How *Saccharomyces cerevisiae* copes with toxic metals and metalloids

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**Abstract**

Toxic metals and metalloids are widespread in nature and can locally reach fairly high concentrations. To ensure cellular protection and survival in such environments, all organisms possess systems to evade toxicity and acquire tolerance. This review provides an overview of the molecular mechanisms that contribute to metal toxicity, detoxification and tolerance acquisition in budding yeast *Saccharomyces cerevisiae*. We mainly focus on the metals/metalloids arsenic, cadmium, antimony, mercury, chromium and selenium, and emphasize recent findings on sensing and signalling mechanisms and on the regulation of tolerance and detoxification systems that safeguard cellular and genetic integrity.

**Introduction**

Metals and metalloids profoundly affect biological systems either because they are essential or because they are toxic or harmful when present in excessive amounts. Living organisms have always dealt with metals, and a very large number of proteins have evolved that require metals for catalytic activity and/or for maintaining protein structure (Waldron et al., 2009). Cells also utilize a wide array of homeostasis and tolerance mechanisms that regulate the availability of essential metals and limit the damaging effects of toxic elements. Malfunction of metal homeostatic or detoxification systems may cause a range of human diseases including cancer. At the same time, many metals are also used as therapeutic agents to treat various ailments and disorders (Thompson & Orvig, 2003; Tamás & Martinoia, 2005; Beyersmann & Hartwig, 2008). The importance of metals for human health as well as their impact on the environment has spurred research on metal biology and led to a significant progress in understanding metal responses and tolerance acquisition mechanisms in many prokaryotic and eukaryotic organisms. Despite this, relatively little is known about toxicity mechanisms at a molecular level. Likewise, our knowledge of metal sensing and how this information is translated into appropriate cellular responses is scarce. *Saccharomyces cerevisiae* (budding yeast) has proved to be a powerful model organism to unravel the molecular details of metal action and detoxification strategies. This review highlights various aspects of metal toxicity and tolerance in *S. cerevisiae*, with a special emphasis on the sensing, signalling and regulatory mechanisms used in response to non-essential metals and metalloids including arsenic, cadmium, antimony, mercury, chromium and selenium.

**Metal-induced damage and toxicity**

The impact of metals and metalloids on biological systems ranges from essential, through beneficial, to nonessential and highly toxic. The toxicity of a given metal is governed by its mechanisms of uptake, oxidation state and speciation, intracellular distribution and interactions with various macromolecules, and depends on its physicochemical properties and ligand preferences. ‘Soft’ transition metals such as silver (Ag), cadmium (Cd) and mercury (Hg) have sulphur as their preferred ligand. ‘Hard’ transition metals such as chromium (Cr), manganese (Mn) and molybdenum (Mo),...
and the metalloids arsenic (As), antimony (Sb), selenium (Se), tellurium (Te) and bismuth (Bi) prefer oxygen in their higher oxidation states, while they prefer sulphur in their lower oxidation states. Lead (Pb), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn) can use oxygen, sulphur or nitrogen as ligands (Summers, 2009). Hence, nearly all nonessential metals and metalloids, including Hg, As, Cd and Pb, display high reactivity with sulphhydryl groups. This property may contribute to their toxicity, but is also exploited by cells for detoxification. Several compounds containing these metals are classified as human carcinogens (e.g. As, Cd, Cr, Ni) or probable carcinogens (e.g. Pb, Co) according to the International Agency for Research on Cancer (http://www.iarc.fr). Metal toxicity may be caused by oxidative stress, impaired DNA repair, inhibition of enzyme function and by disturbing the function of proteins that regulate proliferation, cell cycle progression, apoptosis or differentiation (Stohs & Bagchi, 1995; Ercal et al., 2001; Chen & Shi, 2002; Harris & Shi, 2003; Beyersmann & Hartwig, 2008) (Fig. 1).

**Metals and 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\(^{2-}\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radical (OH\(^\bullet\)). ROS can attack and damage all cellular macromolecules, leading to protein oxidation, lipid peroxidation and DNA damage (Halliwell & Gutteridge, 1984). The mechanisms leading to metal-induced oxidative stress *in vivo* are largely elusive. While Fe, Cu, Cr and Co can undergo redox-cycling reactions, Cd, Hg and Pb are redox inactive. Nevertheless, the latter metals may induce oxidative stress by inhibiting specific enzymes, by depleting pools of antioxidants or through other indirect mechanisms (Stohs & Bagchi, 1995; Ercal et al., 2001; Beyersmann & Hartwig, 2008). Cd has been shown to induce oxidative stress and lipid peroxidation in yeast (Brennan & Schiestl, 1996; Howlett & Avery, 1997). Arsenite [As(III)] – the most toxic form of As – triggers increased ROS production in mammals (Liu et al., 2001), but not to any large extent in wild-type yeast (Menezes et al., 2008). Nevertheless, As(III)-induced oxidative stress and lipid peroxidation were detected in mutants with impaired As(III) detoxification (yap8Δ cells) or oxidative stress defence (yap1Δ cells) systems (Menezes et al., 2008), indicating that As(III) enhances ROS levels also in yeast. While Cd and As have no biological functions, selenium [Se(0)] is an essential trace element and nontoxic at low levels. However, selenate [Se(VI)], selenite [Se(III)] and selenide [Se(II)] are highly reactive and may cause increased ROS production. Indeed, certain reactions of the pathway involved in the reduction of Se(III) into Se(0) appear to produce O\(^{2-}\) and H\(_2\)O\(_2\) (Seko & Imura, 1997; Turner et al., 1998). In a similar way, reduction of chromate [Cr(VI)] – the most toxic form of Cr – into the less toxic form chromite [Cr(III)] produces ROS. In this case, it is the reduction intermediates Cr(V) and Cr(IV) that are thought to trigger OH\(^\bullet\) generation through a Fenton-like mechanism (Shi et al., 1994; Stohs & Bagchi, 1995; Beyersmann & Hartwig, 2008). Fenton-type reactions are described for the nutrient metals Fe and Cu and are supposed to be a major source of hydroxyl radicals and oxidative stress in the cell (Halliwell & Gutteridge, 1984). As mentioned above, several metals are unable to undergo such reactions. Nevertheless, redox-inactive metals may perturb intracellular Fe metabolism (Kitchin & Wallace, 2008), leading to increased levels of free Fe in the cell, which in turn could enhance Fenton-type reactions and elevated ROS levels.

An often-cited mechanism of metal toxicity is depletion of glutathione (GSH) (e.g. Stohs & Bagchi, 1995), which is the main antioxidant molecule in cells. *Saccharomyces cerevisiae* neutralizes many metals and metalloids, for example Cd, As(III), Hg and antimonite [Sb(III)] through chelation to GSH (see later in text); hence, it is possible that this process leads to reduced cytosolic GSH levels. GSH depletion would influence the redox environment and impair the activities of GSH-dependent enzymes, such as glutathione peroxidases, glutathione S-transferases and glutaredoxins, thereby affecting many cellular processes. However, metal concentrations...
that are high enough to be toxic may still be too low to significantly deplete cytosolic GSH. For instance, the GSH concentration is in the millimolar range in yeast, whereas Cd is toxic in the micromolar range (Lafaye et al., 2005). Another observation that argues against GSH depletion as a general toxicity mechanism, at least in S. cerevisiae, is that GSH levels in fact strongly increase in response to Cd (Lafaye et al., 2005) and As(III) (Thorsen et al., 2007) exposure (see later in text). Nevertheless, it cannot be excluded that some metals reduce the GSH pool to an extent where GSH-dependent enzyme activities are affected.

Impact on proteins

Metals and metalloids have the capacity to bind to proteins, often via thiol groups of cysteine residues, and inhibit enzyme function. For instance, methylmercury (MeHg) strongly inhibits the yeast L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT), which catalyses the synthesis of glutamine-6-phosphate. Overexpression of GFAT results in MeHg resistance, suggesting that GFAT is targeted by MeHg (Naganuma et al., 2000). Whether MeHg inhibits this enzyme by binding to a thiol group is unknown. Cd inhibits human thiol transferases [glutathione reductase, thioredoxin reductase (TrxR), thioredoxin] in vitro, possibly by binding to vicinal cysteines in their active sites (Chrestensen et al., 2000). As these proteins protect cells from oxidative stress, their inhibition would lead to increased ROS levels. Cd may also displace Zn and calcium (Ca) ions from metalloproteins (Stobs & Bagchi, 1995; Schützendübel & Polle, 2002; Faller et al., 2005) and zinc finger proteins (Hartwig, 2001), thereby affecting their activity, but it is not known how much this mechanism contributes to Cd toxicity. As(III) has been shown to interact with actin, tubulin, TrxR and many other proteins (Hoffman & Lane, 1992; Menzel et al., 1999; Zhang et al., 2007; Kitchin & Wallace, 2008), and the classical view is that binding leads to enzyme inhibition. This concept is supported by the fact that As(III) binds β-tubulin and inhibits tubulin polymerization (Zhang et al., 2007). In contrast, As(III) binding to pyruvate kinase does not impair enzyme activity (Zhang et al., 2007). Similarly, As(III) binds to the pyruvic acid dehydrogenase (PDH) multienzyme complex, but PDH appears to be more sensitive to inhibition by ROS than by As-containing agents (Samikkannu et al., 2003). Hence, certain proteins may be more susceptible to As(III)-induced protein oxidation than to direct binding of As(III) to critical thiols (Samikkannu et al., 2003). Arsenic trioxide, the form of arsenite used in cancer therapy, has been shown to inhibit mammalian TrxR, probably by direct binding to the enzyme. TrxR inhibition leads to the oxidation of thioredoxin, which is one of the main thiol-dependent electron donor systems in mammalian cells, thereby affecting the regulation of the cellular redox environment and a wide range of cellular activities (Lu et al., 2007). Finally, Cr exposure has been shown to trigger oxidative protein damage (Sumner et al., 2005) and enhanced protein aggregation (Holland et al., 2007) in yeast. Whether protein aggregation is a general feature of metal-exposed cells is not known.

Inhibition of DNA repair

Most metals are weak mutagens and do not damage DNA directly; instead, they may trigger genotoxicity by interfering with DNA repair processes (Beyersmann & Hartwig, 2008). Cd induces recombination events, and base-substitution and frame-shift mutations (Brennan & Schiestl, 1996; Jin et al., 2003; Serero et al., 2008) by inhibiting the DNA mismatch repair system (Jin et al., 2003). This inhibition is a result of Cd blocking the ATPase activity of the Msh2p–Msh6p complex (Banerjee & Flores-Rozas, 2005), but it is not known whether Cd binds to a specific site or displaces a critical Zn ion (McMurray & Tainer, 2003; Banerjee & Flores-Rozas, 2005). As(III) can also trigger various types of DNA damage in mammalian systems and it has been proposed that this metalloid interferes with DNA repair systems (Shi et al., 2004). However, DNA repair functions were not enriched among yeast genes sensitive to As(III) (Haugen et al., 2004; Thorsen et al., 2009) and As(III) treatment did not increase DNA double-strand breaks in yeast (Jo et al., 2009). Se(III) is slightly mutagenic for yeast and the error-prone repair pathway appears to be important for tolerance; the rev3Δ mutant, which is defective in error-prone repair, is strongly Se(III) sensitive while mutations in other DNA repair pathways do not affect tolerance to any large extent (Pinson et al., 2000). How the other metals described here affect DNA repair systems is not known.

Other toxicity mechanisms

Besides the general toxicity mechanisms described above (Fig. 1), other modes of metal action exist. For example, Cr toxicity appears to involve mRNA mistranslation (Holland et al., 2007); As(III) toxicity involves disruption of the actin and tubulin cytoskeleton as well as the folding of de novo synthesized actin and tubulin monomers (Thorsen et al., 2009); Cd toxicity appears to target DNA replication (Serero et al., 2008) and triggers Fe deficiency (Ruotolo et al., 2008; Thorsen et al., 2009). Many metals such as Fe, Cu, Zn, Se, Cr, Cd, Hg and Pb influence membrane fluidity, which in turn could contribute to their toxicity (Garcia et al., 2005). Finally, recent in vitro studies indicate that metals interfere with proper protein folding (Sharma et al., 2008; Ramadan et al., 2009). Even though the above studies provide an insight into toxicity mechanisms, the molecular details of these mechanisms remain poorly understood. Nevertheless,
it is clear that metals and metalloids induce toxicity through many targets, some of which are metal specific while others are common for several metals.

**Metal responses from a genomic perspective**

Yeast cells respond to metal/metalloid exposure by arresting cell cycle progression (Pinson et al., 2000; Yen et al., 2005; Migdal et al., 2008) and by adapting the transcriptome (Gross et al., 2000; Lyons et al., 2000; Momose & Iwahashi, 2001; Fauchon et al., 2002; Stadler & Schweyen, 2002; Jin et al., 2003; Haugen et al., 2004; Shakoury-Elizeh et al., 2004; Thorsen et al., 2007), proteome (Vido et al., 2001; Thorsen et al., 2007; Pereira et al., 2008) and metabolome (Vido et al., 2001; Fauchon et al., 2002; Lafaye et al., 2005; Thorsen et al., 2007). Such responses aim at protecting cells from the damaging effects of these agents (Fig. 1). In addition, a wide variety of basal cellular functions contribute to tolerance acquisition (Haugen et al., 2004; Holland et al., 2007; Dilda et al., 2008; Jin et al., 2008; Jo et al., 2008; Ruotolo et al., 2008; Serero et al., 2008; Thorsen et al., 2009). Perhaps the most comprehensive genome-wide analysis of metal responses to date is the one by Freedman and coworkers; these authors monitored the transcriptome and ‘deletome’ during exposure to seven metals including Ag, Cu, Cd, Hg, Zn, Cr and As (Jin et al., 2008). Most of the other genome-wide studies mentioned above focused on one/two metals and/or one particular aspect (transcriptome, proteome, metabolome, deletome) of the metal response. Because in general, all these studies came to similar conclusions, we will focus the description below on the findings of Freedman and colleagues (Jin et al., 2008). A detailed ‘meta-analysis’ of the transcriptome and deletome during As and Cd exposure was performed in Thorsen et al. (2009). Transcriptional and bioinformatics’ analyses pinpointed two groups of genes responding in a similar way to all metals (Jin et al., 2008). These genes were termed ‘common metal-responsive’ (CMR) genes; induced CMR genes were enriched in biological processes related to metal ion transport and homoeostasis, detoxification of ROS, carbohydrate metabolism, fatty acid metabolism, polyamine transport and RNA polymerase II transcription, whereas repressed CMR genes were enriched in biological processes related to polysaccharide biosynthesis, G-protein signalling, protein targeting and transport (Jin et al., 2008). In fact, these processes are similar to those found to be enriched during exposure to other environmental stresses (Gasch et al., 2000). Non-CMR genes responded in a less uniform fashion, where some metals triggered a certain response while others did not. For example, As and Cu stimulated the expression of genes involved in energy generation and stress responses; As, Hg, Cr and Cd triggered the expression of genes in the sulphur assimilation and GSH biosynthesis pathways; and Cd, Ag, Hg, Zn and Cr induced genes involved in ribosome biogenesis and tRNA modifications (Jin et al., 2008). The same authors also analysed the deletome for metal sensitivities and found that processes related to cell wall integrity, metal chelation, sequestration of metals in vacuoles and oxidative stress defence contributed to tolerance acquisition. In contrast to gene expression that involved a common response to all metals, genes required for tolerance fell into largely distinct clusters. For instance, mutants sensitive to As were enriched in processes related to signal transduction, transcriptional regulation, tubulin folding and the secretory pathway; Cu- and Zn-sensitive genes were enriched in processes related to vesicle-mediated transport; Cd-sensitive genes were enriched in processes related to chromatin modifications, GSH biosynthesis and responses to stress; Cr-sensitive genes were enriched in processes related to sulphur amino acid biosynthesis, ubiquitin-dependent protein sorting and trehalose biosynthesis (Jin et al., 2008). Hence, cells appear to counteract metal toxicity by largely distinct mechanisms, possibly because the chemical properties of a given metal will influence how it affects cells (Haugen et al., 2004; Holland et al., 2007; Dilda et al., 2008; Jin et al., 2008; Jo et al., 2008; Ruotolo et al., 2008; Serero et al., 2008; Thorsen et al., 2009). This is in analogy to oxidative stress tolerance, where S. cerevisiae utilizes separate mechanisms for protection against different forms of ROS (Thorpe et al., 2004). Nevertheless, common metal responses also exist. The most important example is genes involved in sulphur assimilation and GSH biosynthesis; these genes are present in the induced CMR cluster and they are also required for tolerance to As, Cd, Cr and Cu (Jin et al., 2008; Thorsen et al., 2009).

**Metal uptake pathways and their regulation**

Nonessential and toxic metals and metalloids enter cells on the basis of molecular mimicry through plasma membrane permeases and channels evolved for the uptake of essential metals and other nutrients, such as Fe, Mn, Zn, phosphate, sulphate and glycerol (Fig. 2). However, all organisms including yeast have developed mechanisms that can reduce such influx by downregulating the expression of relevant transporters at the transcriptional and post-transcriptional levels and/or by inhibiting their transport activities.

**As uptake systems**

The arsenate [As(V)] oxyanion is a structural analogue of inorganic phosphate and is easily taken up through phosphate transporters in most organisms. Phosphate import into S. cerevisiae is mediated by two high-affinity permeases, Pho84p and Pho89p, and two low-affinity permeases,
Pho87p and Pho90p (Persson et al., 1999; Wykoff & O’Shea, 2001). Deletion of PHO84 and PHO87 confers As(V) tolerance, suggesting that As(V) enters yeast cells through these permeases (Bun-ya et al., 1996; Yompakdee et al., 1996b). Consistently, lack of Pho86p (pho86D), an endoplasmatic reticulum-localized protein involved in trafficking of Pho84p to the plasma membrane, or Gtr1p (gtr1Δ), a cytoplasmic GTPase regulating Pho84p-dependent phosphate transport, also results in increased As(V) tolerance (Bun-Ya et al., 1992, 1996; Yompakdee et al., 1996a; Lau et al., 2000). The pho84Δ mutant also displays enhanced tolerance to Mn, Co, Zn and Cu, indicating that Pho84p may mediate the uptake of these metals as well. Moreover, Pho84p has been shown to play an additional physiological role as a low-affinity Mn transporter (Jensen et al., 2003) and has been implicated in Se(IV) tolerance (Pinson et al., 2004).

In contrast to As(V), the pathway(s) of As(III) entry into cells remained elusive for a long time. Based on genetic data, it was proposed that the aquaglyceroporin GlpF mediates the influx of Sb(III) into Escherichia coli cells (Sanders et al., 1997). Subsequently, we demonstrated that the aquaglyceroporin Fps1p is the main entrance pathway of As(III) and Sb(III) into yeast cells (Wysocki et al., 2001). Deletion of FPS1 results in reduced As(III) uptake and in hypertolerance to both As(III) and Sb(III). The opposite is observed in cells harbouring a hyperactive FPS1 allele; these cells display enhanced As(III) uptake and hypersensitivity to both metalloids (Wysocki et al., 2001). These data clearly establish Fps1p as the main As(III) and Sb(III) entry pathway. Yeast cells lacking FPS1 and two additional major metalloid transporters (acr3Δycf1Δfps1Δ) has been used to identify and characterize As(III) and Sb(III) transporting aquaglyceroporins of mammalian and plant origin (Liu et al., 2002; Bienert et al., 2008; Isayenkov & Maathuis, 2008). Additional studies in bacteria, Leishmania and crop plants (e.g. rice) have confirmed that aquaglyceroporins are metalloid entry pathways in probably most organisms (Gourbal et al., 2004; Meng et al., 2004; Ma et al., 2008). However, it is important to note that aquaglyceroporins are bidirectional channels that may also mediate the efflux of metalloids in certain cases (see later in text). The main form of As(III) in solution is As(OH)3 (Ramirez-Solis et al., 2004). As(OH)3 structurally resembles Sb(OH)3 and glycerol (Ramirez-Solis et al., 2004; Porquet & Filella, 2007), the physiological substrate of E. coli GlpF and S. cerevisiae Fps1p. Hexose permeases also contribute to As(III) uptake in yeast. It has been suggested that three As(OH)3 molecules can polymerize to a ring structure similar to hexose sugars that could be recognized by hexose transporters (Liu et al., 2004).

Fps1p transport activity is regulated, but the mechanisms are not fully understood. Fps1p has a long cytosolic N-terminal tail that is important for gating, and it contains a mitogen-activated protein kinase (MAPK) phosphorylation site (Thr231) (Tamás et al., 1999, 2003; Thorsen et al., 2006). Deletion of the N-terminal domain or changing Thr231 into Ala leads to As(III) and Sb(III) sensitivity due to a high level of unregulated metalloid influx (Wysocki et al., 2001; Thorsen et al., 2006). We have shown that the MAPK Hog1p regulates transport through Fps1p; Hog1p phosphorylates Fps1p on Thr231 and this phosphorylation reduces Fps1p-mediated transport. Hog1p is activated in

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Fig. 2. Transporters mediating the uptake and detoxification of toxic metals in Saccharomyces cerevisiae. See text for an explanation of protein abbreviations.
response to As(III) and Sb(III), and cells lacking Hog1p (hog1Δ) are very sensitive to both metalloids and display an increased rate of Fps1p-dependent As(III) uptake (Thorsen et al., 2006). Two positive regulators of Fps1p activity, the pleckstrin homology (PH) domain proteins Rgc1p and Rgc2p (also called Ask10p), were recently identified. Deletion of RGC1 or RGC2 inactivated Fps1p and results in As(III) tolerance (Beese et al., 2009). However, how these proteins control Fps1p activity remains unclear. Fps1p is also regulated at the transcriptional level; addition of As(III) or Sb(III) to the medium causes a rapid decline in FPS1 mRNA levels (Wysocki et al., 2001). It has been reported that Fps1p is ubiquitylated and targeted for degradation in a Hog1p-dependent fashion in acetic acid-exposed cells. Because Fps1p allows the influx of acetic acid, this mechanism would prevent such influx and toxicity (Mollapour & Piper, 2007). In contrast, Fps1p is not degraded in response to As(III), suggesting different modes of regulation under metalloid/acetic acid stress and/or additional functions of Fps1p during metalloid exposure (Thorsen et al., 2006; Maciaszczyk-Dziubinska et al., 2010). Interestingly, long-term exposure to As(III) results in the upregulation of FPS1 transcription, whereas the expression of FPS1 from a multicopy plasmid surprisingly increases As(III) and Sb(III) tolerance (Maciaszczyk-Dziubinska et al., 2010). These data indicate that Fps1p can mediate both the influx and the efflux of As(III). In support of this notion, we found that cells lacking both Fps1p and the As(III) efflux protein Acr3p were unable to export As(III), while some As(III) export was evident in the acr3Δ mutant. Moreover, fps1Δ cells are As(V) sensitive even though As(V) is not transported by Fps1p (Maciaszczyk-Dziubinska et al., 2010). This dual function of Fps1p in metalloid toxicity (through uptake) and detoxification (through efflux) can be explained by the following model: As(V) enters cells through phosphate permeases and is subsequently reduced to As(III). Next, As(III) is actively extruded as an As(OH)2O− anion by Acr3p or it diffuses out of the cell down the concentration gradient as As(OH)3 through Fps1p. When cells are exposed to As(III), the activity of Fps1p may be inhibited via its N-terminal domain in a Hog1p-dependent manner, thereby reducing As(III) accumulation. However, when the intracellular concentration of As(OH)3 becomes higher than outside the cell, Fps1p may facilitate As(III) export in concert with Acr3p. Interestingly, to prevent the As(III) exported through Acr3p from re-entering via Fps1p, cells appear to decrease the extracellular concentration of As(OH)3 by exporting GSH. Extracellular As(III) and GSH form a complex that cannot enter cells (M. Thorsen, T. Jacobson, R. Vooijs, H. Schat & M.J. Tamás, unpublished data). Besides Fps1p, aquaglyceroporins from specific bacteria and mammals have also been implicated in As detoxification (Yang et al., 2005; Carubrey et al., 2009; McDermott et al., 2010). Moreover, heterologous expression of several plant aquaporins in yeast resulted in increased tolerance to As(V), suggesting that these proteins are bidirectional channels and that they may have a function in metalloid tolerance (Bienert et al., 2008).

**Cd uptake systems**

Cd enters cells through proteins involved in the uptake of essential cations such as Zn (through Zrt1p), Mn (Smf1p, Smf2p), Fe (Fet4p) and Ca (Mid1p). Zrt1p belongs to the Zrt- and Irt-like protein (ZIP) family and mediates high-affinity Zn transport (Zhao & Eide, 1996; Eng et al., 1998). Zrt1p is one of the major pathways through which Cd enters yeast; cells lacking Zrt1p absorb little Cd from the medium, while Zn uptake is strongly inhibited by Cd (Gomes et al., 2002; Gitan et al., 2003). Moreover, Zn-limited cells that upregulate ZRT1 transcription are more sensitive to Cd. Consistently, in the presence of high concentrations of Zn and Cd, Zrt1p is removed from the cell surface to prevent the uptake of toxic Cd and excess Zn (Gitan et al., 1998; Gitan et al., 2003). Zrt1p inactivation involves Rsp5p-dependent ubiquitylation, followed by endocytosis and degradation in the vacuole (Gitan & Eide, 2000; Gitan et al., 2003). Although a putative metal-responsive domain required for Zn- and Cd-induced Zrt1p ubiquitylation has been pinpointed, the molecular details of Zrt1p regulation remain to be established.

Two yeast members of the neutral resistance-associated macrophage protein (Nramp) family, the Mn transporters Smf1p and Smf2p, constitute a second pathway of Cd influx. Smf1p is localized to the plasma membrane and mediates high-affinity Mn uptake (Supek et al., 1996). The substrates of Smf1p also include Cd, Co, Cu, Fe and Zn (Supek et al., 1996; Liu et al., 1997; Chen et al., 1999). Smf2p is also a major Mn import pathway, although localization of Smf2p on the cell surface has never been shown (Portnoy et al., 2000; Luk & Culotta, 2001; Stimpson et al., 2006). Deletion of SMF1 or SMF2 results in similar levels of Cd tolerance, while overexpression of these genes leads to Cd sensitivity (Ruotolo et al., 2008). Hence, Smf1p and Smf2p appear to contribute equally to Cd accumulation. Smf1p and Smf2p are ubiquitylated by the ubiquitin ligase Rsp5p that is targeted to Smf1p and Smf2p by two adaptor proteins: Bsd2p and Tre1p (Stimpson et al., 2006; Sullivan et al., 2007). Cells lacking Bsd2p or Tre1p display Cd sensitivity and elevated Cd uptake due to increased Smf1p and Smf2p protein levels (Liu et al., 1997; Liu & Culotta, 1999; Stimpson et al., 2006). In response to Cd, plasma membrane-localized Smf1p is ubiquitylated by Rsp5p and is rapidly removed from the cell surface by endocytosis (Nikko et al., 2008). Interestingly, Cd-induced sorting of Smf1p does not require Bsd2p, but two arrestin-like proteins, Ecm21p and Crs2p, that recruit Rsp5p to Smf1p. Ecm21p
binds to Smf1p via its constitutively phosphorylated N-terminal domain, which is not necessary for Bsd2p-dependent downregulation (Nikko et al., 2008).

Fet4p is a plasma membrane protein that catalyses low-affinity Fe import (Dix et al., 1994, 1997), and it can also mediate the uptake of Cu and Zn (Hassett et al., 2000; Waters & Eide, 2002). Because Cd and Co inhibit Fet4p transport activity, it may be involved in the uptake of those metals as well (Dix et al., 1994; Hassett et al., 2000). The expression of FET4 is positively regulated at the transcriptional level by the transcription factors Atf1p and Zap1p in response to low levels of Fe and Zn, respectively (Dix et al., 1997; Waters & Eide, 2002), and negatively regulated by Rox1p under aerobic conditions (Jensen & Culotta, 2002; Waters & Eide, 2002). The lack of Rox1p (rox1Δ) results in higher FET4 mRNA levels and Cd sensitivity (Jensen & Culotta, 2002).

Mid1p, a stretch-activated Ca channel, has been suggested to be an additional pathway for Cd uptake into yeast. The lack of Ca uptake in the mid1Δ mutant is restored by heterologous expression of the wheat Ca transporter Lct1 and expression of Lct1 sensitizes yeast to Cd due to elevated Cd accumulation (Clemens et al., 1998). The role of Mid1p in Cd uptake was recently confirmed by direct transport assays (A. Gardarin, S. Chedin, J. Aude, E. Godat, P. Catty & J. Labarre, unpublished data).

**Chromate, molybdate and selenate uptake systems**

Based on transport studies in mammalian cells and structural similarities to sulphate, the uptake of Cr(VI), Se(VI) and molybdate [Mo(VI)] oxyanions via sulphate transporters has been suggested in yeast (Alexander & Aaseth, 1995; Tamás et al., 2005). Indeed, deletion of both high-affinity sulphate permeases, Sul1p and Sul2p, results in enhanced Cr(VI) and Se(VI) tolerance (Cherest et al., 1997), and direct transport assays confirmed that Cr(VI) enters yeast through sulphate permeases (Pereira et al., 2008). A recent genome-wide screen for Cr-sensitive mutants revealed that the actin-mediated endocytosis system is involved in Cr accumulation, and the authors proposed that actin-dependent endocytosis of plasma membrane Cr transporters might contribute to Cr tolerance. However, such a mechanism does not seem to operate on the level of the Cr(VI) transporters Sul1p and Sul2p (Holland & Avery, 2009).

**Metal detoxification systems and their regulation**

The mechanism that provides the highest level of tolerance in microorganisms is metal/metalloid removal from the cytosol through export pathways (Fig. 2). While prokaryotes possess several export routes, there are only two such strains that provide the highest level of metal/metalloid tolerance: (1) Metal detoxification systems involving metal chelation to specific peptides and proteins, such as GSH, phytochelatins (PC) and metallothioneins (MT), and the resulting complexes may be recognized as substrates by transporters for export and/or vacuolar sequestration.

**Plasma membrane metal exporters**

**Acrlp and As tolerance**

As(III) export via Acr3p is probably the most important As detoxification mechanism in *S. cerevisiae* (Wysocki et al., 1997; Ghosh et al., 1999). Acr3p is a prototype member of the arsenical resistance-3 (Acr3) family of transporters, which belongs to the bile/arsenite/riboflavin transporter (BART) superfamily (Mansour et al., 2007). Proteins of the Acr3 family have 10 membrane-spanning helices (Aaltenon & Silow, 2008; Fu et al., 2009) and are widely distributed in prokaryotes and fungi, with the exception of fission yeast *Saccharomyces pombe* (Wysocki et al., 1997; Maciaszczuk et al., 2004; Mansour et al., 2007). Acr3p homologues are also present in three lower plant species (*Physcomitrella patens*, NCBI accession number XP_001761548; *Selaginella moellendorffii*, NCBI accession number AC057621; and *Pteris vittata*, NCBI accession number ACN65413) and in the animal genome of *Danio rerio* (NCBI accession number ACN65413). The ACR3 gene was isolated based on its ability to confer high-level As tolerance to wild-type *S. cerevisiae* when overexpressed (Bobrowicz et al., 1997). Cells lacking ACR3 are highly As sensitive, hyperaccumulate As and display impaired As(III) export, while ACR3 overexpression markedly decreases cytosolic As levels and improves tolerance (Wysocki et al., 1997; Ghosh et al., 1999; Thorsen et al., 2006). These results establish the role of Acr3p in As(III) efflux. Thiol chemistry seems to be crucial for transport through Acr3p; mutagenesis of a conserved cysteine in the fourth membrane-spanning helix of bacterial and yeast Acr3p proteins impaired As(III) export and caused metalloid sensitivity (Fu et al., 2009; E. Maciaszczuk-Dziubinska, D. Wawrzycka, E. Sloma & R. Wysocki, unpublished data).
All members of the Acr3 family characterized to date confer tolerance only to As, and not to the related metalloid Sb (Wysocki et al., 1997; Sato & Kobayashi, 1998; Ghosh et al., 1999; Fu et al., 2009). In contrast, members of the ArsB family of transporters, which are ubiquitously present in bacteria and archaea, but absent in eukaryotes, mediate export of both metalloids (Rosen, 1999). It has been proposed that Acr3p may function as a uniporter that facilitates transport of the As anion As(OH)₂⁻ coupled to the membrane potential (Fu et al., 2009). This model would explain the specificity of Acr3p towards As(III) because at a physiologically neutral pH, little Sb(III) anion would be formed in the cytosol due to the higher pKₐ value of Sb(OH)₃ compared with that of As(OH)₃ (Fu et al., 2009). Nevertheless, phenotypic analyses of yeast mutants lacking proteins involved in metalloid uptake and detoxification suggest that Acr3p may transport Sb(III), at least under specific conditions (Wysocki et al., 2001; E. Maciaszczyk-Dziubinska, D. Wawrzyczka, E. Sloma & R. Wysocki, unpublished data). The acr3Δ mutant is highly sensitive to both As(III) and As(V), but ACR3 overexpression does not lead to high-level As(V) tolerance unless the ACR2 (also called ARR2) gene is present (Bobrowicz et al., 1997; Wysocki et al., 1997). Acr2p is an arsenate reductase that converts As(V) to As(III) (Mukhopadhyay & Rosen, 1998; Mukhopadhyay et al., 2000); hence, As(V) detoxification involves the reduction of As(V) into As(III) for subsequent export out of cells. The identification of Acr2p and Acr3p homologues in P. vittata (Chinese brake fern) indicates that As detoxification involving enzymatic reduction of As(V) to As(III), followed by As(III) export is conserved from bacteria to plants (Ellis et al., 2006; Salt & Norton, 2008). Importantly, P. vittata is capable of hyper-accumulating As in the fronds without toxicity and can therefore potentially be used for phytoremediation of As-contaminated soils (Ma et al., 2001). Nevertheless, the exact role of P. vittata Acr2p and Acr3p in this process remains to be revealed.

Acr3p is regulated at the transcriptional level; the expression of ACR3, and also of ACR2, is tightly controlled by the transcription factor YAP8 (also called ACR1/ARR1) (Bobrowicz & Ulaszewski, 1998; Wysocki et al., 2004; Iлина et al., 2008). In fact, ACR2 and ACR3 share a common promoter and form, together with YAP8, a cluster of As resistance genes (Bobrowicz et al., 1997). The expression of ACR2 and ACR3 is very low in the absence of metalloids, but both genes are simultaneously and robustly induced in a Yap8p-dependent manner in the presence of As(III), As(V) or Sb(III) (Bobrowicz & Ulaszewski, 1998; Bougamim et al., 2001; Haugen et al., 2004; Menezes et al., 2004; Wysocki et al., 2004). Yap8p appears to directly sense As(III) by binding to this metalloid; hence, it couples metalloid sensing to detoxification by regulating the levels of Acr2p and Acr3p. Whether Acr2p and Acr3p are also regulated post-translationally is currently not known.

Pca1p and Cd tolerance

Pca1p constitutes the main route of Cd export in S. cerevisiae and plays a crucial role in Cd tolerance (Adle et al., 2007). The gene was originally identified in a Cd-resistant strain and called CAD2 (Tohoyama et al., 1990), and it was believed that a gain-of-function mutation within CAD2/PCA1 (Arg970Gly) caused enhanced Cd efflux and improved tolerance (Shiraishi et al., 2000). In fact, the PCA1 gene product is not functional in common laboratory strains because of a Gly970Arg mutation in a conserved ATP-binding pocket (Adle et al., 2007). Because the CAD2 allele contains Gly at position 970, it actually represents the wild-type version of Pca1p (Shiraishi et al., 2000). Pca1p belongs to the ubiquitous P₁B-type ATPase super-family including transporters of a wide range of metals such as Ag, Cd, Co, Cu, Pb and Zn (Kühnbrandt, 2004). Expression of wild-type Pca1p confers tolerance by reducing intracellular Cd levels, establishing this protein as a major detoxification pathway. Transcription of PCA1 is constitutive, but the Pca1p protein is not detected under normal conditions due to rapid turnover. In response to Cd, Pca1p is stabilized and targeted to the plasma membrane, where it promotes Cd efflux and tolerance (Adle et al., 2007). The nonfunctional Pca1p-Gly970Arg is also stabilized in the presence of Cd; however, it does not reach the plasma membrane, indicating that the mutation perturbs proper trafficking. Interestingly, Cu and Ag (but no other metal ions) can also stabilize Pca1p, and overexpression of either wild-type Pca1p or Pca1p-Gly970Arg suppresses the Cu toxicity of a Cu-sensitive mutant (ace2Δ). Pca1p-mediated Cu tolerance involves an N-terminal cysteine-rich domain, but not its transport activity. Taken together, Pca1p is a Cd-specific plasma membrane transporter with the ability to sequester Cu when overexpressed (Adle et al., 2007).

The mechanism by which Cd regulates Pca1p has been revealed recently (Adle & Lee, 2008; Adle et al., 2009). The turnover of many plasma membrane proteins involves ubiquitin-mediated endocytosis, followed by vacuolar degradation (Leon & Haguenauer-Tsapis, 2009). In contrast, ubiquitylated Pca1p is targeted for degradation by the proteasome without first going via the plasma membrane. Pca1p has a cysteine-rich metal-sensing degradation signal within its N-terminus that is necessary and sufficient both for rapid degradation and for Cu- and Cd-induced stabilization of proteins (Adle & Lee, 2008). Metal binding to cysteines within this domain induces a conformational change that prevents ubiquitylation and degradation of Pca1p. Interestingly, Pca1p is targeted for ubiquitylation by components of the endoplasmic reticulum-associated
degradation (ERAD) system, like the ubiquitin-conjugating enzyme Ubc7p and the ubiquitin ligase Doa10p (Adle et al., 2009). The ERAD pathway serves as a quality control system involved in the degradation of terminally misfolded secretory proteins (Meuser et al., 2005). Control of Pca1p turnover by ERAD represents a novel regulatory mechanism of plasma membrane protein expression and of responses to metals (Adle et al., 2009).

**Other transporters**

Some additional plasma membrane transporters have been implicated in metal detoxification, but little is known about their regulation or mode of action. Yor1p belongs to the ATP-binding cassette (ABC) transporter family and is structurally related to the human multidrug resistance-associated protein MRP1 and yeast Ycf1p (see later in text). The YOR1 gene was identified by its ability to confer oligomycin resistance when overexpressed and it mediates resistance to a wide range of chemical compounds including anionic drugs, fungicides and lipids (Katzmann et al., 1995; Cui et al., 1996; Decottignies et al., 1998). Deletion of YOR1 results in moderate Cd sensitivity and it probably mediates Cd efflux in the form of Cd(GS)₂ (Cui et al., 1996; Nagy et al., 2006). Alr1p, which is responsible for Mg uptake into cells, may also contribute to Cd tolerance. Mutation of the ALR1 gene results in Cd sensitivity and increased intracellular Cd levels (Kern et al., 2005), but it is not known whether Alr1p mediates Cd export. Ssu1p is a member of the major facilitator superfamily and is involved in sulphite efflux (Park & Bakalinsky, 2000). Overexpression of the SSU1 gene leads to increased tolerance to Se(IV) (Pinson et al., 2000), suggesting that Ssu1p mediates Se(IV) export. However, because deletion of SSU1 causes sensitivity to sulphite and transcription of SSU1 is induced by sulphite, but not by Se(IV), it seems that the main function of Ssu1p is related to sulphite detoxification (Park & Bakalinsky, 2000; Pinson et al., 2000).

**Vacuolar sequestration of metals**

Transport of metals into vacuoles is a common detoxification mechanism in eukaryotes. In S. cerevisiae, the ABC transporter Ycf1p represents a major pathway for vacuolar sequestration of GSH-conjugated metals and xenobiotics, of endogenously produced toxins such as unconjugated bilirubin and the red pigment formed in adenine biosynthetic mutants and of free GSH for degradation within the vacuole (reviewed in Paumi et al., 2009). YCF1 was identified in a screen for genes conferring increased tolerance to Cd when overexpressed (Szczypka et al., 1994). Cells lacking YCF1 are highly sensitive to Cd, Hg, Pb and Sb(III), moderately sensitive to As(III) (Szczypka et al., 1994; Ghosh et al., 1999; Wysocki et al., 2001; Gueldry et al., 2003; Song et al., 2003; Preveral et al., 2006), but grow normally in the presence of other metals/metalloids (Preveral et al., 2006). Ycf1p catalyses active transport of As(GS)₃, Cd(GS)₂ and Hg(GS)₂ into vacuoles in vitro, while the presence of Sb(III) inhibits Ycf1p-mediated transport of GSH conjugates (Li et al., 1997; Ghosh et al., 1999; Gueldry et al., 2003). Together, these data establish the role of Ycf1p-mediated transport of metal–GSH conjugates for metal tolerance.

How Ycf1p-mediated detoxification is regulated is not well understood. YCF1 is transcribed in the absence of metals and its expression is only moderately increased by Cd, Hg, As(III), Sb(III) and Se(IV) (Li et al., 1997; Pinson et al., 2000; Sharma et al., 2002; Gueldry et al., 2003; Wysocki et al., 2004). In fact, strong induction of YCF1 expression is only observed when the transcriptional regulator Yap1p is overexpressed (Wemnie et al., 1994; Sharma et al., 2002) or in mutants that hyperaccumulate As in the cytosol (Wysocki et al., 2004). Ycf1p is subjected to post-translational control at the level of proteolytic processing, intracellular trafficking and phosphorylation (Mason & Michaelis, 2002; Mason et al., 2003; Eraso et al., 2004; Paumi et al., 2008). Ycf1p is positively modulated by phosphorylation and by the guanine exchange factor Tus1p. However, mutating the phosphorylated residues or deleting TUS1 causes only moderate Cd sensitivity (Eraso et al., 2004; Paumi et al., 2007). Ycf1p is also negatively regulated by phosphorylation; mutation of Ser251 increases both its transport capacity in vitro and Cd tolerance in vivo. An integrated membrane yeast two-hybrid screen revealed two candidate kinases, Cka1p and Hal5p, which may act as the negative regulators of Ycf1p (Paumi et al., 2008). Nevertheless, further studies are required to establish whether and how Ycf1p activity is modulated during metal exposure.

Genetic and biochemical studies implicated additional vacuolar transporters in metal tolerance. Two Ycf1p paralogues, Bpt1p and Vmr1p, play a minor role in Cd detoxification (Sharma et al., 2002; D. Wawrzycka & A. Goffeau, unpublished data). Deletion of BPT1 sensitizes cells to Cd only in the absence of YCF1, and the expression of BPT1 is not induced by Cd (Sharma et al., 2002). Similarly, the lack of Vmr1p causes slight sensitivity to Cd (D. Wawrzycka & A. Goffeau, unpublished data). Sequestration of several divalent metal cations, including Cd and Co, into vacuoles requires the presence of Zrc1p and Cot1p, which play important roles in Zn homeostasis (MacDiarmid et al., 2000; 2002). Both transporters belong to the cation diffusion facilitator (CDF) family (Paulsen & Saier, 1997) and share the ability to transport Zn and Co (Conklin et al., 1992, 1994). Zrc1p is also likely to transport Cd and Ni as these metals inhibit Zrc1p-dependent Zn uptake into vacuoles (MacDiarmid et al., 2002). In addition, overexpression of COT1 and ZRC1 confers resistance to
rhodium (Rh) and Cd, respectively (Kamizono et al., 1989; Conklin et al., 1992). Hence, these transporters may contribute to Cd and Co tolerance. Finally, cells lacking the vacuolar P_{7}-type ATPase Ypk9p are slightly sensitive to Cd, Mn, Ni and Se(II) (Gitler et al., 2009; Schmidt et al., 2009). However, how this protein contributes to metal tolerance remains to be established.

Cd tolerance in S. pombe involves the ABC transporter Hmt1, which is localized to the vacuolar membrane (Ortiz et al., 1992). Because fission yeast, like plants, uses PC to chelate metals (see later in text), the major substrate for Hmt1 seemed to be Cd–PC complexes (Ortiz et al., 1995). However, this two-step model involving the formation of metal–PC complexes, followed by vacuolar accumulation via Hmt1-like transporters thought to operate in fission yeast and plants, has been questioned recently (Preveral et al., 2009; Sooksa-Nguan et al., 2009). Firstly, overexpression of S. pombe Hmt1 confers high-level Cd tolerance to S. pombe cells devoid of the enzyme catalysing PC biosynthesis, and also in budding yeast and bacteria that naturally lack PC (Preveral et al., 2009). Secondly, PC-deficient fission yeast display increased sensitivity to As, Cd and Hg, while cells lacking Hmt1 are sensitized only to Cd. Thirdly, Hmt1 proteins appear to transport mainly GSH-conjugated Cd, but little or no metal–PC complexes (Preveral et al., 2009; Sooksa-Nguan et al., 2009). Thus, vacuolar sequestration of metal–GSH conjugates mediated by ABC transporters appears to be conserved from bacteria to humans. In addition, chelation of metals by PC in the cytosol represents a distinct mode of detoxification.

**Metal chelation by MT and PC**

Low-molecular-weight cysteine-rich proteins and peptides such as GSH, MT and PC are important contributors to metal detoxification. In S. cerevisiae, there are two types of MT, Cup1p and Crs5p, which differ structurally and functionally. Cup1p is a 53-amino-acid-long polypeptide, it has limited homology to mammalian MT and it binds Cu, Cd and Zn (Winge et al., 1989). In the absence of metals, CUP1 expression is negligible, but is strongly induced by high Cu levels (Thiele & Hamer, 1986). Cu tolerance can be achieved by genomic CUP1 gene amplification (Fogel & Welch, 1982; Liti et al., 2009) and cup1Δ cells are sensitive to Cu, but not to other metals/metalloids (Ecker et al., 1986; R. Wysocki & M.J. Tamás, unpublished data). Hence, the main physiological role of Cup1p appears to be Cu detoxification. Because high expression of CUP1 leads to enhanced Cd tolerance, it is possible that Cup1p also protects cells from Cd toxicity (Ecker et al., 1986). Crs5p is a 69-residue protein, is more cysteine-rich than Cup1p and shows high similarity to MT from other organisms (Culotta et al., 1994). Crs5p is constitutively expressed, but in the presence of high Cu levels, its expression is elevated, albeit to a lesser extent than CUP1. The crs5Δ mutant is slightly Cu sensitive (Jensen et al., 1996). Recently, Crs5p was shown to bind Cu, Cd and especially well to Zn. Moreover, the protein seems to be essential for yeast viability during mixed Cu/Zn overload. Hence, Crs5p might be a Zn-thionein with a physiological function in Zn homeostasis (Pagan et al., 2007). Likewise, the S. pombe metallothionein Zym1 sequesters Zn, while cells lacking Zym1 are sensitive to Zn and Cd (Borrely et al., 2002). In sum, yeast MT are mainly involved in Cu and Zn homeostasis, with a limited role in Cd tolerance.

PC are peptides found in all eukaryotic kingdoms and in some prokaryotes, and they are composed of (γ-Glu-Cys)_{n}-Gly (PC_{n}; n = 2–11) repeats. These peptides are synthesized from GSH by the enzyme PC synthase (PCS). PC constitute a major pathway of metal detoxification in fission yeast and plants, especially for Cd and As detoxification (Clemens, 2006). PC biosynthesis is stimulated by a wide range of metals and metalloids, including Cd, Cu, Ag, Hg, Zn and As, while deletion of the S. pombe PCS gene results in As(III), Cd and Cu sensitivity (Clemens et al., 1999, 2001; Ha et al., 1999; Wysocki et al., 2003). The formation of Ag+, Cd+, Cu+ and As–PC complexes has been demonstrated in vitro (Maitani et al., 1996; Schmoger et al., 2000). Hence, PC appears to protect cells by chelating metals, thereby reducing their reactivity and toxicity. Importantly, heterologous expression of PCS in S. cerevisiae, or in mutants lacking functional vacuoles, can confer Cd, As(III) and Sb(III) tolerance (Clemens et al., 1999; Wysocki et al., 2003). Thus, chelation of metals by PC in the cytosol is sufficient for improving metal tolerance. As mentioned above, the S. cerevisiae genome does not encode a PCS homologue. Nevertheless, the formation of PC_{2} has been detected in this yeast in response to Cd, Cu and Zn (Kneer et al., 1992). PC_{2} formation involves two vacuolar carboxypeptidases: CPY and CPC (Wünschmann et al., 2007). However, cells lacking these enzymes are not Cd sensitive despite an inability to produce PC_{2}. Thus, naturally produced PC in S. cerevisiae does not appear to contribute to metal tolerance to any large extent.

**Other detoxification systems**

As(V) tolerance involves the arsenate reductase Acr2p; ACR2 deletion sensitize cells only to As(V) and purified Acr2p catalyses the reduction of As(V) into As(III) using GSH and glutaredoxin as electron donors (Mukhopadhyay & Rosen, 1998; Mukhopadhyay et al., 2000). Acr2p shows no similarity to bacterial ArsC arsenate reductases; instead, Acr2p contains a conserved protein tyrosine phosphatase signature HisCys(X)_{3}Arg motif (Fauman et al., 1998). Acr2p has no phosphatase activity, but uses the HisCys(X)_{3}Arg motif as the catalytic centre for As(V) reduction.
(Mukhopadhyay & Rosen, 2001). Interestingly, Acr2p can easily be converted into a phosphatase merely by substituting three amino acids (Mukhopadhyay et al., 2003), and LmACR2 from Leishmania major exhibits both arsenate reductase and protein tyrosine phosphatase activities (Zhou et al., 2006). Hence, Acr2p arsenate reductases probably evolved from protein tyrosine phosphatases as a distinct protein family (Mukhopadhyay et al., 2003).

**Sulphur and GSH metabolism**

Sulphur assimilation and GSH biosynthesis are essential for all organisms. In *S. cerevisiae*, extracellular sulphate is taken up by sulphate transporters and reduced through the assimilation pathway to yield sulphide. Sulphide can then either go through the methyl cycle or into the cysteine/GSH biosynthesis pathway (Fig. 3). Hence, assimilated sulphur will either be incorporated into the sulphur-containing amino acids methionine and cysteine or into the low-molecular-weight thiol molecules *S*-adenosylmethionine and GSH. Transcription of the genes encoding the above activities is regulated by the transcriptional activator Met4p, but in response to metals, Met4p acts in concert with Yap1p to regulate GSH synthesis (see later in text). An essential function of the sulphur pathway is its involvement, through *S*-adenosylmethionine, in the biosynthesis of polyamines and biotin and most transmethylation reactions in the cell (Thomas & Surdin-Kerjan, 1997). Another essential function of this pathway is GSH biosynthesis. GSH is the main redox buffer of the cell and it serves as an electron donor for many enzymes. GSH is also a key factor in the defence against oxidative stress and metal toxicity (Thomas & Surdin-Kerjan, 1997; Grant, 2001), and it contributes to metal detoxification in several ways. Firstly, GSH can bind to metals and the resulting complex is a substrate for proteins that mediate vacuolar sequestration. Secondly, GSH protects cells against metal-induced oxidation. Thirdly, GSH may bind to reactive sulphydryl groups on proteins (protein glutathionylation), thereby shielding them from irreversible metal binding and/or oxidative damage (Grant, 2001; Pompella et al., 2003). While the first two mechanisms are well characterized, less is known about the role of protein glutathionylation in metal tolerance. Recently, we discovered a fourth detoxification function for GSH involving extracellular metal chelation. As(III)-exposed yeast cells export significant amounts of GSH and extracellular GSH forms a complex with As(III) outside cells. This complex does not readily enter cells; consequently, cells that produce and export GSH accumulate less As in the cytosol and grow better during As(III) exposure (M. Thorsen, T. Jacobson, R. Vooijs, H. Schat & M.J. Tamás, unpublished data).

The expression of genes in the sulphate assimilation and GSH biosynthesis pathways is stimulated by As(III), Cd, Hg and Cr(VI) (Momose & Iwahashi, 2001; Vido et al., 2001; Fauchon et al., 2002; Haugen et al., 2004; Thorsen et al., 2007; Jin et al., 2008). The levels of the corresponding enzymes also increase in As(III)-, Cd- and Cr(VI)-exposed cells (Vido et al., 2001; Thorsen et al., 2007; Pereira et al., 2008). In response to As(III) and Cd, the cells also boost sulphur metabolite pools, the GSH synthesis rate, as well as

**Fig. 3.** Sulphur and GSH 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 GSH synthesis. See text for details.
zymes have a lower content of sulphur-containing amino acids compared with those expressed under nonstress conditions, while expression of the latter (sulphur-rich) enzymes is repressed. The isoenzyme switch was linked to the necessity to spare sulphur to allow cells to strongly enhance GSH levels during Cd exposure (Fauchon et al., 2002). Optimal sulphur sparing also involves a decrease in the global protein synthesis rates coupled to the reduced sulphur amino acid composition of the newly synthesized proteome. These responses might allow an overall sulphur amino acid saving of up to 30% (Fauchon et al., 2002; Lafaye et al., 2005). The transcription factor Met4p regulates the isoenzyme switch and plays a major role in the sulphur-sparing response, indicating that the same regulator controls GSH synthesis and the mechanisms that save sulphur for enhanced GSH production (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 (Baudouin-Cornu et al., 2001). As mentioned above, Cr(VI) and sulphur starvation also trigger the sulphur-sparing response. However, in these cases, the cellular pools of sulphur metabolites and GSH are significantly reduced, and the sulphur-sparing response might be a consequence of sulphur deprivation (Lafaye et al., 2005; Pereira et al., 2008) rather than a need to spare sulphur for incorporation into GSH.

Cd detoxification in S. pombe involves GSH and PC; however, the levels of the enzymes of the sulphate assimilation, GSH and PC biosynthesis pathways do not increase in the presence of Cd (Chen et al., 2003; Bae & Chen, 2004). Despite this, PC synthesis is elevated because binding of Cd to PCS strongly activates the enzyme (Grill et al., 1991; Vatamaniuk et al., 2000; Maier et al., 2003). Sulphide synthesis is also increased in S. pombe (Chen et al., 2003; Bae & Chen, 2004) and sulphide participates in the production of the high-molecular-weight PC–Cd–S complex that has a high Cd-binding capacity (Ow et al., 1994). A similar mechanism has been described in Candida glabrata (Dameron et al., 1989). Interestingly, PC-based detoxification of Cd, As(III) and Sb(III) seems to be more efficient than the GSH-based detoxification mechanism operating in S. cerevisiae because the expression of S. pombe or Arabidopsis thaliana PCS in S. cerevisiae improved its tolerance to these metals (Clemens et al., 1999; Wysocki et al., 2003).

Signalling and transcriptional regulation

Signalling proteins and transcriptional regulators are key mediators of the cell’s response to metals (Fig. 4). These proteins sense the presence of metals, and regulate various tolerance and detoxification systems. In recent years, several such regulators have been identified, and the molecular mechanism by which they are activated and contribute to tolerance is starting to emerge.
Yap1p: protection against oxidants and metals

Yap1p (yeast AP-1) is one of eight AP-1-like transcription factors (Yap1p–Yap8p) in *S. cerevisiae*. These proteins contain a basic leucine zipper (bZIP) DNA-binding domain and a number of conserved cysteine residues (Moye-Rowley et al., 1989; Fernandes et al., 1997; Toone et al., 2001). In Yap1p, these cysteines are clustered into two domains – a C-terminal cysteine-rich domain (cCRD) composed of residues Cys598, Cys620 and Cys629 and an N-terminal cysteine-rich domain (nCRD) composed of residues Cys303, Cys310 and Cys315 – and they are important for Yap1p regulation (Toone et al., 2001; D’Autreaux & Toledano, 2007). Yap1p mediates the cell’s response to peroxides and other oxidants, chemicals with electrophilic properties, and metals by controlling expression of about 70 genes. The majority of those genes encode proteins that maintain a favourable redox balance in the cell, enzymes involved in detoxification of ROS and proteins conferring metal and drug resistance (Lee et al., 1999; Gasch et al., 2000; Haugen et al., 2004; Thorsen et al., 2007). Cells lacking YAP1 fail to properly induce the expression of those genes and are sensitive to a broad range of oxidants, chemical agents and metals. Yap1p regulates target-gene expression principally by binding to the so-called Yap1p recognition element (YRE) with the consensus sequence TT/GAC/GTAA (Kuge & Jones, 1994; Wu & Moye-Rowley, 1994; Fernandes et al., 1997; Harbison et al., 2004; Tan et al., 2008), but it may also act through alternative YRE sequences (He & Fassler, 2005).

Yap1p can be activated by a multitude of stress signals including peroxides, diamide (Kuge & Jones, 1994), menadione (Stephen et al., 1995; Stephen & Jamieson, 1997), the electrophiles diethylmaleate (Kuge & Jones, 1994), benomyl and MMS (Nguyen et al., 2001), and the metals Cd (Hirata et al., 1994; Stephen & Jamieson, 1997), As(III) (Menezes et al., 2004; Wysocki et al., 2004), Sb(III) (Wysocki et al., 2004), Se(III) (Azevedo et al., 2003) and Hg (Westwater et al., 2002). Moreover, the yap1Δ mutant displays sensitivity to these agents. Importantly, all these signals control Yap1p through regulated nuclear export by stress-induced post-translational modifications. In the absence of stress, Yap1p is mainly localized to the cytosol due to rapid nuclear export by the nuclear export receptor Crm1p. Crm1p interacts with a nuclear export signal (NES) embedded within the Yap1p cCRD (Kuge et al., 1997, 1998; Yan et al., 1998). Upon exposure to stress signals, the Yap1p–Crm1p interaction is lost due to redox- or chemical-dependent modifications of Yap1p cysteines, resulting in the nuclear accumulation of Yap1p (Kuge et al., 1997; Yan et al., 1998). H$_2$O$_2$-induced Yap1p activation involves the formation of an intramolecular disulphide bond between the cCRD Cys598 and the nCRD Cys303 (Delaunay et al., 2000), and possibly also between Cys310 (nCRD) and Cys629 (cCRD) (Wood et al., 2003). Disulphide linkage between these N- and C-terminal cysteines triggers a conformational change that masks the Yap1p NES and results in the nuclear accumulation of the protein. Reduction of these disulphide bonds causes structural modifications that lead to NES exposure and redistribution of Yap1p to the cytosol (Wood et al., 2003).
et al., 2004). Yap1p activation by H₂O₂ involves Gpx3p (also called Orp1p), which acts as the actual H₂O₂ sensor (Delaunay et al., 2002; Paulsen & Carroll, 2009), and a second protein Ybp1p (Veal et al., 2003; Gulshan et al., 2004), whose function is not understood. The Gpx3p–Yap1p H₂O₂ sensor operates as a cysteine-redox relay, where Gpx3p–Cys36 senses the H₂O₂ signal and oxidizes to a Cys–SOH. Oxidized Gpx3p transduces this signal to Yap1p by engaging the latter into a Cys36–Cys598 intermolecular disulphide, which is then converted to the intramolecular Cys303–Cys598 disulphide of active Yap1p (Delaunay et al., 2002; Paulsen & Carroll, 2009). Yap1p oxidation is rapid and the formation of interdomain disulphides results in an active form of Yap1p that is more resistant to reduction/deactivation by thioredoxin (Izawa et al., 1999; Delaunay et al., 2000; Carmel-Harel et al., 2001; Okazaki et al., 2007).

Although metals and chemicals also activate Yap1p by regulating its nuclear export, these compounds are sensed through a distinct mechanism that does not involve inter-CRD disulphide bonds and bypass the requirement of Gpx3p and Ybp1p (Kuge et al., 1997; Delaunay et al., 2000; Azevedo et al., 2003; Veal et al., 2003; Gulshan et al., 2004). The electrophile N-ethylmaleimide activates Yap1p by the covalent modification of cCRD Cys598, Cys620 and Cys629 (Azevedo et al., 2003) while Yap1p activation by diamide involves the formation of intra-CRD disulphide bonds between either one of Cys598, Cys620 and Cys629 (Kuge et al., 2001). Cd- and Se(III)-triggered activation of Yap1p proceeds through the cCRD (Azevedo et al., 2003), whereas As(III)-triggered activation may be more complex. In the latter case, Yap1p mutants with modified cCRD cysteines or nCRD cysteines together with modified Cys620 displayed perturbed nuclear retention and reduced ability to confer As(III) tolerance (Wysocki et al., 2004). The exact details by which metals interact with or modify Yap1p, for example by direct binding or through oxidative modifications, and whether those modifications disrupt the Yap1p–Crm1p interaction, remain to be revealed.

Interestingly, Yap1p appears to act together with other transcription factors to orchestrate responses to various stress conditions. Yap1p controls the oxidative stress response in cooperation with Skn7p (Lee et al., 1999); it regulates GSH biosynthesis together with Met4p (Wheeler et al., 2003; Thorsen et al., 2007); it acts together with Pdr1p and Pdr3p to regulate multidrug resistance pathways (Wendler et al., 1997); and it is involved in regulating the expression of RPN4 (Owsianik et al., 2002; Haugen et al., 2004), a transcription factor controlling proteasomal gene expression. Together, these transcription factors form an interconnected transcriptional network that mediates the cellular response to the toxic and damaging effects of environmental stress agents.

Many different fungi possess functional homologues of Yap1p, for example S. pombe Pap1 (Toone et al., 2001) and Candida albicans Cap1 (Alarcó & Raymond, 1999). Similar to Yap1p, S. pombe Pap1 is activated by H₂O₂-induced disulphide bond formation involving a peroxiredoxin, Tpx1, that transfers the redox signal to and hence activates Pap1 (Vivancos et al., 2004, 2005). Moreover, Pap1 is required for Cd and As(III) tolerance because deletion of the pap1 gene results in sensitivity to these metals (Rodriguez-Gabriel & Russell, 2005; Kennedy et al., 2008). The mechanisms by which metals activate Pap1 are unknown.

Yap8p: an arsenite-sensing transcription factor

Yap8p (also called Acr1p/Arr1p) is another metal-responsive AP-1-like transcription factor. In contrast to Yap1p, which protects cells in response to many stress conditions, Yap8p is exclusively required for As tolerance. Yap8p is necessary for As(III)-induced expression of ACR2 and ACR3, which appear to be its only gene targets (Wysocki et al., 2004; Ilina et al., 2008). The shared ACR2-ACR3 promoter contains an extended pseudopalindromic YRE (TGAATATTACTA) sequence that is recognized by Yap8p, but not by Yap1p. We have shown that this sequence is essential for Yap8p binding and for As(III)-induced ACR2 and ACR3 expression (Wysocki et al., 2004; Ilina et al., 2008). Yap8p is not regulated at the level of localization; instead, it resides in the nucleus bound to the ACR2-ACR3 promoter both in untreated and in As(III)-exposed cells (Wysocki et al., 2004; Di & Tamás, 2007). Yap8p is regulated by the ubiquitin–proteasome pathway; Yap8p is present in low levels in untreated cells due to degradation, whereas Yap8p is stabilized in As(III)-exposed cells and stimulates enhanced transcription of its target genes (Di & Tamás, 2007). Yap8p degradation involves the ubiquitin-conjugating enzyme Ubc4p while the ubiquitin ligase that acts on Yap8p remains to be identified. How As(III) activates Yap8p is not fully understood. Proper Yap8p function requires cysteine residues that are conserved in Yap1p and other fungal AP-1 proteins; the mutation of Yap8p Cys132, Cys137 or Cys274 affects Yap8p stabilization, disrupts As(III)-triggered ACR3 expression and results in As sensitivity (Menezes et al., 2004; Wysocki et al., 2004; Di & Tamás, 2007). Based on these data, we proposed that Yap8p might be activated by direct binding to As(III) (Wysocki et al., 2004; Di & Tamás, 2007). Indeed, analysis with X-ray spectroscopy revealed that As(III) binds to purified Yap8p with high affinity in an S₃ site, pointing to the involvement of three cysteine residues. A Yap8p mutant lacking Cys132 and Cys274 shows dramatically reduced As(III) binding ability, indicating that these are two of the three cysteines comprising the three-coordinate binding site (J. Yang & B.P. Rosen, unpublished data). Hence, Yap8p can be viewed as a sensor protein that efficiently couples As(III) sensing to
detoxification. Nevertheless, the mechanism by which As(III) binding enables Yap8p to trigger ACR2-ACR3 expression remains to be unveiled.

Yap8p homologues are only present in the genomes of three closely related fungal species: *Saccharomyces paradoxus*, *Saccharomyces kudriavzevii* and *Kluyveromyces lactis* (Ilina et al., 2008). *Kluyveromyces lactis* possesses a functional Yap8p orthologue; this protein can fully complement the As(III) sensitivity of *S. cerevisiae yap8Δ*, and the *K. lactis yap8Δ* mutant is As sensitive. Interestingly, *K. lactis* Yap8p may play an additional role in mediating tolerance to Cd and peroxides (J. Veide-Vilg, Yap8p is currently under investigation.

or DNA-binding capacity compared with that of

of

logue; this protein can fully complement the As(III) sensitivity of about 45 genes; the expression of the core genes requires

expression remains to be unveiled.

Met31p or Met32p, whereas the loss of Cbf1p affects only a subset of gene targets (Lee et al., 2010; Pereira et al., 2007; Chandrasekaran et al., 2006; Flick et al., 2006). In response to Cd, both mechanisms of Met4p inactivation described above are lost, resulting in the rapid induction of Met4p-dependent genes. Cd inhibits SCFMet30 activity by triggering the dissociation of Met30p from the ubiquitin ligase complex. This is followed by a deubiquitylation step that removes ubiquitin from Met4p, thereby fully restoring Met4p activity (Barbey et al., 2005; Yen et al., 2005). However, neither the mechanism by which Cd triggers dissociation of Met30p from the ubiquitin ligase complex nor the identity of the deubiquitylating enzyme that acts on Met4p is known. Similar effects on Met4p ubiquitylation have been observed with As(III) (Yen et al., 2005), which is consistent with the importance of Met4p for As(III)-induced expression of sulphur/GSH genes (Thorsen et al., 2003; Kobayashi et al., 2006). The observation that Cd- and As(III)-treated cells arrest cell cycle progression (Yen et al., 2005; Migdal et al., 2008) suggests a possible role for Met30p–Met4p in this control.

**Met4p: a regulator of sulphur and GSH metabolism**

The bZlP protein Met4p is the principal transcriptional activator of the sulphur assimilation and GSH biosynthesis pathways (Fig. 3). Met4p controls the expression of its gene targets in response to variations in the intracellular pool of an organic sulphur compound, possibly cysteine (Thomas & Surdin-Kerjan, 1997; Hansen & Johannsen, 2000; Menant et al., 2006), in response to metals such as As(III), Cd and Cr(VI), and it also controls the aforementioned sulphur-sparing programme (Faucount et al., 2002; Thorsen et al., 2007; Pereira et al., 2008). Met4p is unable to bind to DNA directly; instead, it is recruited to target promoters by the DNA-binding proteins Met31p, Met32p and Cbf1p. Another cofactor, Met28p, stabilizes the DNA-bound Met4p-containing complexes (Thomas & Surdin-Kerjan, 1997). The Met4p–Cbf1p–Met28p complex is targeted to the regulatory sequence CAGCTGA by the centromere-binding factor Cbf1p, while the Met4p–Met28p–Met31p–Met32p complex is targeted to the DNA sequence AAACTGTGGC by the redundant Met31p–Met32p proteins (Blaiseau et al., 1997; Kuras et al., 1997; Thomas & Surdin-Kerjan, 1997; Blaiseau & Thomas, 1998). The core Met4p regulon consists of about 45 genes; the expression of the core genes requires Met31p or Met32p, whereas the loss of Cbf1p affects only a subset of gene targets (Lee et al., 2010).

Met4p regulation involves Met30p, the F-subunit of the SCFMet30 ubiquitin ligase complex that targets Met4p for ubiquitylation and inactivation. Interestingly, ubiquitylated Met4p can encounter different fates depending on the growth condition; it can be targeted by the 26S proteasome for degradation or it can be maintained in its ubiquitylated form without degradation (Kaiser et al., 2000; Rouillon et al., 2000; Kuras et al., 2002; Chandrasekaran et al., 2006; Flick et al., 2006). When methionine is added back to sulphur-starved cells grown in minimal medium, SCFMet30 targets Met4p for poly-ubiquitylation and degradation (Rouillon et al., 2000; Kuras et al., 2002). In rich medium where sulphur compounds are abundant, Met4p is oligo-ubiquitylated (one to four ubiquitin moieties), but not degraded. It has been shown that a ubiquitin-binding motif within Met4p protects the protein from degradation and that oligo-ubiquitylated Met4p fails to form functional transcriptional complexes with its auxiliary factors (Kaiser et al., 2000; Kuras et al., 2002; Chandrasekaran et al., 2006; Flick et al., 2006). An oligo-ubiquitylated Met4p fails to form functional transcriptional complexes with its auxiliary factors (Kaiser et al., 2000; Kuras et al., 2002; Chandrasekaran et al., 2006; Flick et al., 2006). In response to Cd, both mechanisms of Met4p inactivation described above are lost, resulting in the rapid induction of Met4p-dependent genes. Cd inhibits SCFMet30 activity by triggering the dissociation of Met30p from the ubiquitin ligase complex. This is followed by a deubiquitylation step that removes ubiquitin from Met4p, thereby fully restoring Met4p activity (Barbey et al., 2005; Yen et al., 2005). However, neither the mechanism by which Cd triggers dissociation of Met30p from the ubiquitin ligase complex nor the identity of the deubiquitylating enzyme that acts on Met4p is known. Similar effects on Met4p ubiquitylation have been observed with As(III) (Yen et al., 2005), which is consistent with the importance of Met4p for As(III)-induced expression of sulphur/GSH genes (Thorsen et al., 2007). However, this mode of Met4p regulation does not seem to apply in response to Co, Ni and Pb (Yen et al., 2005). Interestingly, fully activated Met4p triggers a cell cycle arrest (Patton et al., 2000; Su et al., 2005). The observation that Cd- and As(III)-treated cells arrest cell cycle progression (Yen et al., 2005; Migdal et al., 2008) suggests a possible role for Met30p–Met4p in this control.

**Schizosaccharomyces pombe** induces the expression of genes encoding transporters of sulphur compounds in response to Cd (Chen et al., 2003) via the bZlP transcription factor Zip1 (Harrison et al., 2005). Zip1 is regulated by the SCFPZip1 ubiquitin ligase by a mechanism similar to how SCFMet30 regulates Met4p in *S. cerevisiae* involving Cdm-mediated Zip1 stabilization and induced expression of Zip1 gene targets (Harrison et al., 2005). Remarkably, this mechanism of regulation seems to be conserved in mammals; the SCF ubiquitin ligase complex Keap1/Cul3/Rbx1 controls degradation of the bZlP transcription factor Nrf2. This factor is stabilized by Cd and oxidative stress (Stewart et al., 2003; Kobayashi et al., 2004), and Nrf2 also controls GSH biosynthesis (Chan & Kwong, 2000; Sun et al., 2005), indicating an evolutionary conservation of this feature among eukaryotes.

**Rpn4p: regulating proteasomal degradation of damaged proteins**

Many metals may interfere with enzyme/protein activity, and the ubiquitin–proteasome pathway provides a mechanism
to remove damaged or nonfunctional proteins (Goldberg, 2003). The transcription factor Rpn4p is a central regulator of proteasomal abundance in the cell. In addition, Rpn4p can associate with the 19S proteasomal cap, although the purpose of this association is unknown. Rpn4p regulation is complex and involves both ubiquitin-dependent and ubiquitin-independent degradation by proteasomes. Moreover, the expression of RPN4 is induced under various stress conditions that may require higher proteasome levels (Hanna & Finley, 2007). Rpn4p and Rpn4p-dependent processes have been implicated in As(III) tolerance: (1) expression of proteasomal genes are strongly induced by As(III), (2) this induction requires Rpn4p and (3) cells lacking RPN4 are highly As(III) sensitive. Expression of the RPN4 gene is also enhanced by As(III) and this induction requires the transcription factor Yap1p (Haugen et al., 2004; Thorsen et al., 2007, 2009). These data suggest that cells may increase the number of proteasomes and that enhanced protein degradation is important for As(III) tolerance. The latter is supported by the observation that mutants defective in the ubiquitin–proteasome pathway display severe As(III) sensitivity (Di & Tamás, 2007). Rpn4p is also required for Cd tolerance (Thorsen et al., 2009) and Cd-treated cells display increased protein degradation rates (Medicherla & Goldberg, 2008). Finally, Cr triggers mRNA mistranslation and protein aggregation, and proteasomal activity is required for Cr tolerance (Holland et al., 2007). Together, these data suggest that metal-treated cells accumulate damaged proteins and that cells need to remove those proteins for optimal tolerance. Intriguingly, cells do not only control proteasome abundance in response to environmental stress, but proteasomes may also be subject to other modes of regulation (Hanna & Finley, 2007). The mammalian AIRAP protein has been shown to be specifically induced by As(III) and to associate with the 19S proteasomal cap. The exact role of AIRAP in proteasome function is unclear, but it may enhance proteasomal stability and/or activity when bound to the 19S cap (Stanhill et al., 2006). Whether Rpn4p would have a similar role is unknown.

**Hog1p has a dual role in As(III) tolerance: As(III) influx and cell cycle control**

Eukaryotic cells respond to various stress conditions by activating a family of serine/threonine kinases called MAPKs. It is well known that the mammalian p38 MAPK pathway plays a major role in As(III) tolerance by modulating gene expression via an AP-1 transcription factor (Elbirt et al., 1998; Verma et al., 2002). Interestingly, As(III) resistance of myeloma cell lines is associated with increased activation of the p38 pathway (Wen et al., 2008). We have shown that the MAPK Hog1p, a yeast homologue of p38, is phosphorylated in response to As(III) and Sb(III), while cells lacking Hog1p are highly sensitive to these metalloids (Thorsen et al., 2006). In contrast to rapid, robust and transient activation of Hog1p by osmotic stress (the main trigger of Hog1p activation), As(III) and Sb(III) cause a delayed and prolonged profile of Hog1p phosphorylation (Sotelo & Rodríguez-Gabriel, 2006; Thorsen et al., 2006). Importantly, Hog1p does not accumulate in the nucleus and does not affect transcription in As(III)-exposed cells (Thorsen et al., 2006), suggesting that it mediates tolerance through cytoplasmic targets. One such target is the aquglyceroporin Fps1p; Hog1p phosphorylates Fps1p and downregulates its transport activity, thereby reducing As(III) influx. Deletion of HOGL or expression of an Fps1p mutant lacking the MAPK phosphorylation site at Thr231 results in increased As(III) uptake and metalloid sensitivity (Thorsen et al., 2006). Hog1p may also influence Fps1p indirectly by modulating Rgc1p and Rgc2p. Rgc2p is phosphorylated in the presence of As(III) and this phosphorylation is partially Hog1p-dependent and epistasis analysis places RGC1 and RGC2 between FPS1 and HOG1 (Beese et al., 2009). Hence, Hog1p affects Fps1p activity in two ways: by directly phosphorylating Fps1p on Thr231 (Thorsen et al., 2006) and indirectly by downregulating the positive regulators of Fps1p activity (Beese et al., 2009).

Hog1p also plays a crucial role under stress conditions by controlling cell cycle progression. Under mild osmotic stress, Hog1p contributes to cell cycle delay in all phases by downregulating G1, S and G2 cyclins, by blocking the degradation of Sic1p, which is an inhibitor of S-phase cyclin-dependent kinase (CdK)–cyclin complexes, by delaying the origin of replication firing and by stabilizing the Swe1p kinase, which negatively regulates M-phase CdK–cyclin complexes (Escoté et al., 2004; Clotet et al., 2006; Yaakov et al., 2009). The role of Hog1p in cell cycle regulation is manifested by accelerated cell cycle progression in the hog1Δ mutant exposed to osmotic stress or permanent cell cycle arrest during sustained activation of Hog1p. Surprisingly, we found that the hog1Δ mutant is permanently arrested in G1 in the presence of As(III) (Migdal et al., 2008). This result suggests that Hog1p not only promotes cell cycle arrest but also the recovery from stress-induced cell cycle delay. Under prolonged As(III) exposure, G1-synchronized hog1Δ cells exhibit prolonged stabilization of the CDK-inhibitor Sic1p. Consistently, deletion of SIC1 in the hog1Δ background suppresses persistent G1 delay. Hence, Hog1p promotes recovery from As(III)-induced cell cycle arrest in G1 by inducing the degradation of Sic1p and releasing the activity of S-phase CdK–cyclin complexes. However, cells expressing a Sic1p mutant that cannot be phosphorylated by Hog1p (Sic1p–Thr173Ala) do not display a cell cycle recovery defect, suggesting that Hog1p indirectly regulates Sic1p stability in the presence of As(III) (Migdal et al., 2008).
Hog1p is also phosphorylated upon Cd exposure with a kinetics and magnitude that resembles its phosphorylation by As(III) (Bilsland et al., 2004), but specific targets through which Hog1p mediates Cd tolerance remain to be identified. Hog1p homologues in *C. albicans* (CaHog1) and *S. pombe* (Sty1/Spc1) are activated by As(III) and Cd (Smith et al., 2004; Rodriguez-Gabriel & Russell, 2005). Moreover, deletion of Hog1p homologues in *S. pombe* and the fungal pathogens *C. albicans* and *Candida lusitaniae* results in Cd and As(III) sensitivity (Toone et al., 1998; Smith et al., 2004; Rodriguez-Gabriel & Russell, 2005; Boisnard et al., 2008). In contrast to *S. cerevisiae*, Cd treatment in *C. albicans* triggers the osmostress-like response including rapid, but transient phosphorylation, followed by nuclear accumulation of CaHog1 and a major reprogramming of transcription (Smith et al., 2004; Enjalbert et al., 2006). Similarly, the response to As(III) and Cd in *S. pombe* requires the transcription factor Pap1, which is a downstream target of the MAPK Sty1/Spc1 (Toone et al., 1998; Rodriguez-Gabriel & Russell, 2005; Kennedy et al., 2008). However, the mechanisms of metal and metalloid tolerance induced by stress-activated MAP kinases have not yet been revealed in these organisms.

**Target of rapamycin (TOR) pathway and protein kinase A (PKA): regulation of general stress responses and ribosomal proteins**

The TOR pathway is a major signalling network that mediates temporal control of cell growth in eukaryotes. The *S. cerevisiae* TOR pathway consists of two branches formed by multiprotein complexes called TOR complex 1 (TORC1) and TOR complex 2 (TORC2). At the heart of each complex is a serine/threonine protein kinase (Tor1p and Tor2p), belonging to the phosphatidylinositol kinase-related kinase (PIKK) family. Under favourable growth conditions, TORC1 stimulates transcription, translation, ribosome biogenesis and nutrient uptake, and at the same time, inhibits protein degradation and autophagy as well as the transcription of specific sets of genes involved in stress and nutrient starvation responses. In contrast, TORC2 is specifically required for cell cycle-dependent polarization of the actin cytoskeleton, which is crucial for bud formation (Wullschleger et al., 2006; Soulard et al., 2009). The downstream effectors of TORC1 in the activation of ribosomal protein and ribosomal biogenesis gene transcription include positive regulation of the Sch9p kinase, the forkhead transcription factor Fhl1p and the zinc finger-containing transcription factor Sfp1p (Marion et al., 2004; Martin et al., 2004; Urban et al., 2007). In response to nitrogen starvation and stress, TORC1 signalling is inhibited, leading to down-regulation of protein synthesis, promotion of autophagy and activation of several stress-responsive transcription factors, including Msn2p and Msn4p (Wullschleger et al., 2006). The TOR pathway acts in concert with another major nutrient-responsive signal transduction pathway: the Ras/cyclic AMP (cAMP)/PKA pathway. PKA consists of two catalytic and two inhibitory subunits, and is activated by the presence of cAMP to regulate cell growth and responses to nutrients and stress. PKA controls a wide range of processes, including transcription, energy metabolism and cell cycle progression, and acts by modulating transcription factors, enzymes and other regulatory kinases (Santangelo, 2006). Recent findings implicate the TOR and PKA pathways in metal tolerance by modulating the expression of genes involved in the general stress response and protein synthesis (Hosiner et al., 2009).

Genome-wide expression analyses, coupled to searches for cis-regulatory elements in coregulated genes, implicated Msn2p and Msn4p in the transcriptional response to As(III) (Haugen et al., 2004; Thorsen et al., 2007; Kristiansson et al., 2009). These proteins are partially redundant transcriptional activators with Cys2–His2 zinc finger DNA-binding motifs, and they stimulate the expression of genes in response to many stress conditions. Activation of these ‘general stress responsive’ transcription factors involves translocation from the cytoplasm to the nucleus and binding to promoters containing the DNA sequence AAGGGG (Martínez-Pastor et al., 1996; Schmitt & McEntee, 1996; Görner et al., 1998; Gasch et al., 2000). The activity of Msn2p/Msn4p is regulated by growth conditions and the nutritional status of cells, and involves the PKA and TOR pathways (Görner et al., 1998; Beck & Hall, 1999). In response to As(III), Msn2p and Msn4p translocate to the nucleus and stimulate the expression of target genes. Interestingly, deletion of both Msn2 and Msn4 results in increased tolerance to As(III), whereas their overexpression enhances sensitivity (Hosiner et al., 2009). Based on these data, it was suggested that As(III) toxicity might be a consequence of chronic activation of stress-responsive genes via Msn2p/Msn4p (Hosiner et al., 2009). In line with this notion, hyperactivation of these proteins has a negative effect on cell growth (Durchschlag et al., 2004). Similarly, overexpression of Msn2 sensitizes yeast cells to MeHg, whereas Msn2 deletion slightly improves growth in the presence of this agent (Hwang et al., 2005).

Genome-wide expression and computational analyses also implicated Sfp1p, Fhl1p and Rap1p in controlling As(III)-repressed genes (Haugen et al., 2004; Thorsen et al., 2007; Kristiansson et al., 2009). These transcription factors principally regulate the expression of genes involved in ribosomal function. Sfp1p has been shown to be regulated in response to nutrients and stress by the kinases TOR and PKA (Marion et al., 2004; Hosiner et al., 2009). Sfp1p is localized to the nucleus, where it binds to target-gene promoters and promotes the expression of ribosomal proteins under optimal growth conditions. In response to
inhibition of TOR, changes in nutrient availability or stress conditions, Sfp1p is released from DNA and leaves the nucleus, which results in the downregulation of ribosomal protein-gene expression (Marion et al., 2004). Accordingly, As(III), but also Hg and Ni, inhibits TORC1 and reduces Sfp1p-regulated gene expression. Moreover, As(III) treatment leads to Sfp1p dephosphorylation, dissociation from chromatin and nuclear exit (Hosiner et al., 2009). Cells lacking SFP1 cannot downregulate ribosomal protein-gene expression in response to As(III). Curiously, while tor1Δ cells are As(III) sensitive, SFP1 deletion results in enhanced As(III) tolerance. Moreover, SFP1 overexpression sensitizes cells to As(III) (Hosiner et al., 2009). Currently, it is not completely clear how these apparently contradictory views on the role of Sfp1p and TORC1 can be reconciled into a coherent model.

## Concluding remarks

We have learned a great deal in recent years about how *S. cerevisiae* and other yeasts cope with toxic metals and metalloids. Nevertheless, we still lack a molecular insight into many aspects of metal biology. For example, genome-wide phenotype screens and molecular work pinpointed many proteins that protect cells from metal toxicity; yet, the exact mechanism(s) by which these proteins mediate tolerance are largely elusive. We know very little about how metals activate transcription factors and signalling proteins, and how these proteins in turn activate their gene/protein targets. We also know little about the post-translational regulation of various transporters involved in metal tolerance. Importantly, how these proteins interact in space and time as they orchestrate the cell’s response to metals on a whole-cell or systems level has remained largely unexplored. Other aspects of metal biology that have received limited attention is whether cells respond differently under acute and chronic exposure and whether colonies use strategies that are distinct from those used by cells in (liquid) culture. Finally, the insight into how metal tolerance systems have evolved is only starting to emerge. We believe that *S. cerevisiae* is an excellent model system for exploring these fundamental aspects of metal biology; many large-scale techniques and knock-out/overexpression collections are available for identifying toxicity targets and tolerance systems, yeast is easily used as a heterologous host to study proteins from other organisms and yeast is a front-runner when it comes to the availability of (novel) systems biology approaches to unveil fundamental mechanisms on a whole-cell and/or a single-cell level. Because many mechanisms involved in metal toxicity and detoxification appear to be conserved in various eukaryotic organisms, such work in yeast may prove of value for identifying similar mechanisms in other organisms and have important implications for the use of metals in medical therapy.

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Yeast metal tolerance


