the inhibition of yeast thiol transferases has not been tested experimentally, this hypothesis is consistent with the Cd(II) hypersensitive phenotype of the *trr1Δ*, *glr1Δ* and *trx1Δ trx2Δ* mutants (Vido et al. 2001).

Cd(II) may also displace zinc and calcium ions from metalloproteins (Stohs and Bagchi 1995; Schützendübel and Polle 2002; Faller et al. 2005) and from zinc finger proteins (Hartwig 2001) leading to inhibition of essential proteins. However, although this hypothesis is interesting, there is no experimental evidence indicating that such a mechanism is involved in cadmium toxicity. In particular, it is not known whether the inhibition by cadmium of the ATPase activity of the MMR

system is due to cadmium binding to a specific site or to the replacement of an unidentified zinc site (McMurray and Tainer 2003).

2.3.2 Depletion of free glutathione pools

Cd(II), As(III), Hg(II), and Sb(III) are detoxified, at least in part, through chelation of the metal to glutathione (GSH) and subsequent transport of the complex into the vacuole (Li et al. 1997; Ghosh et al. 1999; Gueldry et al. 2003). This detoxification pathway may thus contribute to reduce or deplete free GSH pools from the cytosol. Cytosolic GSH depletion would reduce the activity of GSH dependent enzymes, such as glutathione peroxidases, glutathione S-transferases, and glutaredoxins that are involved in oxidative stress defence and perform essential functions in the cell. Among these enzymes, the activity of the glutaredoxin Grx5 is particularly important for the cell since it is required for the activity of mitochondrial iron/sulphur enzymes (Rodriguez-Manzaneque et al. 2002). Interestingly, another protein, the yeast prion Ure2, showing a glutathione peroxidase activity (Bai et al. 2004) is essential for cadmium tolerance (Rai et al. 2003) indicating that its glutathione peroxidase activity may play a role in combating cadmium toxicity. However, metal concentrations that are high enough to be toxic may still be too low to significantly deplete glutathione; glutathione is estimated to be in the mM range (Lafaye et al. 2005a) whereas cadmium is toxic in the micromolar range. Furthermore, the cellular glutathione pool has been shown to increase in response to cadmium (Lafaye et al. 2005a). Nevertheless, it cannot be excluded that metal exposure decreases the available pool of glutathione to an extent where the activity of GSH dependent enzymes become affected.

2.3.3 Fenton reactions

Fenton-type reactions are described for the nutrient metals Fe(II) and Cu(I) and are supposed to be a major source of hydroxyl radicals and oxidative stress in the cell (Halliwell and Gutteridge 1984).

$$M(n+1) + O_2^- \text{------>} M(n) + O_2$$

$$M(n) + H_2O_2 \text{------>} M(n+1) + OH^\bullet + OH^-$$

In contrast to iron and copper, Cd(II) and Hg(II) are unable to undergo such reactions. However, it cannot be excluded that Cd(II) may perturb intracellular iron metabolism (Lesuisse and Labbe 1995). An increased level of free iron in the cell would enhance Fenton-type reactions and ROS production.

2.3.4 Arsenite and oxidative stress

Arsenite exposure leads to increased ROS production in mammals (Liu et al. 2001), however, the source of ROS is unknown. It has been proposed that arsenite can damage the mitochondrial membrane, which in turn may result in increased intracellular superoxide ($O_2^{\bullet-}$) levels. Moreover, As(III) may activate NADH oxidase which leads to elevated cellular $O_2^{\bullet-}$ levels (Huang et al. 2004; Shi et al. 2004). Similarly, NADPH oxidase, an enzyme complex formed in response to

immune challenge and other stressors, appears to play an important role in arsenic-induced superoxide formation (Chou et al. 2004). In turn, the formation of $O_2^{\bullet-}$ leads to other ROS such as $OH^{\bullet}$ and H_2O_2. As(III) may also increase H_2O_2 production as a result of As(III) oxidation or formation of hydroxyl radicals during the release of iron from ferritin triggered by arsenicals. Cellular H_2O_2 levels may also increase as a result of As(III) inhibition of glutathione peroxidase (Huang et al. 2004; Shi et al. 2004).

2.4 Selenium and chromium salts

In vitro studies (Turner et al. 1998) have shown that reduction of selenite involves reactions with sulphhydryl groups of thiol-containing molecules such as glutathione, leading to production of the intermediate metabolites selenodiglutathione (GS-Se-SG), glutathioselenol (GS-SeH) and hydrogen selenide (HSe^-) and finally to elemental selenium. Certain reactions of this pathway produce hydrogen peroxide and superoxide anion (Seko and Imura 1997). In a similar way, the reduction of chromate Cr(VI) in the cell is supposed to involve reactions with cellular glutathione (Liu et al. 1997a). The reduction intermediates Cr(V) and Cr(IV) are thought to be responsible for the generation of $OH^{\bullet}$ through a Fenton like mechanism (Shi et al. 1994; Stohs and Bagchi 1995). The final product of the reduction pathway is Cr(III), which is thought to present low toxicity.

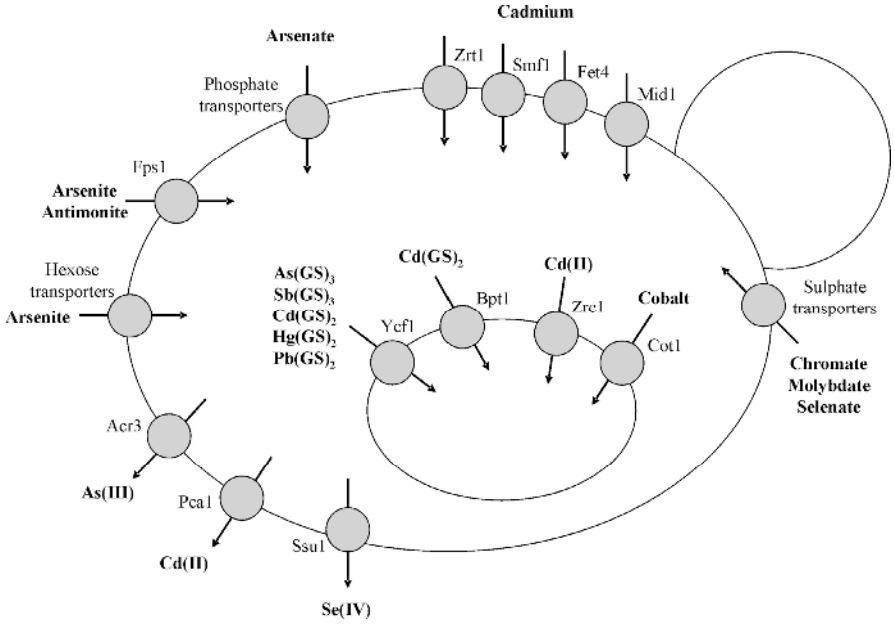

Fig. 1. Pathways of uptake and detoxification of toxic nonessential metals in *Saccharomyces cerevisiae*. See text and Table 1 for explanation of protein abbreviations.

3 Metal uptake pathways

A rapidly increasing number of proteins are being discovered that allow passage of nonessential metals across cell membranes and organelles inside the cell (Fig. 1; see also Chapter 14). While nonessential toxic metals appear to cross cell membranes through transporters responsible for nutrient metal acquisition, specific transporters contribute to their removal from the cytosol involving either metal export and/or sequestration in (an) internal organelle(s). Certain metals are transported in the free ionic form while others may be transported as complexes with various ligands. Inactivation of the uptake pathways in conjunction with enhanced activity of detoxification systems protects cells against metal toxicity.

3.1 Arsenic and antimony

Arsenic exists in two biologically important oxidation states; pentavalent arsenate As(V) and trivalent arsenite As(III). Arsenate is structurally similar to phosphate and competes with phosphate for transport. As(V) uptake in *Escherichia coli* is catalysed by the PhoS-PstABC phosphate translocating ABC-type ATPase complex and bacteria with defective phosphate transport accumulate little As(V) and display enhanced As(V) tolerance (Bennett and Malamy 1970; Willsky and Malamy 1980; Gatti et al. 2000). Similarly, arsenate enters plant roots via phosphate transporters (Meharg and Macnair 1990, 1992; Abedin et al. 2002; Wang et al. 2002) and a common arsenate/phosphate entry pathway may also exist in mammals (Huang and Lee 1996). In *S. cerevisiae*, arsenate uptake is likely to be mediated by the high-affinity phosphate transporter Pho84. Mutations in *PHO84* or in the *PHO87* and *PHO88* genes encoding respectively a low affinity phosphate transporter and a putative phosphate transporter, confer arsenate tolerance (Bun-ya et al. 1996; Yompakdee et al. 1996b). In addition, mutation of the *PHO86* gene also produces cellular arsenate tolerance (Bun-ya et al. 1996; Yompakdee et al. 1996a). Pho86 is an endoplasmic reticulum resident protein that is required for packaging of Pho84 into vesicles for subsequent transport to the plasma membrane (Lau et al. 2000).

The first arsenite influx pathway described was the *S. cerevisiae* aquaglyceroporin Fps1. In addition to arsenite, this protein also mediates antimonite entry into cells (Wysocki et al. 2001). Fps1 is a 74 kDa protein with six transmembrane helices and it belongs to the aquaporin family of channel proteins found in virtually all organisms. Whereas classical aquaporins are thought to be highly specific water channels, aquaglyceroporins may be permeated by a variety of substrates including glycerol and urea (Hohmann et al. 2000; Maurel et al. 2002; King et al. 2004). Fps1 plays a central role in yeast osmoregulation (Tamás and Hohmann 2003). Fps1 is inactive under hyperosmotic stress to permit glycerol accumulation and possibly turgor recovery. Upon hypoosmotic shock, Fps1 is rapidly activated and mediates glycerol export in order to prevent cell bursting and death (Tamás et al. 1999). Regulation of Fps1 activity requires a short domain in the cytoplasmic N-

terminal extension: an Fps1 protein lacking this domain cannot restrict transport and becomes hyperactive (Tamás et al. 1999, 2003).

Inactivation of Fps1, either by increasing external osmolarity or by deleting the *FPS1* gene, confers high level of arsenite and antimonite tolerance to yeast cells. In addition, deletion of *FPS1* reduces the uptake of arsenite into cells. Conversely, cells expressing a hyperactive Fps1 protein accumulate large amounts of arsenite and presumably also antimonite. As a consequence, such cells are highly As(III) and Sb(III) sensitive (Wysocki et al. 2001). Interestingly, this metalloid entry pathway appears to be controlled: expression of the *FPS1* gene is strongly repressed when cells are exposed to As(III) or Sb(III) (Wysocki et al. 2001). While the identity of the protein/signal transduction pathway that controls *FPS1* expression in the presence of these metalloids remains elusive, it was recently shown that the mitogen-activated protein kinase (MAPK) Hog1 modulates Fps1-mediated As(III) and probably also Sb(III) influx into cells (see further). However, repression of *FPS1* in the presence of As(III) occurs independently of Hog1 (Thorsen, Tängemo, Wagner, Wysocki, Boman, and Tamás: in preparation). Importantly, the capacity to transport arsenite and antimonite is not restricted to Fps1 but encompasses other aquaglyceroporins; the GlpF protein mediates As(III) and Sb(III) uptake in *E. coli* (Meng et al. 2004); mammalian AQP9 is permeated by both As(III) and Sb(III) whereas mammalian AQP7 transports As(III) (Liu et al. 2002, 2004b); LmAQP1 mediates uptake of both As(III) and Sb(III) into various *Leishmania* species and disruption of an *LmAQP1* allele in *L. major* results in increased Sb(III) resistance (Gourbal et al. 2004).

The predominant form of arsenite in solution at neutral pH appears to be the polyhydroxylated form $As(OH)_3$ (Ramirez-Solis et al. 2004). Similarly, the chemical form of Sb(III) recognised by aquaglyceroporins is thought to be $Sb(OH)_3$ (Baes and Mesmer 1976). Interestingly, polymerisation of three $As(OH)_3$ molecules is predicted to form a six-membered ring structure that may be similar to the six-membered ring structures of arsenious oxide (As_4O_6) and of hexose sugars (Liu et al. 2004a). In line with this notion, a recent study provided evidence that glucose carriers catalyse the uptake of As(III) in yeast: a strain lacking all glucose carriers exhibited low level of As(III) uptake (Liu et al. 2004a) and improved tolerance (Tängemo and Tamás: unpublished data).

Although Fps1 and hexose transporters are mediating the majority of As(III) influx into cells, genetic evidence (Wysocki et al. 2001) and transport data (Liu et al. 2004a) suggest that additional uptake routes exist. The molecular identity of the protein(s) catalysing residual arsenite uptake remains to be unveiled.

Arsenic and antimony-containing drugs are currently employed in the treatment of a variety of diseases. Arsenic trioxide (Trisenox) is used as a treatment for acute promyelocytic leukaemia and it might also be employed for other haematological and solid cancers (Evens et al. 2004; Ravandi 2004). Drugs containing arsenic or antimony are also employed to treat diseases caused by the protozoan parasites *Trypanosoma* and *Leishmania* (Murray 2001; Barrett et al. 2003). Knowledge of how arsenite and antimonite enters cells is imperative for the understanding of metalloid toxicity as well as of their ability to serve as chemotherapeutic agents. Indeed, recent studies have demonstrated that aquaglyceroporins

can modulate drug sensitivity in leukaemia (Bhattacharjee et al. 2004) as well as in *Leishmania* (Gourbal et al. 2004).

3.2 Cadmium

The molecular identity of the pathways mediating cadmium uptake has for long been elusive, although it was assumed that cadmium enters cells through transport systems for other essential elements such as calcium and iron. For instance, dietary iron and calcium deficiency was shown to promote increased accumulation of cadmium via the intestinal tract (reviewed in: Himeno et al. 2002). Recently, it has become clear that cadmium enters eukaryotic cells through a number of divalent cation transport systems. Cadmium uptake in yeast is mediated by various transport proteins involved in uptake of essential divalent cations including Zrt1 (zinc), Smf1 (manganese, copper, iron), and Fet4 (iron).

Zinc uptake in *S. cerevisiae* is primarily mediated by the high-affinity Zrt1 and low-affinity Zrt2 zinc transporters (Zhao and Eide 1996a, 1996b; see also Chapter 3). Zrt1 and Zrt2 are members of the ZIP (ZRT, IRT-like Protein) family of metal transporters present in bacteria, yeast, plants, and mammals. The ZIP proteins are capable of transporting a variety of cations including cadmium, iron, manganese, and zinc (Guerinot 2000; Gaither and Eide 2001). In addition to zinc, cadmium also appears to be a substrate for Zrt1; cells deleted for *ZRT1* accumulate less cadmium than wild type cells (Gomes et al. 2002) and Zrt1-mediated zinc uptake is strongly inhibited in the presence of cadmium (Gitan et al. 2003). Zrt1 is controlled at multiple levels. Firstly, transcription of the *ZRT1* gene is strongly induced under zinc-limiting conditions (Zhao and Eide 1996a, 1996b). Secondly, Zrt1 is inactivated by ubiquitination and subsequent endocytosis in zinc-replete cells thereby preventing further zinc uptake (Gitan et al. 1998; Gitan and Eide 2000). Interestingly, Zrt1 is also ubiquitinated and removed from the cell surface in the presence of cadmium. This inactivation may be an important mechanism to protect zinc-limited cells from cadmium toxicity (Gitan et al. 2003).

A second pathway of cadmium uptake into cells is through the Smf1 protein, which is a yeast member of the Nramp (neutral resistance-associated macrophage protein) family of metal transporters found in bacteria, fungi, plants, and mammals. As with the proteins of the ZIP family, the Nramp transporters can potentially recognize a broad range of substrates including copper, cadmium, manganese, and iron (Cellier et al. 1995; Thomine et al. 2000; Forbes and Gros 2001). *S. cerevisiae* expresses three functionally distinct members of the Nramp family encoded by the *SMF1*, *SMF2*, and *SMF3* genes (Portnoy et al. 2000). Smf1 was originally described as a high-affinity manganese transport system (Supek et al. 1996) and was later demonstrated to contribute to cellular accumulation of cadmium and copper (Liu et al. 1997b). Smf1 also stimulates iron uptake into *Xenopus* oocytes (Chen et al. 1999). The transport activity of Smf1 is regulated in response to metals; Smf1 is targeted to the vacuole when cells are replete with manganese whereas Smf1 fails to enter the vacuole under manganese starvation

and is instead inserted into the plasma membrane where it mediates metal uptake (Liu and Culotta 1999).

The function of Smf1 in cadmium uptake was revealed by analysis of the *BSD2* gene product. *BSD2* was first isolated as a gene, when inactivated by mutation, suppressed oxidative damage in yeast cells devoid of the copper/zinc superoxide dismutase (Liu and Culotta 1994). Cells lacking *BSD2* exhibited an increased level of Smf1-dependent manganese uptake, accumulated high levels of cadmium and cobalt, and displayed cadmium and cobalt hypersensitivity (Liu and Culotta 1994; Liu et al. 1997b). Interestingly, inactivation of *SMF1* in *bsd2Δ* cells eliminated the cadmium sensitivity of the *bsd2Δ* mutant but not its cobalt sensitivity. Instead, cobalt accumulation in *bsd2Δ* was reduced by deleting another Nramp encoding gene *SMF2* (Liu et al. 1997b). Smf2 is regulated by manganese and Bsd2 in a similar way as Smf1. However, while Smf1 moves to the plasma membrane in response to manganese starvation, Smf2 redistributes to intracellular vesicles. Moreover, Smf2 does not show any cell surface localisation (Portnoy et al. 2000; Luk and Culotta 2001). Hence, it is unclear how Smf2 contributes to cobalt influx into cells. Bsd2 is localised to the membrane of the endoplasmic reticulum. In the presence of manganese, Bsd2 is required to direct Smf1 and Smf2 to the vacuole (Liu et al. 1997b; Liu and Culotta 1999). In fact, Bsd2 is involved in Rsp5-mediated ubiquitination and sorting of various transmembrane proteins (Hettema et al. 2004). Hence, vacuolar targeting of Smf1 might also involve Rsp5.

The *FET4* gene encodes a low affinity iron transporter localised to the plasma membrane (Dix et al. 1994, 1997). In addition to its role in iron uptake, Fet4 has also been shown to be a physiologically relevant copper transporter (Hassett et al. 2000; Portnoy et al. 2001) and may be capable of transporting zinc, cadmium, and cobalt as well (Dix et al. 1994; Li and Kaplan 1998; Waters and Eide 2002). Transcription of the *FET4* gene is tightly regulated by environmental factors; the Atf1 iron responsive transcription factor induces *FET4* expression under iron-limiting conditions, the Zap1 transcription factor controls *FET4* expression in response to zinc and the Rox1 repressor controls *FET4* expression in response to oxygen (Jensen and Culotta 2002; Waters and Eide 2002). Repression of *FET4* in the presence of oxygen may protect cells against cadmium toxicity.

Finally, cadmium may also enter cells through calcium uptake systems such as the wheat LCT1-encoded calcium transporter. Interestingly, wheat LCT1 can restore calcium influx in a yeast strain deleted for the *MID1* gene encoding a stretch-activated calcium-permeable channel (Clemens et al. 1998). However, whether Mid1 indeed mediates influx of cadmium has not been tested.

Also plant members of the ZIP and Nramp protein families catalyse nonessential metal transport in addition to essential metals. For instance, the *Arabidopsis* IRT1 transporter (Korshunova et al. 1999; Rogers et al. 2000) as well as AtNramp1, AtNramp3, and AtNramp4 metal transporters are permeated by cadmium ions (Thomine et al. 2000). Similarly, the mammalian divalent metal transporter 1 DCT1 (also called DMT1), a member of the Nramp family, exhibits broad substrate specificity towards a variety of divalent cations including cadmium and lead (Gunshin et al. 1997).

3.3 Mercury

Mercury is a highly toxic, nonessential metal and the methylated form, *i.e.* methylmercury, is the most important form of mercury in terms of toxicity. Gram-negative bacteria protect themselves against the toxic effects of mercury by transporting Hg(II) into the cell via a specific uptake system. The periplasmic Hg(II)-binding protein MerP binds mercury and delivers it to the mercury transporter MerT for transport into the cell (Hobman and Brown 1996; Qian et al. 1998). An additional route of mercury uptake may involve the MerC protein (Sahlman et al. 1997). Once inside the cell, Hg(II) is reduced using NADPH to Hg(0) by the MerA reductase. Hg(0) is believed to leave the cell by passive diffusion (reviewed in: Nies 1999; Brown et al. 2002).

Mercury has a high affinity for reduced sulphhydryl groups, including those of cysteine and glutathione. It is in form of a methylmercury-cysteine complex that methylmercury enters mammalian cells through amino acid carriers. Methylmercury-L-cysteine is structurally similar to methionine and this complex is a substrate for transport systems that catalyse methionine uptake across cell membranes (reviewed in: Ballatori 2002). How mercury enters eukaryotic microorganisms such as yeast and fungi is not known.

3.4 Other metals

Other micronutrients with a potential for toxicity include selenate, molybdate and chromate. These metals cross cell membranes though sulphate transporters in mammalian cells (Ballatori 2002) whereas cobalt may be transported into yeast cells through the phosphate transporter Pho84 (Jensen et al. 2003). Phosphate and sulphate transporters are likely to carry the metals across the plasma membrane in form of oxyanions.

4 Metal transport and detoxification systems

Cells evade metal toxicity through metal export from the cell, sequestration within internal organelles, chelation by metal-binding proteins and peptides and reduction of uptake. Export of toxic metals and compartmentalisation in specific organelles such as the vacuole are the most efficient detoxification mechanisms found in microbes. Toxic metals may also bind to glutathione, metallothionein, and phytochelatin compounds and the resulting complexes are often substrates for transport systems. In this way, cells reduce the cytosolic concentration of reactive metal ions to sub-toxic levels, which leads to a better ability to survive and to proliferate in a polluted environment. The molecular identity of such detoxification systems may differ between metals and also between organisms.

4.1 Efflux-mediated tolerance systems

Metal export is a common detoxification strategy in prokaryota and a large number of transport proteins catalysing metal export have been characterised (Silver 1998; Rosen 1999b; Nies 2003). In contrast, there are very few such systems known in lower eukaryota. The most well-characterised metal exporter in *S. cerevisiae* is the plasma membrane protein Acr3 (Wysocki et al. 1997; Ghosh et al. 1999).

The *ACR3* gene (also called *ARR3*) was isolated in a screen for genes that confer high-level arsenic resistance to cells when overexpressed (Bobrowicz et al. 1997). *ACR3* encodes a 46 kDa protein with 10 potential membrane-spanning helices. Deletion of *ACR3* sensitises cells to arsenite and arsenate but not to the related metalloid antimony or any other metal tested including cadmium (Wysocki et al. 1997). Based on the fact that Acr3 is not able to contribute to arsenate tolerance without the activity of the arsenate reductase Acr2 (also called Arr2; see Section 4.4), it was suggested that Acr3 is a specific arsenite export protein (Bobrowicz et al. 1997). Indeed, cells expressing multiple copies of the *ACR3* gene accumulated little arsenite (Wysocki et al. 1997), whereas the *acr3Δ* mutant was deficient in arsenite export (Ghosh et al. 1999). The mechanism by which Acr3 transports As(III) is not understood but the lack of an ATP-binding cassette in the Acr3 sequence may indicate that As(III) export is coupled to the membrane potential (Rosen 1999).

The activity of Acr3 is controlled at the level of transcription; *ACR3* expression is strongly induced by As(III), As(V) and to a lesser extent by Sb(III). Metalloid-stimulated expression of *ACR3* requires the AP-1-like transcription factor Yap8 and possibly also Yap1 (Bobrowicz and Ulaszewski 1998; Bouganim et al. 2001; Haugen et al. 2004; Menezes et al. 2004; Wysocki et al. 2004).

The fact that *ACR3* expression is stimulated by antimonite may suggest that arsenite is not the sole substrate for Acr3. However, yeast cells lacking *ACR3* are not Sb(III) sensitive (Wysocki et al. 1997). In addition, an Acr3 homologue from the *Bacillus subtilis ars* operon confers resistance only to arsenicals (Sato and Kobayashi 1998). On the other hand, antimonite sensitivity of double *acr3*Δ *ycf1*Δ mutant (*YCF1* encodes a vacuolar ABC-transporter involved in metal detoxification: see Section 4.2) was only slightly improved by deletion of *FPS1*, while the *ycf1*Δ *fps1*Δ double mutant showed much higher increase in Sb(III) tolerance (Wysocki et al. 2001). This observation suggests that Acr3 might contribute to antimony tolerance under certain conditions.

The *ACR3* gene is located in a cluster of arsenical resistance genes that in terms of function resembles the prokaryotic *ars* operon (Bobrowicz et al. 1997). However, Acr3 shows no structural or sequence similarities to the well-studied arsenite/antimonite transporter ArsB from the *E. coli* plasmid R773 or the *Staphylococcus aureus* plasmid pI258. As a result of sequencing of numerous prokaryotic and fungal genomes, a novel and distinct family of Acr3-like transporters has emerged (Wysocki et al. 2003). Interestingly, *ACR3* homologues are widely distributed in both *Bacteria* and *Archea*, but are not found in eukaryotes except for a few fungal species, including some *Saccharomyces* yeasts, *Kluyveromyces lactis*,

Candida albicans, and *Neurospora crassa*. Phylogenetic analysis of the Acr3-like family suggests that horizontal gene transfer has played a major role in the evolution of the *ACR3* gene and that fungi might have acquired the *ACR3* homologue directly from a prokaryotic organism relatively late in evolution (Wysocki et al. 2003).

The *ars* operon of the *E. coli* plasmid R773 encodes an ATP-driven metalloid pump ArsAB consisting of two proteins; the arsenite- and antimonite-stimulated ATPase ArsA bound to the inner-membrane protein ArsB (Rosen 1999). Interestingly, in the absence of ArsA, ArsB uses the membrane potential for arsenite and antimonite export. No homologues of *E. coli* ArsB transporter have been found in eukaryotic genomes. In contrast, ArsA homologues are ubiquitous but the physiological function of eukaryotic ArsA remains elusive (Kurdi-Haidar et al. 1998; Zuniga et al. 1999; Bhattacharjee et al. 2001; Shen et al. 2003). For instance, the *S. cerevisiae* ArsA homologue encoded by the *ARR4* (*YDL100c*) gene is not associated with the plasma membrane and the ATPase activity of Arr4 is not stimulated by metalloids (Shen et al. 2003). Deletion of *ARR4* results in a slight sensitivity to several metals like copper, zinc, cobalt, chromium, vanadate, and arsenic (Zuniga et al. 1999; Shen et al. 2003) and this sensitivity is enhanced at 37°C suggesting a general role of Arr4 in stress response that is not related to a specific metal detoxification pathway.

The plasma membrane protein Ssu1 belongs to the major facilitator superfamily and overexpression of *SSU1* increases selenite tolerance (Pinson et al. 2000). Ssu1 has also been implicated in sulphite efflux from yeast cells (Park and Bakalinsky 2000) and due to analogous structures, both selenite and sulphite are likely to be recognised by Ssu1 for export. However, expression of *SSU1* is not induced by selenite and the primary physiological substrate of this transporter has not been determined. Moreover, selenite can efficiently be reduced by glutathione to less toxic elemental selenium that precipitates as red granules within the cell (Pinson et al. 2000). Hence, the mechanism by which Ssu1 affects selenite tolerance remains unclear.

PCA1 encodes a P-type metal-transporting ATPase with a role in copper and iron homeostasis (Rad et al. 1994; De Freitas et al. 2004) and a single amino acid substitution in *PCA1* (Arg970Gly) has been reported to increase cadmium tolerance (Shiraishi et al. 2000). Cells expressing *PCA1*-Arg970Gly accumulated less cadmium than either wild type or *pca1Δ* cells. Overexpression of *PCA1*-Arg970Gly further increased cadmium tolerance by lowering the intracellular cadmium level compared to cells with a single copy of this allele. Collectively, Pca1-Arg970Gly appears to contribute to cadmium tolerance, possibly by increasing cadmium export (Shiraishi et al. 2000). However, how this is achieved remains to be revealed.

4.2 Vacuolar sequestration of toxic metals

In *S. cerevisiae*, the yeast cadmium factor 1 (Ycf1) constitutes the major pathway of toxic metal sequestration in the vacuole. The *YCF1* gene was isolated in a

screen for genes conferring cadmium resistance to cells when present in multiple copies. Cells that overexpress *YCF1* exhibit elevated cadmium resistance while the *ycf1Δ* mutant is hypersensitive to this metal (Szczypka et al. 1994). Ycf1 is an ATP-binding cassette (ABC) transporter and it shares strong sequence similarity to the human cystic fibrosis conductance regulator (CFTR) and to the multidrug-associated proteins MRP1 and MRP2 (Szczypka et al. 1994; Buchler et al. 1996). Ycf1 is a 171 kDa protein present in the vacuolar membrane and it catalyses ATP-dependent uptake of a range of glutathione-conjugated metals and xenobiotics into the vacuole (Li et al. 1996, 1997; Tommasini et al. 1996). Other MRP family members involved in metal detoxification includes *Leishmania* PgpA that transports $As(GS)_3$ into intracellular vesicles (Légaré et al. 2001), mammalian MRP2 that, in the human liver, catalyses the extrusion of arsenic-glutathione complexes into bile (Kala et al. 2000) and human MRP1 that mediates export of $As(GS)_3$ in several tissues (Leslie et al. 2004).

In addition to its role in cadmium detoxification, Ycf1 has been shown to confer resistance to the metalloids arsenic and antimony. The *ycf1Δ* mutant is moderately sensitive to arsenite whereas it displays very strong antimonite sensitivity (Ghosh et al. 1999; Wysocki et al. 2001). In contrast, cells lacking *ACR3* are As(III) and As(V) sensitive but do not show any antimonite sensitivity. Deletion of both *ACR3* and *YCF1* genes results in additive arsenite sensitivity indicating that yeast cells possess two distinct metalloid detoxification pathways with different specificities (Ghosh et al. 1999).

Transport of $As(GS)_3$ into vacuoles is inhibited not only by cadmium and antimony but also by mercury, suggesting an additional role of Ycf1 in mercury tolerance (Ghosh et al. 1999). In agreement with this hypothesis, deletion of *YCF1* sensitises cells to mercury and active transport of $Hg(GS)_2$ across the membrane of vesicles isolated from *YCF1*-overexpressing cells has been demonstrated (Gueldry et al. 2003). Ycf1 also confers resistance to lead; the *ycf1Δ* mutant is Pb(II)-sensitive whereas cells overexpressing *YCF1* are more Pb(II)-resistant (Song et al. 2003).

How Ycf1 is regulated by various metals and xenobiotics is not fully understood. Expression of the *YCF1* gene is not induced to any large extent by metal treatment; cadmium exposure results in a twofold increase in *YCF1* mRNA levels (Li et al. 1997; Sharma et al. 2002), a twofold increase under selenite exposure (Pinson et al. 2000) whereas arsenite and antimonite exposure does not seem to affect *YCF1* expression (Wysocki et al. 2004). It is possible that the basal level of Ycf1 in the vacuolar membrane is sufficient to mediate metal tolerance. Thus, conjugation of metals to GSH may represent the rate-limiting step in tolerance acquisition. In that case, increased GSH synthesis might be sufficient to promote increased vacuolar sequestration of GSH-conjugated metals by the action of Ycf1 (Wysocki et al. 2004). A similar mechanism of arsenic tolerance has been proposed in *Leishmania*, where increased synthesis of trypanothione, the major source of reduced thiols in trypanosomatidae, is required to produce resistance as a result of formation and extrusion of metalloid-thiol complexes (Mukhopadhyay et al. 1996). It has been shown that Ycf1 is phosphorylated at two residues, Ser908 and Thr911, and that mutation of these sites severely impairs its transport activity

(Szczypka et al. 1994; Eraso et al. 2004). Neither the kinase responsible for Ycf1 phosphorylation nor whether phosphorylation affects Ycf1 activity under metal exposure is known. Finally, Ycf1 is also controlled at the level of proteolytic processing and intracellular trafficking (Mason and Michaelis 2002; Mason et al. 2003). Again, whether these processes are regulated under metal exposure is unknown.

Besides Ycf1, five additional *S. cerevisiae* members of the ABC transporter superfamily have been identified based on sequence similarities: Bpt1, Ybt1/Bat1, Yor1, Vmr1 (Yhl035), and Nft1 (Ykr103/Ykr104w) (Decottignies and Goffeau 1997; Mason et al. 2003). Bpt1 is localised in the vacuolar membrane and mediates transport of unconjugated bilirubin and glutathione conjugates into the vacuole (Petrovic et al. 2000; Klein et al. 2002; Sharma et al. 2002). In addition, Bpt1 plays a role in cadmium detoxification. However, the contribution of Bpt1 in vacuolar sequestration of cadmium is less important than that of Ycf1. In fact, the function of Bpt1 in cadmium resistance is only detectable in cells lacking *YCF1* (Sharma et al. 2002). Ybt1/Bat1 transports free bile acid (Ortiz et al. 1997) and, together with Bpt1 and Ycf1, Ybt1/Bat1 contributes to vacuolar accumulation of the toxic red pigment that is produced by adenine biosynthetic mutants (Sharma et al. 2002, 2003). Yor1 is a plasma membrane protein that participates in the detoxification of several unrelated compounds, like oligomycin, cadmium and a wide spectrum of organic anions (Cui et al. 1996; Decottignies et al. 1998; Katzmann et al. 1999). The function and cellular localisation of Vma1 and Nft1 remain to be characterized (Mason et al. 2003), although Vma1 appears to be in the vacuolar membrane and to mediate cadmium resistance (D. Wawrzycka, personal communication). Since these proteins share structural similarities and overlapping substrate specificities, it is reasonable to assume that the yeast MRP1-like ABC transporters provide resistance to a wide range of toxic metals and metalloids. Detailed phenotypic analysis of metal tolerance in various multiple deletion mutants in conjunction with transport studies will be required to support this hypothesis.

Two additional transporters appear to have a function in vacuolar sequestration of toxic metals; Zrc1 and Cot1. Zrc1 and Cot1 are both vacuolar membrane proteins (Li and Kaplan 1998) belonging to the CDF (cation diffusion facilitator) family of transporters (Paulsen and Saier 1997). Overexpression of *ZRC1* or *COT1* confers zinc tolerance, suggesting an increased capacity to sequester zinc in the vacuole (MacDiarmid et al. 2000, 2002). The *COT1* gene was originally isolated as a dosage-dependent suppressor of cobalt toxicity and overexpression of *COT1* increases cobalt and rhodium tolerance (Conklin et al. 1992). In fact, Cot1 and Zrc1 share significant sequence similarity and Zrc1 was originally isolated as a high-copy suppressor of zinc and cadmium toxicity (Kamizono et al. 1989). Hence, in addition to zinc, these transporters are also capable of mediating vacuolar cobalt and cadmium uptake.

Cadmium detoxification involves an ABC-type transporter also in *S. pombe*. Fission yeast Hmt1 is located in the vacuolar membrane and contributes to cadmium tolerance (Ortiz et al. 1992). In contrast to *S. cerevisiae* Ycf1, *S. pombe* Hmt1 does not transport cadmium-glutathione conjugates, but instead catalyses

the transport of glutathione-derived phytochelatins and phytochelatin-cadmium complexes into the vacuole (Ortiz et al. 1995).

4.3 Metal-binding peptides and proteins: metallothioneins and phytochelatins

Toxic metals may inactivate cellular proteins by reacting with sulphhydryl groups of cysteine residues and thiol-mediated defence mechanisms are commonly employed by eukaryotic cells. These include small cysteine-rich proteins known as metallothioneins and metal-binding peptides such as phytochelatins and glutathione (see Section 5).

Yeast metallothioneins encoded by *CUP1* were shown to maintain copper homeostasis and to confer cadmium tolerance by directly chelating metal ions (Winge et al. 1985). In contrast to mammalian metallothioneins, yeast Cup1 does not seem to protect cells against mercury, zinc, or arsenic toxicity (Ecker et al. 1986; Wysocki and Tamás: unpublished data). Moreover, deletion of the second metallothionein gene *CRS5*, exhibiting high similarity to mammalian metallothioneins and to a lesser extent to *CUP1*, renders cells sensitive only to copper (Culotta et al. 1994). In support of these findings, Crs5 was reported to sequester copper ions in a similar manner as mammalian metallothioneins (Jensen et al. 1996). Taken together, the contribution of metallothioneins to metal tolerance in yeast appears to be limited to copper and possibly to cadmium. However, although overexpression of *CUP1* increases cadmium tolerance, *cup1Δ* cells are not cadmium sensitive indicating a minor contribution of metallothioneins to cadmium detoxification. Furthermore, expression of *CUP1* is not induced in response to cadmium (Vido et al. 2001) suggesting that cadmium detoxification by chelation to metallothionein may not be a physiologically relevant mechanism in *S. cerevisiae*.

In *S. pombe*, resistance to a wide range of metals is associated with phytochelatins (Cobbett 2000b, 2000a). The general structure of these polypeptides is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2-11) and their synthesis from glutathione is mediated by the enzyme phytochelatin synthase (*PCS*). Production of phytochelatins is induced in response to cadmium, copper, arsenic, zinc, silver, and nickel exposure (Clemens et al. 2001). The enzymatic activity of phytochelatin synthase is furthermore stimulated by cadmium, copper, silver, zinc, lead, and mercury (Ha et al. 1999). However, only phytochelatin complexed with cadmium, copper, silver, and arsenic have been detected *in vitro* (Maitani et al. 1996; Schmoger et al. 2000).

Identification of the *PCS* gene encoding phytochelatin synthase in *S. pombe* and plants was an important step in understanding the physiological function of phytochelatins (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999). Phenotypic analysis of *S. pombe* cells lacking the *PCS* gene showed that the mutant was sensitised to cadmium, arsenate, arsenite, and copper (Clemens et al. 1999; Ha et al. 1999; Wysocki et al. 2003). Heterologous expression of the *S. pombe PCS* gene in *S. cerevisiae* wild type and the *acr3Δ ycf1Δ* double mutant not only confirmed an important role of phytochelatins in cadmium and arsenite de-

toxification but also revealed their ability to confer antimonite resistance (Clemens et al. 1999; Ha et al. 1999; Wysocki et al. 2003).

Chelation of cadmium by phytochelatins in the *S. pombe* cytosol is followed by the sequestration of phytochelatin-Cd complexes in the vacuole by the ABC transporter Hmt1 (Ortiz et al. 1992, 1995). Moreover, the existence of an additional phytochelatin-independent pathway of cadmium accumulation into the vacuole based on a $Cd(II)/H^+$ antiporter activity has been proposed (Ortiz et al. 1995). Sulphite metabolism also contributes to phytochelatin-mediated cadmium tolerance. It is believed that sulphite incorporation into phytochelatin-Cd complexes leads to their stabilisation and increases the number of cadmium molecules per complex (Reese and Winge 1988).

4.4 Arsenate reduction – a pathway leading to tolerance and drug activation

The initial step in arsenate detoxification in most organisms is the enzymatic reduction of As(V) to As(III). Two distinct prokaryotic families of arsenate reductases have been identified and extensively studied at the molecular level; one family includes the ArsC from the *E. coli* plasmid pR773 and the other one is represented by the ArsC encoded by the plasmid pI258 from *S. aureus* (Mukhopadhyay and Rosen 2002). In contrast, no arsenate reductase was known in eukaryotic organisms until the isolation of the *ACR* gene cluster in *S. cerevisiae* (see above; Bobrowicz et al. 1997). Overexpression of the arsenite export protein-encoding gene *ACR3* conferred resistance only to As(III), while the presence of both *ACR2* and *ACR3* genes on a multicopy plasmid resulted in increased tolerance to As(III) and As(V). This observation suggested that *ACR2* might encode an arsenate reductase (Bobrowicz et al. 1997). Indeed, *ACR2* deletion sensitises cells only to the pentavalent form of arsenic (Mukhopadhyay and Rosen 1998) and the purified homodimeric protein exhibits As(V) reductase activity (Mukhopadhyay et al. 2000).

Despite the lack of sequence identity, Acr2 and the *E. coli* R773 arsenate reductase share functional and mechanistic similarities. Both enzymes use glutathione and glutaredoxin as electron donors for arsenate reduction, and Acr2 complements the arsenate sensitivity of *E. coli* cells bearing an *arsC* deletion (Mukhopadhyay et al. 2000). Furthermore, a single cysteine residue (Cys12 in ArsC and Cys76 in Acr2) is required for catalytic activity (Mukhopadhyay and Rosen 2001). This may suggest that, as in the case of *E. coli* R773 ArsC, a mixed disulphide between the Acr2 enzyme and glutathione is formed, followed by glutaredoxin binding associated with reduction of arsenate to the dihydroxy monothiol As(III) intermediate; the two last steps in this reaction would involve the formation of a monohydroxy intermediate containing positively charged arsenic and the final release of free $As(OH)_3$, as determined for the crystal structure of *E. coli* R773 ArsC (DeMel et al. 2004).

Cys76 of Acr2 is a part of the $HC(X)_5R$ motif that is conserved in the Cdc25 family of dual-specific protein phosphatases (PTPs) (Fauman et al. 1998) and in

rhodanase, a thiosulphate sulphurtransferase (Hofmann et al. 1998). In PTPases, the $HC(X)_5R$ consensus, called the P-loop, serves as a phosphate binding pocket, where the active-site cysteine forms the phosphoenzyme intermediate that is stabilized by the adjacent arginine residue (Jackson and Denu 2001). The Cys76Ala and Arg82Ala mutations in the $HC(X)_5R$ consensus site of Acr2 resulted in loss of arsenate resistance and loss of reductase activity *in vitro*, suggesting that a phosphatase-active site is used as the catalytic centre for arsenate reduction in yeast (Mukhopadhyay and Rosen 2001).

Despite having the phosphatase active site, Acr2 does not exhibit phosphatase activity towards the substrate *p*-nitrophenyl phosphate (Mukhopadhyay and Rosen 2001). However, the P-loop in Acr2 lacks the GXGXXG motif that is found in a variety of PTPs (Mukhopadhyay et al. 2003). Interestingly, Acr2 was converted into a phosphatase at the expense of its arsenate reductase activity when three glycine residues were introduced at positions 79, 81, and 84 within the phosphatase motif (Mukhopadhyay et al. 2003). The conserved cysteine and arginine residues of the $HC(X)_5R$ were also required for the acquired phosphatase activity of the mutated Acr2 suggesting that both PTPs and Acr2 share a similar mechanism of catalysis. Based on these results and sequence similarities of Acr2 and I258 ArsC family to phosphatases, Mukhopadhyay et al. (2003) hypothesised that all three families of arsenate reductases, including *E. coli* R773 ArsC, *S. aureus* I258 ArsC, and *S. cerevisiae* Acr2, may have evolved independently for at least three times from an ancestral phosphatase (Mukhopadhyay et al. 2003). Due to the anaerobic atmosphere, the dominant form of arsenic in water was originally a trivalent arsenite. Thus, microorganisms first developed specific arsenite exporters to cope with arsenic toxicity. Next, when the atmosphere became oxidizing, the phosphatase would have been converted by only a couple of simple point mutations into arsenate reductases, which could produce substrates for the existing arsenite transporters (Rosen 1999; Mukhopadhyay et al. 2003).

Until recently, Acr2 was the only known eukaryotic arsenate reductase. The sequence of Acr2 was used to find an arsenate reductase-encoding gene in *Leishmania major* (Zhou et al. 2004). *LmACR2* is able to restore arsenate tolerance to *E. coli* and *S. cerevisiae* cells lacking arsenate reductases. Furthermore, the purified *Lm*Acr2 reduces not only arsenate but also antimonate [Sb(V)] in the presence of glutathione and glutaredoxin. Most importantly, expression of *LmACR2* in *L. infantum* amastigotes resulted in increased sensitivity to the Sb(V)-containing drug Pentostam (Zhou et al. 2004). Pentavalent antimony-containing drugs are used in the treatment of protozoan infections and reduction of Sb(V) to the more toxic Sb(III) is believed to be a pathway to activate the drug in *Leishmania* cells (dos Santos Ferreira et al. 2003; Wyllie et al. 2004; Zhou et al. 2004). Identification of an arsenate/antimonate reductase in *Leishmania* could be of major importance for predicting treatment efficacy of patients with leishmaniasis as well as in designing new strategies to sensitise pathogenic protozoa to available drugs.

Chromate (VI) is believed to be reduced non-enzymatically to the more stable trivalent form (Arslan et al. 1987). However, a chromate-resistance phenotype in *Candida maltosa* strain has recently been associated with active NADPH-dependent chromate reduction (Ramirez-Ramirez et al. 2004). The chromate re-

ductase enzyme remains to be identified. In most prokaryota, Hg(II) is reduced enzymatically to an inert monoatomic form Hg(0) by a MerA mercuric reductase located in the plasmid-encoded *mer* operon (reviewed in: Nies 1999; Brown et al. 2002). No mercuric reductase is known in eukaryotic organisms and the Acr2 arsenate reductase seems to be the sole example of toxic metal-detoxification reductase in lower eukaryota.

5 Sulphur and glutathione metabolism

A major chemical property of many toxic metals including Cd(II), As(III), Hg(II), and Pb(II) is their capacity to strongly bind to thiol residues. This property is used by most organisms for chelation, sequestration, and detoxification. For detoxification of nonessential toxic metals, yeasts overexpress the thiol-containing peptides glutathione and/or phytochelatins depending on the species (see above).

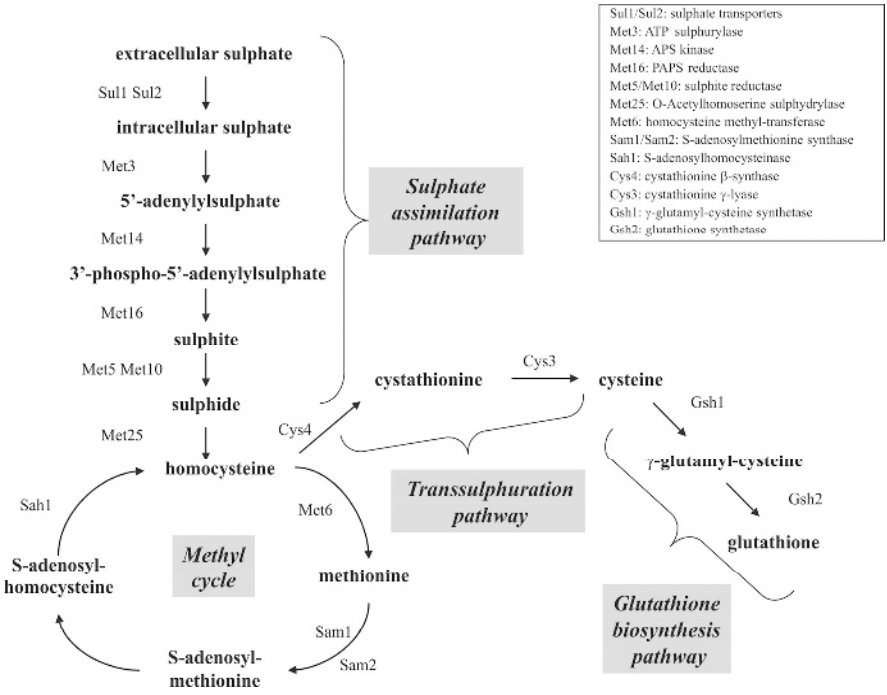

Fig. 2. Sulphur and glutathione metabolism in *Saccharomyces cerevisiae*. The sulphur pathway can be divided into three parts: the sulphate assimilation pathway, the methyl cycle, and the branch leading to cysteine and glutathione synthesis. See the text for details.

5.1 The sulphur pathway of *S. cerevisiae*

The sulphur pathway is composed of the sulphate assimilation pathway, the methyl cycle, the transsulphuration pathway and the glutathione biosynthesis pathway (Fig. 2). Inorganic sulphate (SO_4^{2-}) or sulphite (SO_3^{2-}) is reduced through the assimilation pathway resulting in production of sulphide (S^{2-}) and its incorporation into homocysteine or cysteine. Most yeast species are able to incorporate sulphide into both homocysteine and cysteine (Paszewski 2001). In *S. cerevisiae*, however, sulphide cannot be incorporated into cysteine leaving homocysteine the only precursor of the two sulphur amino acids; methionine through the methyl cycle and cysteine through the transsulphuration pathway (Thomas and Surdin-Kerjan 1997). Cysteine or a derivative of cysteine is probably the sensor of the metabolic state in the pathway (Hansen and Johannsen 2000; Paszewski 2001). Cysteine is also required for glutathione synthesis. Another essential function of the sulphur pathway is its involvement, through S-adenosylmethionine, in the biosynthesis of polyamines, biotin, and for almost all transmethylation reactions in the cell. The *S. cerevisiae* sulphur pathway and its regulation has been reviewed in detail by Thomas and Surdin-Kerjan (1997).

5.2 Toxic metals induce the synthesis of glutathione

Genome-wide transcriptional analyses in *S. cerevisiae* show that Cd(II), As(III) and Hg(II) induce the expression of genes of the sulphate assimilation and of the glutathione biosynthesis pathways (Momose and Iwahashi 2001; Vido et al. 2001; Fauchon et al. 2002; Haugen et al. 2004; Thorsen et al. in preparation). Proteome analysis also evidenced strong induction of the enzymes of the sulphur pathway in response to cadmium (Vido et al. 2001). Consistent with these data and the importance of glutathione for cadmium detoxification, the glutathione synthesis rate, and the flux in the pathway are strongly increased in response to this metal (Vido et al. 2001; Fauchon et al. 2002). In addition, all the intermediate metabolites of the glutathione pathway increase under cadmium exposure (Lafaye et al. 2005a, 2005b). Importantly, the cadmium response has been analysed at the level of the transcriptome, proteome and metabolome and the data correlate nicely (Vido et al. 2001; Fauchon et al. 2002; Lafaye et al. 2005a, 2005b).

Arsenite treatment also induces expression of genes and enzymes of the sulphur pathway (Haugen et al. 2004) but the induction levels seem less pronounced than with cadmium. The flux in the sulphur pathway and the glutathione synthesis rate are also strongly increased (Thorsen et al. in preparation). Reduced glutathione forms a complex with Cd(II), As(III) and other toxic metals which are transported into the vacuole by the ABC transporter Ycf1 as outlined above.

Interestingly, the two major transcriptional activators of the cadmium response, Met4 and Yap1 (see Section 6), control different parts of the detoxification pathway. Met4 controls the first part (*CYS4*, *CYS3*, *GSH1*) (Dormer et al. 2000; Fauchon et al. 2002) whereas Yap1 regulates the second part of the glutathione biosynthesis pathway (*GSH1*, *GSH2*). Yap1 also controls expression of glutathione

metabolism genes (*GTT2*, *GLR1*, *YCF1*) (Wemmie et al. 1994b; Wu and Moye-Rowley 1994; Grant et al. 1996; Gasch et al. 2000; Sugiyama et al. 2000). Both factors contribute to induced *GSH1* expression (Wheeler et al. 2003).

5.3 Sulphur sparing in proteins

Cadmium detoxification by GSH necessitates high amounts of sulphur. To cope with this vital sulphur requirement, the sulphur metabolism, normally directed to the production of methionine and cysteine for protein synthesis, is redirected to glutathione biosynthesis (Fig. 3; Fauchon et al. 2002). For optimal sulphur sparing, yeast cells both decrease their global protein synthesis rate (Lafaye et al. 2005a) and reduce the sulphur amino acid composition of the newly synthesized proteome. Remarkably, some abundant glycolytic enzymes rich in sulphur amino acids are replaced by sulphur-depleted isoenzymes. For example, the pyruvate decarboxylase enzyme Pdc1 containing 16 sulphur amino acids is strongly repressed whereas the sulphur-poor isoenzyme Pdc6 (5 sulphur amino acids) is dramatically induced. This global change in protein expression allows an overall sulphur amino acid saving of 30%. Interestingly, Met4, the main transcriptional activator of the sulphate assimilation pathway, is responsible for the isoenzyme switches and

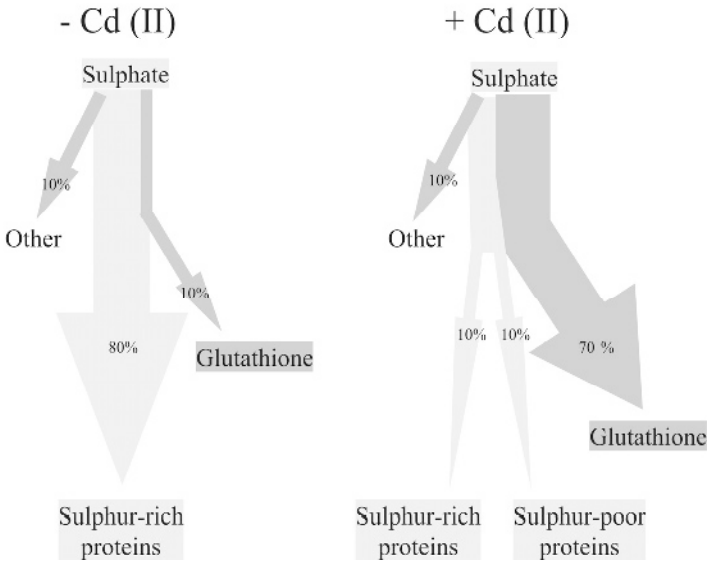

Fig. 3. Sulphur sparing in response to cadmium. Cd(II) exposure leads to a strong increase in glutathione levels, a decreased synthesis of many sulphur-rich proteins and the replacement of these proteins by sulphur-poor proteins.

plays a major role in the sulphur-sparing response, indicating that the same regulator controls both glutathione synthesis and the mechanisms to save sulphur in order to produce more glutathione (Fauchon et al. 2002). This regulation emphasizes the importance of sulphur sources in detoxification and indicates that a selective pressure may act on the atomic composition of proteins as also showed by Baudouin-Cornu et al. (2001).

5.4 Other yeasts

Induction of the (genes in the) glutathione biosynthesis pathway could also be expected in *S. pombe* since the main detoxification mechanism in this yeast involves chelation of Cd(II) to glutathione and to phytochelatins (PC). However, transcriptome and proteome analyses indicated that enzymes of the sulphate assimilation, glutathione and the PC synthesis pathways are not induced upon exposure to this metal (Chen et al. 2003; Bae and Chen 2004). Only some transporters of sulphur compounds are transcriptionally induced by Cd(II) (Chen et al. 2003), particularly a sulphate transporter, and this induction is dependent on the bZIP transcription factor Zip1 (Harrison et al. 2005). Although expression of genes encoding enzymes of the sulphur metabolic pathway are not induced, a strong increase in PC synthesis is observed in response to cadmium as well as to many other metals including Hg(II) (Ow et al. 1994). Binding of Cd(II) to PC synthase strongly activates the enzyme (Grill et al. 1991; Vatamaniuk et al. 2000; Maier et al. 2003). Sulphide synthesis is also increased without any transcriptional induction of enzymes in the sulphur assimilation pathway except the sulphate transporter-encoding gene (Bae and Chen 2004). Sulphide participates in the production of the high molecular weight PC-Cd-S complex with high cadmium binding capacity (Ow et al. 1994). A similar mechanism has been described in *Candida glabrata* (Dameron et al. 1989). Detoxification of Cd(II), As(III) and Sb(III) through PC-based chelation seems to be more efficient than the GSH-based chelation mechanism operating in *S. cerevisiae* since the expression of *S. pombe* or *A. thaliana* PC synthase in *S. cerevisiae* results in a strong increase in tolerance to these metals (Clemens et al. 1999; Wysocki et al. 2003).

5.5 Selenate and chromate interferes with the sulphate assimilation pathway

Selenate and chromate salts probably enter cells through sulphate transporters (Marzluf 1970; Breton and Surdin-Kerjan 1977). Interestingly, mutants resistant to selenate or chromate (*sul1Δ*, *sul2Δ*, *met3Δ*, *met14Δ*, and *met16Δ*) are all mutants of the sulphate assimilation pathway (Cherest et al. 1997) suggesting that metabolization of chromate and selenate through this pathway is necessary to cause toxicity. It is conceivable that selenate [Se(VI)] is toxic due to its conversion to selenite [Se(III)]. Another possibility to explain the phenotype of these mutants is that a sulphur-precursor metabolite accumulates in the mutant cells as a consequence of

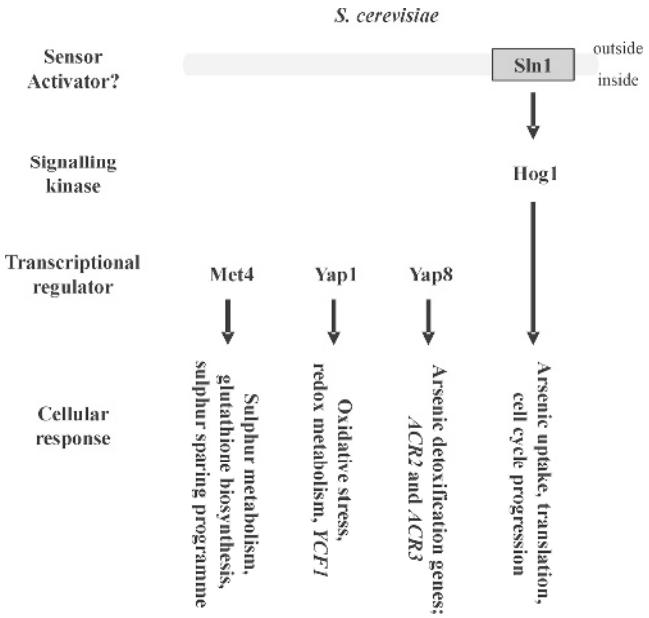

Fig. 4. Signalling proteins and transcriptional regulators involved in metal tolerance.

the lacking enzyme activity and causes a repression/inhibition of sulphate transporters and then resistance to the toxic analogues selenate and chromate.

6 Signalling and transcriptional regulation

The activity of various detoxification systems that contribute to cellular tolerance is controlled by signalling proteins and transcriptional regulators; yet, our understanding of the mechanisms by which eukaryotic cells sense the presence of non-essential metals, and activate such tolerance systems is rudimentary. However, recent work has identified a number of players involved (Fig. 4). Below, we will outline what is known about the mechanisms through which these transcription factors and signalling proteins are activated and contribute to tolerance.

6.1 Yap1 protects cells from a variety of oxidants and metals

One of the most well-characterised transcription factors involved in metal tolerance acquisition is the *S. cerevisiae* transcription factor Yap1 (yeast AP-1). Yap1 regulates the main peroxide (H_2O_2 and organic peroxides) metabolism pathway and contributes to stress responses elicited by specific metals and by several chemicals with electrophilic properties. Over the last few years, Yap1 has provided a unique model to understand how cells specifically sense and respond to

oxidants and to molecules of seemingly different chemical nature. Moreover, identification of the Yap1 gene-targets has provided basic insights into the mechanisms of oxidative, metal and chemical stress and the specific cellular defences used to counter these threats. The extended role of Yap1 in stress tolerance acquisition can be rationalised as an important cellular device for survival in soil habitats or during successful host invasion, *i.e.*, for providing protection against hostile environments and host-defence mechanisms that include both oxidative stress and a variety of fungicidal molecules. The recent description of a Yap1-operated indoleacetic response (Prusty et al. 2004), a plant hormone that cause yeast to differentiate into an invasive form, highlights the role of Yap1 and more generally of fungal stress responses in plant-pathogen interactions. True functional homologues of Yap1 have now been identified in many different fungi, including *S. pombe* Pap1 (Toone et al. 2001), *Candida albicans* Cap1 (Alarco and Raymond 1999), *Kluyveromyces lactis* KLYap1 (Billard et al. 1997) and Chap1 of the maize pathogen *Cochliobolus heterostrophus* (Lev et al. 2005). Genes homologous to *YAP1* are also present in *Aspergillus nidulans* (GeneBank accession no EAA62093) and *Neurospora crassa* (CAB91681). In addition to Yap1, *S. cerevisiae* contains another seven AP-1-like proteins: Yap2 to Yap8 (Fernandes et al. 1997; Toone et al. 2001).

6.1.1 Yap1, an AP-1-like bZIP transcription factor

Yap1 was initially identified as a protein capable of activating the SV40 AP-1 recognition-element (ARE) and purified by virtue of its ARE-specific DNA-binding affinity as a 90 kDa protein (Harshman et al. 1988). The cloning of the *YAP1* gene revealed the presence of a basic-leucine zipper (bZIP)-domain, which in contrast to other bZIP family members is amino- rather than carboxy-terminal (Moye-Rowley et al. 1989). This domain also differs from AP-1 factors at two of the five highly conserved residues that contact DNA, conferring a distinctive DNA-binding specificity to Yap1 (Fernandes et al. 1997). The identification of several natural Yap1 binding sites (Kuge and Jones 1994; Wu and Moye-Rowley 1994) and analysis of Yap1 DNA-binding properties (Fernandes et al. 1997) have established the Yap1 recognition element (YRE) as either TTACTAA or TGACTAA, which appear distinct from the canonical AP-1 recognition site (TGACTCA). There may be one or more additional Yap1 recognition sites since many *bona fide* Yap1 target gene promoters (*e.g. TSA1*) lack a YRE, although they directly bind Yap1 (Lee et al. 1999). Whether Yap1 homodimerizes to bind DNA as most other bZIP proteins is not established with certainty (Fernandes et al. 1997).

6.1.2 Yap1 regulates the yeast peroxide detoxification pathway in conjunction with Skn7

Yap1 regulates the yeast peroxide detoxification pathway. Such regulation meant to prevent oxidative stress-induced cellular damage, is essential for aerobic life and has the hallmarks of a homeostatic control. This function, first observed by

Schnell et al. (1992) on the basis of the hypersensitivity of *yap1Δ* cells to H_2O_2, *tert*-butylhydroperoxide (*t*-BOOH), cumene hydroperoxide and to redox cycling drugs (Schnell et al. 1992), was initially missed due to lack of a *yap1Δ* phenotype under normal laboratory growth conditions. Kuge and Jones (1994) subsequently established this function by identifying *TRX2,* encoding one of the two cytoplasmic thioredoxins, as a Yap1-dependent gene induced by H_2O_2, *t*-BOOH, diamide and diethylmaleate and by showing that *yap1Δ* is hypersensitive to diamide and diethylmaleate in addition to peroxide (Kuge and Jones 1994). Importantly, the response to peroxide and to the prooxidant drugs diamide and diethylmaleate relates to distinct modes of Yap1 activation (see further). Since then, an important number of peroxide-inducible Yap1-dependent genes have been identified that comprise most cellular antioxidants, catalases, superoxide dismutases, peroxiredoxins, glutathione peroxidases, cytochrome c peroxidase, activities of the glutathione, thioredoxin and pentose phosphate pathways, and other reductase systems such as the ATP-dependent sulphinic reductase sulphiredoxin (Lee et al. 1999; Gasch et al. 2000; Biteau et al. 2003). The nature of the peroxide-inducible Yap1 regulon fully explains the inability of *yap1Δ* to adapt to and to grow in the presence of peroxides (Stephen et al. 1995). The *yap1Δ* mutant is also hypersensitive to the lipid peroxidation by-products malondialdehyde (Turton et al. 1997) and linoleic acid hydroperoxide (LoaOOH) (Evans et al. 1998). The crucial function of Yap1 in peroxide detoxification is further supported by its identification in a genome-wide screen for genes that suppress the accumulation of mutations (Huang et al. 2003) and by the effect of *YAP1* overexpression in delaying chronological aging (Herker et al. 2004); two cellular functions largely affected by oxygen stress.

Yap1 controls the peroxide response in cooperation with Skn7, a transcription factor sharing similarities with the receiver domain of prokaryotic two-component systems (Brown et al. 1993) and to the helix-turn-helix DNA-binding domain of the heat shock transcription factor Hsf1 (Brown et al. 1994). Skn7 was identified in a genetic screen for peroxide sensitive (POS) mutants (Krems et al. 1995, 1996) and was later shown to be required for Yap1-dependent activation of *TRX2* and *TRR1* in response to H_2O_2 (Morgan et al. 1997; Lee et al. 1999). Skn7 actually only operates on a subset of the peroxide-inducible Yap1 regulon that includes mainly antioxidants and activities of the thioredoxin pathway (Lee et al. 1999). Interestingly, Yap1-dependent genes that do not require Skn7 often coincide with those needed for metal and chemical tolerance such as those encoding activities of the GSH pathway and efflux pumps (see below). Skn7 may cooperate with Yap1 through formation of a heteromeric transcription factor, based on the simultaneously binding of the two factors at target promoters (Morgan et al. 1997; Lee et al. 1999). The gene-promoter information that discriminates between Yap1 regulation versus Yap1-Skn7 co-regulation is not clearly established.

Skn7 also operates in a Yap1-independent peroxide-inducible pathway through interaction with Hsf1 (Raitt et al. 2000) and has ramifications in several other cellular pathways, including cell cycle control, cell wall synthesis, osmotic stress, and the calcineurin pathway. Together, these data suggest that one function of Skn7 might be to coordinate the responses elicited by these multiple pathways (reviewed in: Toledano et al. 2003).

6.1.3 Role of Yap1 in the tolerance to metal and chemical stress

Yap1 is also required for cellular tolerance to metals and to unrelated chemicals having in common, at least for most of them, demonstrated electrophilic properties. The very early identification of the *YAP1* gene in several independent high-copy plasmid screens for resistance to several unrelated drugs [4-nitroquinoline-N-oxide (4-NQO), N-methyl-N'-nitro-N-nitrosoguanine (MNNG), triaziquone, sulphomethuron methyl, cycloheximide, the iron chelators o-phenanthroline, 1-nitroso-2-naphtol] (Leppert et al. 1990; Hertle et al. 1991; Hussain and Lenard 1991; Schnell and Entian 1991) strongly hinted to a function of Yap1 in some aspects of drug-stress responses. Hypersensitivity of *yap1Δ* to methylglyoxal (Wu et al. 1993), 4-NQO and to a lesser extent to cycloheximide, MNNG and sulphomethuron methyl (Hertle et al. 1991) further supported this notion. Finally, this assumption gained full credence upon the observation that Yap1 is activated by several of the toxic chemicals for which it confers tolerance, including benomyl and MMS (Nguyen et al. 2001), and upon the identification of several Yap1 target genes that operate in the multiple drug resistance mechanism. These genes include those encoding the membrane-associated transporters *YCF1*, *ATR1*, and *FLR1* that operate as drug-efflux pumps. As already described, Ycf1 functions as a GSH-conjugate pump in the detoxification of several metals and of diazaborine (Jungwirth et al. 2000). Atr1 and Flr1 are transporters of the major facilitator family. Atr1 is involved in resistance to 4-NQO and aminotriazole (Coleman et al. 1997) and Flr1 in the resistance to fluconazole (Alarco et al. 1997; Alarco and Raymond 1999), the pro-oxidant drugs diamide, diethylmaleate and menadione (Nguyen et al. 2001), cerulenin (Oskouian and Saba 1999), benomyl, methotrexate (Broco et al. 1999; Tenreiro et al. 2001), and diazaborine (Jungwirth et al. 2000).

Yap1 regulates an integral cadmium detoxification pathway distinct from but overlapping with the bZIP transcription factor Met4 (see below) at several target genes (Dormer et al. 2000; Fauchon et al. 2002; Wheeler et al. 2003). This function was initially identified by the cadmium hypersensitivity of *yap1Δ* (Hirata et al. 1994; Wemmie et al. 1994b) and by the identification of *GSH1,* encoding γ-glutamyl synthase, as a Yap1 target (Wu and Moye-Rowley 1994). Several other Yap1-dependent cadmium inducible genes important for cadmium tolerance have been identified, including the already mentioned Ycf1 (Wemmie et al. 1994a), other GSH biosynthesis and sulphur amino acid metabolism genes (Hirata et al. 1994; Stephen and Jamieson 1997; Takeuchi et al. 1997; Vido et al. 2001). Yap1 also plays an important role in arsenite and antimonite tolerance; Yap1 is activated by these metalloids and is responsible for induced expression of GSH biosynthesis and sulphur amino acid metabolism genes and genes with oxidative stress defence and detoxification function (Haugen et al. 2004; Wysocki et al. 2004; Thorsen et al. in preparation). Moreover, growth of *yap1Δ* is impaired in the presence of these metalloids. Yap1 is also involved in mercury tolerance (Westwater et al. 2002).

The function of Yap1 in cadmium and, more generally, chemical stress tolerance does not require Skn7; the *skn7Δ* mutant is not sensitive but rather more resistant to diamide and cadmium (Morgan et al. 1997; Lee et al. 1999). Such resistance could be explained by the selection of genes operated by Skn7 in the Yap1

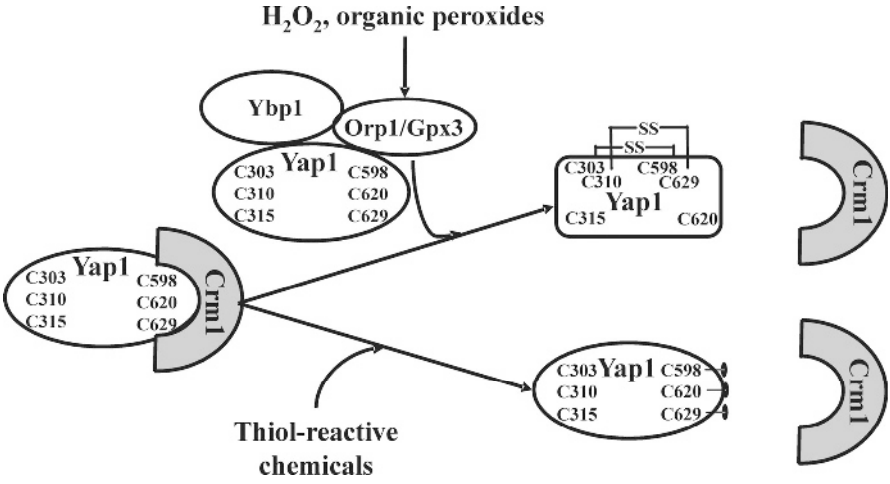

Fig. 5. A model of the activation of Yap1 by peroxides (H_2O_2 and organic peroxide) and thiol reactive chemicals (cadmium, mercury, diethylmaleate, N-ethylmaleimide etc.) that highlights the existence of two redox centres within the protein (adapted from: Azevedo et al. 2003; Wood et al. 2004). Unmodified Yap1 is recognized by Crm1 and permanently exported out of the nucleus. Peroxide will lead to Yap1 intramolecular disulphide bond formation through an Orp1 and Ybp1-dependent mechanism (see text), resulting in a change of conformation concealing the Yap1 nuclear export signal (NES) and, hence, its interaction with Crm1 and export out of the nucleus. Thiol reactive agents, by modifying Yap1 C-terminal cysteines lead to a presumable modification of the Yap1 NES. The later mechanism is Orp1 and presumably also Ybp1-independent.

regulon (see above) (Lee et al. 1999; Vido et al. 2001). Moreover, Skn7 has a negative effect on this response, based on the super-induced levels of some Yap1 target genes in cadmium treated *skn7Δ* cells (Vido et al. 2001).

6.1.4 Molecular control of Yap1

An intriguing feature of Yap1 resides in its activation by a multitude of stress signals, including peroxides (H_2O_2, *t*-BOOH), diamide (Kuge and Jones 1994), menadione (Stephen et al. 1995; Stephen and Jamieson 1997), the electrophiles diethylmaleate (Kuge and Jones 1994), benomyl and MMS (Nguyen et al. 2001), cadmium (Hirata et al. 1994; Stephen and Jamieson 1997), arsenite (Menezes et al. 2004; Wysocki et al. 2004), antimonite (Wysocki et al. 2004), and mercury (Westwater et al. 2002). In fact, all these signals control Yap1 through regulated nuclear export by stress-induced post-transcriptional modification(s).

Under non-stress conditions, Yap1 is restricted to the cytoplasm (Kuge et al. 1997) by virtue of rapid nuclear export by the nuclear export receptor Crm1/Xpo1 (Fig. 5). Crm1 recognises and non-covalently interacts with a non-canonical hydrophobic leucine-rich nuclear export signal (NES) located in a carboxy-terminal domain, named C-terminal cysteine rich domain (cCRD) that also carries three re-

peats of the cysteine motif CSE (Kuge et al. 1997, 1998; Yan et al. 1998). Upon exposure to stress signals, Yap1 accumulates in the nucleus due to loss of the Yap1-Crm1 interaction (Kuge et al. 1998; Yan et al. 1998). In contrast to its regulated nuclear export, Yap1 import into the nucleus is constitutive and is mediated by the nuclear import receptor Pse1/Kap121 (Isoyama et al. 2001). Hence, a key step in Yap1 activation resides in the regulation of a Crm1-dependent nuclear export controlling subcellular location. Stress-induced redox or chemical modification of Yap1 cysteines appeared as the likely mechanism disrupting the interaction between the Yap1-NES and Crm1. Although this early model was correct, it did not consider how peroxides, metal or chemical signals reach Yap1, a process actually more complex than initially thought, which underlie the true sensing function operated at the level of Yap1 and its specificity.

6.1.5 The S. cerevisiae peroxide sensor

In response to H_2O_2, Yap1 is activated by oxidation to an intramolecular disulphide bond formed between the cCRD Cys598 and Cys303 located within a second CRD at the N-terminal portion of the protein (nCRD) (Delaunay et al. 2000; for a detailed review see Toledano et al. 2004). A second intra-molecular disulphide bond between Cys310 and Cys629 has been identified in purified and air-oxidized Yap1 (Wood et al. 2003). Disulphide linkage between N- and C-terminal Yap1 cysteine residues promotes a conformational change concealing the Yap1 C-terminal NES, as recently established by a detailed NMR structural analysis of an oxidized truncated form of Yap1 encompassing both N- and C-terminal CRDs (Wood et al. 2004).

Yap1 oxidation by H_2O_2 is not direct, involving Orp1 (Oxidant Receptor Peroxidase) that acts as the actual H_2O_2 sensor of the pathway (Delaunay et al. 2002). Yap1 oxidation involves a third subunit Ybp1 (Yap1-Binding Protein) (Veal et al. 2003; Gulshan et al. 2004), a protein with no discernable functional domain, likely acting by chaperoning the Orp1-Yap1 interaction. Orp1, also known as Hyr1/Gpx3, is a 20 kDa protein with sequence homology to glutathione peroxidases (GPx) (Inoue et al. 1999; Avery and Avery 2001). Orp1 has *in vitro* peroxidase activity operated by a mechanism distinct from classical GPxs, involving a catalytic disulphide between the conserved peroxidatic Cys36 and Cys82 and a reduction by thioredoxin and not by GSH (Delaunay et al. 2002). Although the Orp1 disulphide forms in cells exposed to H_2O_2, Orp1 only contributes to H_2O_2 tolerance by regulating Yap1 but not by acting as an H_2O_2 peroxide reductase. However, Orp1 is active for the detoxification of lipid peroxides *in vivo* (Avery and Avery 2001). Further, in some strain backgrounds, *orp1Δ* is hypersensitive to cadmium (Avery et al. 2004), a result taken as an indication that the toxicity of this metal is, at least in part, due to production of lipid hydroperoxides.

The Orp1-Yap1 H_2O_2 sensor operates as a cysteine-redox relay (Delaunay et al. 2002; Toledano et al. 2004). The Orp1 catalytic Cys36 senses the H_2O_2 signal and oxidizes to a Cys-SOH. Oxidized Orp1 transduces this signal to Yap1 by engaging the latter into a Cys36-Cys598 intermolecular disulphide, which is then converted to the intramolecular Cys303-Cys598 disulphide of active Yap1. The second Yap1

intramolecular disulphide bond probably forms similarly through recruitment of a second Orp1 oxidized molecule. Importantly, since both alternate Orp1 intra- and intermolecular disulphides are observed in H_2O_2-treated cells, the nascent Orp1 Cys36 sulphenic acid is poised to react with either Orp1 Cys82 to complete its peroxidatic cycle or with Yap1 Cys598. In this process, Ybp1, the absence of which prevents Orp1-Yap1 disulphide linkage, may act as a scaffold bringing Orp1 and Yap1 into a non-redox complex, and may also chaperon mixed-disulphide formation by guiding Orp1 Cys36-SOH towards Yap1 Cys598 and/or preventing formation of the competing Orp1 Cys36-Cys82 disulphide bond.

Yap1 oxidation occurs rapidly, within 1 minute and transiently, lasting about 30-45 minutes, indicating that it is deactivated by an efficient reductase system (Delaunay et al. 2000). This system is probably thioredoxin as suggests the partial Yap1 oxidation in cells lacking this reductase (Izawa et al. 1999; Delaunay et al. 2000; Carmel-Harel et al. 2001) although *in vivo* evidence of a Yap1-thioredoxin interaction could not be obtained (Izawa et al. 1999; Delaunay et al. 2000). Alternatively, lack of thioredoxin may maintain Yap1 activation by the increase in intracellular H_2O_2 resulting from impairment of the H_2O_2 scavenging capacity of thioredoxin-dependent peroxiredoxins (Carmel-Harel et al. 2001). In contrast to thioredoxin, the GSH pathway does not seem to interfere with Yap1 regulation, as attests lack of a Yap1 phenotype upon inactivation of GSH reductase or of both dithiol glutaredoxins (Izawa et al. 1999; Delaunay et al. 2000). However, GSH depletion caused by *GSH1* inactivation results, to some extent, in Yap1 activation as observed by some (Wheeler et al. 2003) but not by other authors (Izawa et al. 1999; Delaunay et al. 2000). This difference may either be due to the paucity of the effect or to the difficulty of total cellular depletion of GSH, an essential molecule in yeast.

6.1.6 A second Yap1 redox centre for metals and electrophiles

Although metals and chemicals also activate Yap1 by regulating its nuclear export (Kuge et al. 1997), these compounds are sensed through a distinct mechanism (Azevedo et al. 2003). This distinction was initially suggested by Wemmie et al. (1997) who observed that Yap1 activation by diamide only relied on cCRD cysteines, whereas peroxide required additional cysteines in the nCRD (Wemmie et al. 1997; Coleman et al. 1999). In addition, all known non-peroxide Yap1 inducers tested were unable to promote the oxidation of Yap1 to the intramolecular disulphides characteristic of its activation by peroxides (Delaunay et al. 2000; Azevedo et al. 2003). Further, Yap1-activation by these compounds by-passed the requirement of Orp1 (Delaunay et al. 2000; Azevedo et al. 2003) and Ybp1 (Veal et al. 2003; Gulshan et al. 2004). The observation that most, if not all, non-peroxide Yap1 inducers had thiol-reactive properties in common suggested that they could directly activate Yap1 by binding to critical NES vicinal cysteines, either covalently as in the case of electrophiles or non-covalently as in the case of metals, thereby altering the Yap1-Crm1 interaction. This hypothesis was confirmed using the model electrophile N-ethylmaleimide, shown to activate Yap1 by covalent modification of the Yap1 C-terminal Cys598, Cys620 and Cys629 (Azevedo et al.

2003). Activation of *S. pombe* Pap1 by diethylmaleate has also been proposed to operate through covalent cysteine adduct formation (Castillo et al. 2002). Yet, one more distinct mechanism has been proposed for Yap1 activation by diamide, involving formation of either one of the three possible disulphide bonds within C-terminal cysteines (Kuge et al. 2001). However, this model seems unlikely since Yap1 activation by diamide only requires either one of the Yap1 C-terminal cysteines (Kuge et al. 1997, 1998; Yan et al. 1998), which rather suggests covalent modification of single cysteines, as diamide may also form stable cysteine adducts. Interestingly, the two modes of Yap1 sensing were shown to operate simultaneously with the quinone menadione (Azevedo et al. 2003), a drug that is both a superoxide anion generator and a highly reactive electrophile. Here, superoxide anions are believed to activate the Orp1-dependent activation pathway through their dismutation to H_2O_2.

6.2 Yap8: a specific arsenic resistance factor

Yap8 is a 33 kDa protein of the yeast AP-1 family of transcription factors and exhibits about 15% identity to Yap1. The *YAP8* (also called *ACR1*/*ARR1*) gene was isolated together with *ACR2* and *ACR3* on a genomic DNA fragment that increased arsenic tolerance when expressed from a multicopy plasmid. Based on genetic data and on the similarity of Yap8 to other yeast AP-1-like proteins, it was suggested that Yap8 is a transcriptional regulator of the *ACR3* gene (Bobrowicz et al. 1997). Later, several labs provided evidence that Yap8 contributes to arsenic tolerance by controlling expression of the *ACR2* and *ACR3* genes (Bobrowicz and Ulaszewski 1998; Bouganim et al. 2001; Haugen et al. 2004; Maciaszczyk et al. 2004; Menezes et al. 2004; Wysocki et al. 2004).

Although Yap8 is a member of the yeast AP-1 protein family and shares sequence homology with other fungal AP-1-like transcription factors, it appears to behave differently from the most well-characterised yeast AP-1 protein Yap1. Firstly, deletion of *YAP8* produces hypersensitivity to As(III), As(V) and a weak Sb(III) sensitivity whereas growth of the *yap8Δ* mutant is unaffected by the presence of oxidants including methyl viologen (paraquat), *t*-BOOH, menadione and diamide (Wysocki et al. 2004). Secondly, Wysocki et al. (2004) showed that Yap8 resides predominantly in the nucleus by monitoring a GFP-Yap8 fusion protein as well as by detecting a genomic copy of a myc-tagged Yap8 in nuclear extracts. Chromatin immunoprecipitation (ChIP) assays further strengthened the notion that at least a portion of Yap8 is nuclear since Yap8 was found to be associated with the *ACR3* promoter in both untreated as well as As(III)-exposed cells (Wysocki et al. 2004). In contrast to Wysocki et al. (2004), Menezes et al. (2004) reported that GFP-Yap8 is regulated at the level of nuclear export in a Crm1-dependent fashion, suggesting that Yap8 is controlled in a similar way as Yap1. Whether the discrepancy between these two studies is due to the use of different *Saccharomyces* strains has yet to be clarified. Thirdly, Yap8 may bind to a DNA sequence that is different from that of Yap1 (Wysocki et al. 2004). Arsenic-induced expression of *ACR2* and *ACR3* requires the presence of a TTAATAA promoter sequence (Yap1

binds to and regulates expression primarily through a TTACTAA sequence) as well as Yap8: deletion of TTAATAA from the *ACR2*/*ACR3* promoter or deletion of *YAP8* reduces induction of *ACR2* and *ACR3* expression in response to As(III) exposure. In contrast, *ACR2* and *ACR3* expression is largely unaffected by *YAP1* deletion (Wysocki et al. 2004) although other studies suggested that Yap1 may affect expression under certain conditions (Bouganim et al. 2001; Haugen et al. 2004; Menezes et al. 2004). Since ChIP assays evidenced the presence of Yap8 on the *ACR3* promoter (Wysocki et al. 2004), it is tempting to speculate that Yap8 binds to the TTAATAA sequence. However, whether this is indeed the case has yet to be demonstrated. It is important to note that Yap8 was not found to be bound to its own promoter despite the presence of a TTAATAA element (Wysocki et al. 2004). The nucleotides flanking the TTAATAA element are different for the two promoters; TTGATTAATAATCAA in the *ACR3* promoter and TTCTTAATAAATT in the *YAP8* promoter, and it is possible that this difference affects DNA binding and expression of target genes (Wysocki et al. 2004). The fact that mutations of nucleotides flanking the Yap1 and Yap2 DNA binding-sites decreased expression of their respective target genes (Cohen et al. 2002) would support this notion.

The finding that Yap8 is permanently associated with the *ACR3* promoter suggests that arsenic-induced activation of Yap8 is neither exerted at the level of localisation nor at the level of As(III)-stimulated binding to the *ACR3* promoter (Wysocki et al. 2004). Yap8 activation requires a number of cysteine residues (Cys132 and Cys274) that are conserved in several fungal AP-1-like proteins; the Yap8 Cys132Ala and Yap8 Cys274Ala mutants were unable to stimulate induction of *ACR3* expression and, consequently, failed to promote cellular arsenic tolerance (Wysocki et al. 2004). In contrast to *yap1Δ*, *yap8Δ* cells are not sensitive to chemical oxidants. Moreover, overexpression of *YAP8* does not restore H_2O_2 tolerance to *yap1Δ* cells. Although Yap8 activation by metalloid-induced oxidative modifications cannot be excluded solely based on these data, such an activation mechanism may appear less likely. Instead, it is conceivable that these Yap8 cysteines directly bind to As(III) inducing a conformational change such that the modified Yap8 can trigger gene expression (Wysocki et al. 2004). The exact mechanism of arsenic-mediated Yap8 activation remains to be unveiled.

6.3 Hog1 mediates As(III) tolerance through multiple mechanisms

Mitogen-activated protein kinase (MAPK) pathways are of central importance for all eukaryotic cells since they are critically involved in controlling cell growth, differentiation as well as in establishing stress responses. MAPKs are activated by dual phosphorylation of adjacent threonine and tyrosine residues by a wide variety of environmental cues (Widmann et al. 1999; Kyriakis and Avruch 2001). For instance, As(III) exposure results in activation of mammalian p38, *S. pombe* Sty1 and *S. cerevisiae* Hog1 (Tamás and Wysocki 2001; Qian et al. 2003). However, the molecular mechanisms through which these proteins mediate As(III) tolerance are not fully understood.

Hog1 is the MAPK of the high osmolarity glycerol (HOG) pathway that mediates the response to high osmolarity in *S. cerevisiae* (reviewed in: Hohmann 2002; Tamás and Hohmann 2003). Increased extracellular osmolarity leads to activation of Hog1 through two independent upstream branches, the Sln1-Ssk1 branch and the Sho1 branch, converging at the MAPKK Pbs2 (Brewster et al. 1993). In turn, Hog1 activates a number of targets including various transcription factors (Rep et al. 1999; Alepuz et al. 2001; Proft et al. 2001; de Nadal et al. 2003) and the MAPK activated protein kinase (MAPKAPK) Rck2 involved in translation control (Bilsland-Marchesan et al. 2000; Teige et al. 2001). Hog1 has an additional role in controlling cell cycle progression under osmotic stress (Alexander et al. 2001; Belli et al. 2001; Yaakov et al. 2003; Escoté et al. 2004).

Besides its role in osmoprotection, Hog1 has an important function in mediating arsenic and antimony tolerance; cells lacking the *HOG1* gene or that express *HOG1* alleles that cannot be phosphorylated or are deficient in kinase activity, are highly sensitive to As(III) and Sb(III). In line with the observed phenotypes, Hog1 is phosphorylated in As(III) or Sb(III) exposed cells. Interestingly, metalloid-induced phosphorylation, and hence activation of Hog1, displays both quantitative and qualitative differences to osmotic activation: phosphorylation is clearly weaker, reaches its maximum at a later time point and lasts longer. As(III)-stimulated Hog1 phosphorylation is furthermore fully dependent on Pbs2 and Ssk1; Hog1 is not phosphorylated in As(III)-exposed *pbs2Δ* and *ssk1Δ* cells and these mutants are basically as As(III) sensitive as the *hog1Δ* mutant. In contrast, mutants in the Sho1 branch of the pathway affect metalloid signalling and tolerance only to a minor extent (Wysocki, Van der Does, Johansson, and Tamás: unpublished data). Collectively, these data suggest that As(III)-stimulated activation of Hog1 mainly occurs through the Sln1 branch of the pathway.

A striking feature of As(III)-induced activation of Hog1 is the lack of nuclear accumulation of the MAPK and an almost complete absence of Hog1-dependent transcriptional responses (Thorsen, Kristiansson, Nerman, and Tamás: unpublished data). Hog1 controls expression of about 150 genes under osmotic stress (Posas et al. 2000; Rep et al. 2000) whereas only four genes display Hog1-dependent expression changes under As(III) exposure. Strains lacking these genes display wild type As(III)-tolerance and are therefore not likely to play a major role in metalloid protection (Thorsen, Kristiansson, Nerman, and Tamás: unpublished data). Instead, Hog1 appears to mediate As(III) tolerance by other mechanisms.

Firstly, Hog1 contributes to As(III)-tolerance by affecting Fps1-mediated transport: *hog1Δ* cells accumulate more arsenic than wild type cells whereas *hog1Δ fps1Δ* accumulate little As(III). Additional deletion of *FPS1* in a *hog1Δ* background suppresses the As(III) and also the Sb(III) sensitivity of this mutant (Thorsen, Tängemo, Wagner, Wysocki, Boman, and Tamás: unpublished data). It was previously reported that osmotic inactivation of Fps1 is independent of Hog1 (see Section 3.1) (Luyten et al. 1995; Tamás et al. 1999). Similarly, *FPS1* expression in metalloid-treated cells is reduced to similar extents both in wild type and *hog1Δ* cells. However, the basal Fps1-dependent transport rate is elevated in a *hog1Δ* mutant: deletion of *HOG1* increases Fps1-dependent uptake of both glyc-

erol and As(III). Hence, metalloid sensitivity of *hog1Δ* cells can largely be attributed to increased metalloid influx through Fps1 (Thorsen, Tängemo, Wagner, Wysocki Boman, and Tamás: unpublished data).

Secondly, Hog1 may activate the MAPKAPK Rck2 under As(III) exposure. Rck2 is phosphorylated in a Hog1-dependent manner both upon osmotic (Bilsland-Marchesan et al. 2000; Teige et al. 2001) and oxidative (Bilsland et al. 2004) stress treatments. Rck2 is also required for optimal As(III) tolerance and genetic evidence suggests that the protein is a Hog1 target also under these conditions; growth of *rck2Δ* is impaired in the presence of As(III) and *RCK2* overexpression partially suppressed the As(III) sensitivity of *hog1Δ* (Wysocki and Tamás: unpublished data). Hence, epistasis analysis indicates that the two proteins act in the same pathway. The molecular function of Rck2 is not well understood although the protein has been implicated in downregulation of protein synthesis under osmotic stress by affecting phosphorylation of elongation factor 2 (Teige et al. 2001). Regulation of protein synthesis appears to be an important response to environmental stresses (Sheikh and Fornace 1999). Although the effect of arsenic on protein synthesis has not been explored, the arsenic-sensitive phenotype of mutants lacking regulators of protein synthesis suggests that this may be the case.

Thirdly, Hog1 affects cell cycle progression under As(III) exposure. A temporary cell cycle arrest is part of the physiological response to various environmental stress conditions since such a delay provides time to repair damage and to change metabolism so that cells can adapt to new growth conditions (Mendenhall and Hodge 1998; Wilson and Roach 2002). It was recently reported that Hog1 phosphorylates the CDK-inhibitor protein Sic1 in response to osmotic stress, resulting in Sic1 stabilisation and inhibition of the cell cycle at the G_1/S phase (Escoté et al. 2004). After short-time exposure to arsenite, however, loss of *HOG1* and *SIC1* does not affect an initial cell cycle arrest in G_1. Interestingly, Hog1 and Sic1 seem to be required for maintaining G_1 arrest as both *hog1*Δ and *sic1*Δ mutants resume cell cycle faster then wild type cells. In addition, the double *hog1*Δ *sic1*Δ mutant displays higher arsenite sensitivity and a stronger checkpoint defect (Wysocki and Tamás: unpublished data). These results suggest that Hog1 and Sic1 may have independent roles in cell cycle regulation after short-time arsenic treatment.

Surprisingly, in asynchronous cultures containing As(III), *hog1*Δ cells accumulated with 1N DNA content, while wild type preferentially arrested in G_2/M phase. Analysis of cells synchronized in G_1 and cultivated continuously in the presence of As(III) confirmed that the *hog1*Δ mutant was defective in abrogation of the As(III)-induced G_1 arrest (Wysocki and Tamás: unpublished data). This observation suggests that Hog1 plays an additional role in adaptation to long-term metalloid stress. Interestingly, Hog1 functions in recovery from G_1 arrest under higher concentrations of salt (Belli et al. 2001). The possible mechanism of cell division resumption under arsenic and osmotic stresses might include restoring transcription and/or translation leading to the production of factors required for the relief of inhibitory mechanisms or stimulation of G_1/S transition. The *hog1*Δ mutant is defective in translation initiation after osmotic stress-induced inhibition (Uesono and Toh 2002). Thus, lack of adaptation to arsenic-induced G_1 delay could be ex-

plained by a failure to re-start translation. Since *rck2Δ* did not show any cell cycle defect, Rck2 is not likely to be involved in this process (Wysocki and Tamás: unpublished data).

Hog1 is also required for optimal cadmium tolerance and Hog1 is phosphorylated in cadmium exposed cells (Bilsland et al. 2004). However, the targets of Hog1 under cadmium stress as well as the mechanisms by which Hog1 controls cadmium tolerance are unknown.

6.4 Met4

The *de novo* synthesis of sulphur amino acids and S-adenosylmethionine from inorganic sulphur source, sulphate, or sulphite (Fig. 2) is controlled by the bZIP transcriptional activator Met4. This metabolic pathway is tightly regulated probably because sulphate reduction necessitates high levels of energy (ATP). Met4 activity strongly decreases when methionine is added into the culture medium. This regulation is the result of methionine conversion into cysteine which is the probable sensor of the pathway (Hansen and Johannsen 2000). Different factors modulate the activity of Met4 including Met28, Met31, Met32, Cbf1, and Met30 (Thomas and Surdin-Kerjan 1997). Met30 is the F subunit of the SCF^{Met30} ubiquitin ligase. SCF ubiquitin ligases are multisubunit complexes and the budding yeast SCF consists of Skp1, the scaffold protein Cdc53, the RING-finger protein Rbx1/Roc1/Hrt1 and one member of the family of F-box proteins (Deshaies 1999). In minimal medium, the function of Met30 is to target the ubiquitylation and the degradation of Met4 when the genes of the sulphur pathway should not be expressed, *i.e.*, upon addition of methionine to the medium (Rouillon et al. 2000). In rich medium, Met4 is oligo-ubiquitinated but is not degraded. This modification alleviates transactivation properties for most *MET* genes but gives transcriptional activation specificity to the *SAM1* and *SAM2* genes (Kuras et al. 2002). In response to Cd(II), both mechanisms of inhibition are over-ridden to enable rapid Met4-dependent induction of the sulphur metabolic pathway necessary to increase glutathione production (Barbey et al. 2005). In rich medium, a deubiquitylation step rapidly removes inhibitory ubiquitin moieties from Met4, which fully activates the transactivator. In minimal medium, Cd(II) inhibits cysteine-dependent degradation of Met4 through dissociation of the Skp1-Met30 interaction (Barbey et al. 2005).

Although Met4 contains a bZIP domain, the transactivator is not able to bind DNA without association to its cofactors, Cbf1, Met28, Met31, and Met32, which in different combinations tether Met4 to DNA. A first complex (Cbf1-Met4-Met28) contains the bZIP factor Met28 and the centromere factor Cbf1 (Thomas and Surdin-Kerjan 1997). This complex recognizes a specific DNA sequence (TCACGTG) also present in yeast centromeres and recognized by Cbf1 at the centromeres. Moreover, this sequence is found in most *MET* genes and is necessary for activation of *MET16* and *MET25* (Kuras et al. 1997; Thomas and Surdin-Kerjan 1997). Alternatively, Met4 and Met28 can associate to the homologous factors Met31 or Met32. These complexes recognize the DNA sequence

AAACTGTG (Blaiseau and Thomas 1998) found in the promoter of many *MET* genes (Thomas and Surdin-Kerjan 1997). These factors are dispensable for *MET25* expression but required for expression of both *MET3* and *MET14* (Blaiseau et al. 1997). The variety of transactivating complexes acting on different DNA sequences suggests the flexibility of Met4 to modulate expression of its target genes in response to different environmental conditions.

The two DNA binding sites cited above were also found at proximity (both 370 to 350 nt upstream from ATG) in the promoter region of *GSH1*. This DNA sequence as well as the presence of Met4 and Met31/Met32 is important for Cd(II)-induced *GSH1-lacZ* reporter gene expression (Dormer et al. 2000). Thus, the Met4-Met28-Met31/Met32 complex is thought to play an important role in the transcriptional induction of *GSH1* expression by cadmium. The contribution of the different Met4 complexes in the expression of the numerous other Met4-dependent genes that are induced by cadmium (Fauchon et al. 2002) is still unknown.

The Met4-dependent genes induced by cadmium can be classified into three different groups according to their function: (1) enzymes of the sulphur metabolic pathway; (2) transporters of sulphur compounds and; (3) other proteins with functions unrelated to sulphur metabolism. This latter group includes sulphur depleted proteins and particularly isoenzymes of some glycolysis enzymes. Interestingly, the most abundant proteins induced in a Met4-dependent way have a particularly low sulphur content indicating that Met4 controls the global saving of sulphur in proteins in response to Cd(II) (Fauchon et al. 2002).

In *S. pombe*, among the enzymes involved in sulphur metabolism, only the transporters of sulphur compounds are induced upon Cd(II) exposure (Chen et al. 2003). Induction of these genes is controlled by the bZIP transcription factor Zip1 (Harrison et al. 2005). The level of Zip1 in the cell is tightly controlled by the SCF^{pof1} ubiquitin ligase by a mechanism very similar to the regulated degradation of Met4 by SCF^{Met30} ubiquitin ligase in *S. cerevisiae*. Upon cadmium exposure, Zip1 is stabilized and expression of Zip1-dependent genes is induced (Harrison et al. 2005). However, the functions of Met4 and Zip1 present some differences; Zip1 controls the expression of only a small number of genes compared to Met4. Moreover, Met4 is important both for the synthesis of sulphur amino acids in unstressed conditions and for glutathione synthesis in response to metals whereas Zip1 seems to be required only for the Cd(II) response. However, it cannot be excluded that Zip1 controls the detoxification pathways of other toxic compounds. Strikingly, this mechanism of regulation seems conserved in mammals; the SCF ubiquitin ligase complex Keap1/Cul3/Rbx1 controls the degradation of the bZIP transcription factor Nrf2. This factor is stabilized by Cd(II) and other oxidative stress conditions (Stewart et al. 2003; Kobayashi et al. 2004) and it also controls glutathione synthesis (Chan and Kwong 2000; Sun et al. 2005) indicating an evolutionary conservation of this feature among eukaryotes.

6.5 Other transcriptional regulators

Genome-wide transcriptional and subsequent bioinformatics analysis has identified additional proteins that may affect transcription under metal exposure. Network mapping of As(III) exposed cells resulted in the identification of a number of proteins that are likely to mediate part of the transcriptional response to As(III) (Haugen et al. 2004). The proteins identified include the general stress-responsive transcription factors Msn2 and Msn4, the heat shock factor Hsf1, the transcriptional activator of the ubiquitin-proteasome pathway Rpn4 as well as Fhl1 involved in the control of rRNA processing (Haugen et al. 2004). The molecular details of As(III)-induced activation of these proteins and the details of how the systems whose expression they regulate contribute to tolerance remain unknown. Among the yeast AP-1-like proteins, Yap2 is most closely related to Yap1. Although overexpression of *YAP2* confers increased Cd(II) tolerance (Wu et al. 1993; Hirata et al. 1994) and Cd(II) exposure results in Yap2 nuclear retention (Bilsland et al. 2004), *yap2Δ* has no Cd(II) sensitive phenotype. Similarly, Yap2 does not appear to have a role in As(III) tolerance (Haugen et al. 2004; Wysocki et al. 2004). Hence, besides Yap1 and Yap8, the function of the yeast AP-1-like proteins is unknown.

7 Conclusions and future perspectives

S. cerevisiae is extensively used as a eukaryotic model organism for both fundamental and applied studies. From genetic and physiological points of view, yeast is a favourite organism for many molecular cell biologists. Also with respect to the cellular defence mechanisms against toxic metals described in this review, yeast appears to be a very suitable model system. For instance, the proteins mediating metal influx and efflux seem to be conserved in various eukaryotic organisms. Similarly, one can predict that sulphur metabolism and glutathione biosynthesis will play an equally important role in other eukaryotes as it does in yeast. Finally, the signalling and regulatory mechanisms that the cell utilises to control the tolerance systems have counterparts in higher organisms. However, it is important to keep in mind that, while general strategies employed by cells to evade metal toxicity may be conserved through evolution, it is likely that the molecular details differ between organisms. One such example is the usage of different thiol-based detoxification systems by *S. cerevisiae* and *S. pombe*: while the former employs glutathione the latter utilises phytochelatin for detoxification purposes. In any case, the study of metal responses in budding and fission yeast are complementary and have revealed important information with relevance to plant and mammalian cells.

Although our understanding of how eukaryotic cells respond to nonessential toxic metals has increased considerably in recent years, there is still a long way to go before we have a comprehensive insight into the molecular details of such defence systems. Firstly, we have not yet identified all the players involved in metal

tolerance acquisition. Moreover, we need to know the exact mechanism by which these proteins mediate tolerance. For instance, what is the nature of the (metal-induced) signal that activates transcription factors and signalling proteins? How do these proteins, in turn, activate their respective targets? What is the substrate (metal) specificity of plasma membrane permeases and ABC-transporters that are involved in metal tolerance? Secondly, the dynamic nature of signalling events and transcriptional responses during metal exposure remains poorly explored. For instance, the mechanisms controlling the subcellular localisation of individual signalling proteins and transcriptional regulators are only starting to emerge. Moreover, how these proteins interact in space and time as they orchestrate the cellular response during metal exposure remains to be investigated. One should also keep in mind that cells are likely to respond differently under acute and chronic exposure and/or use different mechanisms to cope with sudden or chronic exposure. Thirdly, a global whole-cell or organism-level understanding of metal responses requires integration of various 'omic' approaches including transcriptomic, proteomic and metabolic profiling. In this way, it is possible to achieve a systems-level comprehension of the protective mechanisms used by cells or organisms during exposure to various cytotoxic agents and drugs. Fourthly, there is a clear need for technological developments that will allow the measurement of metal concentrations in different organelles or accurate measurements of ROS in the cell and/or organelles. A detailed understanding of metal tolerance mechanisms in yeast may prove of value for identifying similar mechanisms in other organisms and have important implications for the use of these metals in medical therapy.

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