INSTITUTIONEN FÖR KEMI OCH MOLEKYLÄRBIOLOGI

Cellular Responses to Arsenite and Cadmium - Mechanisms of Toxicity and Defense in *Saccharomyces cerevisiae*

Therese Jacobson

Institutionen för kemi och molekylärbiologi

Naturvetenskapliga fakulteten

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Cellular Responses to Arsenite and Cadmium - Mechanisms of Toxicity and Defense in *Saccharomyces cerevisiae*

Doctoral thesis

Department of Chemistry and Molecular Biology
University of Gothenburg
Box 462, SE-405 30 Göteborg, Sweden

Cover picture:
Top: *S. cerevisiae* under arsenite stress. Left: Hsp104-GFP foci, right: Bright field.
Bottom left: Growth curves of *S. cerevisiae* affected by arsenite.
Bottom right: Glutathione rescue of *gsh1Δ* mutant cells.

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FSC

Men mamma måste du räkna celler?
Kan du inte räkna Pokémons istället?
- Robin

Mamma är biolog. Hon jobbar med gift.
-Pontus

Populärvetenskaplig sammanfattning

Arsenik och kadmium är två giftiga grundämnen som finns naturligt i berggrunden. Arsenik förekommer i höga koncentrationer på vissa ställen i världen och kontaminerar grundvattnet, vilket leder till att lokalbefolkningen ständigt utsätts för arsenik. Vissa grödor, särskilt ris tar upp arsenik mycket effektiv eftersom proteinerna på rötterna inte kan skilja på arsenik och det livsnödvändiga grundämnet fosfor. Tack vare sina giftiga egenskaper används arsenik som aktiv beståndsdel i läkemedel som används mot specifika blodcancer- och infektionssjukdomar. Kadmium sprids i naturen främst genom konstgödning och avfall från elektronikindustrin, och tas upp i kroppen bl.a. genom föda och cigarettrök. För att kartlägga grundläggande toxicitetsmekanismer hos arsenik och kadmium, och för att förstå hur celler försvarar sig mot dessa toxiska ämnen, har vi studerat hur jästsvampen *Saccharomyces cerevisiae* reagerar på exponering. Dessa studier har resulterat i en rad intressanta upptäkter.

Vi har identifierat en försvarsmekanism där cellerna utsöndrar molekylen glutation som består av tre aminosyror och som binder till arsenik utanför cellerna. Genom denna bindning hindras arseniken från att tas upp i cellerna. Vi har också utvecklat en matematisk modell som beskriver hur olika proteiner bidrar till cellens respons och försvar mot arsenik. Med hjälp av denna modell har vi kunnat uppskatta att arsenik som ändå tar sig in i cellerna primärt binder till olika proteiner, men att arseniken över tid skiftar till att istället binda till intracellulärt glutation. Vidare har vi kunnat konstatera att både arsenik och kadmium bidrar till att proteiner i jästcellerna bildar aggregat, dvs. att proteinerna klumpar ihop sig. Proteinaggregering är negativt för cellerna och många neurodegenerativa sjukdomar hos människor såsom Alzheimers och Parkinsons är kopplat till proteinaggregering. Arsenik och kadmium inducerar proteinaggregering både genom liknande och olika mekanismer. Arsenik hindrar funktionaliteten hos chaperoner, en sorts hjälpproteiner som har till uppgift att hjälpa nybildade proteiner att anta sin rätta tredimensionella form. Om nybildade proteiner inte antar sin rätta form är sannolikheten stor att de klumpar ihop sig med andra proteiner och bildar aggregat. Kadmium påverkar inte chaperoner, utan påverkar en specifik grupp av proteiner, nämligen zink-bindande proteiner. Zinkjoner är viktiga för att vissa proteiner skall anta sin rätta tredimensionella form, och kadmium tycks ersätta zink i dessa proteiner och därmed leda till aggregering. Vi har i detta arbete således identifierat både cellulära toxicitets- och försvarsmekanismer.

Abstract

All biological systems have to cope with a wide range of metals that are present in the environment. Metals can be essential or beneficial for life, inert or non-essential and toxic, often depending on their chemical form and concentration. Most organisms have evolved defense mechanisms in order to deal with toxic metals. The toxic effect of a certain metal depends on cellular uptake, the mode of action inside the cell, on the efficacy of cellular defense systems, and on the intracellular localization of the compound. In this thesis, the main focus has been to investigate toxicity mechanisms and cellular responses to arsenite and cadmium. Arsenite is a trivalent, abundant and highly toxic form of arsenic found in nature and used in medical therapy. Cadmium is a heavy metal that has been used e.g. in paint, batteries and electronic industry with an increasing use during the industrialization. As a biological model system the budding yeast *Saccharomyces cerevisiae* has been used in this study, since it is a powerful and versatile tool to uncover fundamental traits in eukaryote cells.

First we identified a novel extracellular defense mechanism to arsenite; yeast cells export glutathione that chelates arsenite in the extracellular environment and prevents arsenite from entering the cell. We next measured intracellular arsenic content in a variety of mutants and used the data to create a mathematical model. This model predicted the role and contribution of different proteins in the cellular response to arsenite, and predicted that intracellular arsenite is mainly protein-bound upon acute exposure, while the main intracellular pool of arsenite after chronic exposure is bound to glutathione. Finally, we found a novel mode of action of arsenite and cadmium, namely the induction of widespread protein aggregation. We show that both arsenite and cadmium target newly synthesized proteins for aggregation. Arsenite also affected chaperone activity *in vivo*. Cadmium does not seem to inhibit chaperone activity *in vivo*. Instead, displacement of zinc in proteins seems to play an important role in the induction of protein aggregation upon cadmium exposure. Proteasomal degradation is involved in the clearance of protein aggregates induced upon arsenite and cadmium exposure.
Thus, we have provided new insights regarding both mechanisms of toxicity and defense.

Keywords: arsenic, arsenite, cadmium, glutathione, extracellular defense, protein aggregation, chaperones, proteasome, *Saccharomyces cerevisiae*.

List of papers

I Michael Thorsen*, Therese Jacobson*, Riet Vooijs, Clara Navarrete, Tijs Bliek, Henk Schat, Markus J. Tamás
*Equal contribution
"Glutathione serves an extracellular defence function to decrease arsenite accumulation and toxicity in yeast"
Molecular Microbiology 2012, 84(6), 1177–1188
doi:10.1111/j.1365-2958.2012.08085.x

II Soheil Rastgou Talemi, Therese Jacobson, Vijay Garla, Clara Navarrete, Annemarie Wagner, Markus J. Tamás, Jörg Schaber
"Mathematical modelling of arsenic transport, distribution and detoxification processes in yeast"
Molecular Microbiology 2014, 92(6), 1343–1356
doi:10.1111/mmi.12631

III Therese Jacobson*, Clara Navarrete*, Sandeep K. Sharma, Theodora C. Sideri, Sebastian Ibstedt, Smriti Priya, Chris M. Grant, Philipp Christen, Pierre Goloubinoff, Markus J. Tamás
*Equal contribution
"Arsenite interferes with protein folding and triggers formation of protein aggregates in yeast"
Journal of Cell Science 2012, 125(21): 5073–5083
doi: 10.1242/jcs.107029

IV Therese Jacobson, Robbe Thange, Arghavan Assouri, Markus J. Tamás
"Cadmium causes misfolding and aggregation of cytosolic proteins in yeast"
Manuscript.

Paper contributions

Paper I-III

I performed the major part of the experimental work and contributed to a minor part to the writing of the manuscripts.

Paper IV

I performed the major part of the experimental work and wrote the major part of the manuscript.

Papers not included

Markus J. Tamás, Sandeep K. Sharma, Sebastian Ibstedt, Therese Jacobson and Philipp Christen
"Heavy Metals and Metalloids As a Cause for Protein Misfolding and Aggregation"
Biomolecules 2014, 4(1): 252-267
doi:10.3390/biom4010252

Elzbieta Petelenz-Kurdziel, Clemens Kuehn, Bodil Nordlander, Dagmara Klein, Kuk-Ki Hong, Therese Jacobson, Peter Dahl, Jörg Schaber, Jens Nielsen, Stefan Hohmann, Edda Klipp
"Quantitative Analysis of Glycerol Accumulation, Glycolysis and Growth under Hyper Osmotic Stress"
PLoS Computational Biology 9(6): e1003084
doi:10.1371/journal.pcbi.1003084

Table of contents

1 Preface

We live longer lives and develop more diseases compared to earlier generations. Industry, technology, agriculture, food additives, flame retardants… Our daily exposure to chemicals increases and the incidence of cancer and neurodegenerative diseases follows the same trend. Limit doses for different compounds are stated by authorities, but a chronic exposure to even low levels of certain compounds such as toxic metals will result in an accumulation in our tissues over time if there is no efficient extrusion pathway.

Metals occur naturally in the earth´s crust. Some metals that are essential or beneficial for life can be toxic in overdoses, some metals are inert and some are toxic. Toxic metals can affect cells in both common and distinct ways, causing the cells to react differently. Differences and similarities of the toxic properties of specific metals can be compared in order to add more details to the understanding of each metal. In our studies we have focused on arsenic and cadmium; two toxic metals that are both affecting the health of humans and animals and that are interfering with plants and microbes through a variety of modes of action at a cellular level. Since these elements are ubiquitous in nature, living organisms have evolved systems to handle their presence. Even though many people only relate to arsenic as a substance used for committing homicide during past times, the research on arsenic toxicity is pre-eminently up-to-date; arsenic today is a threat to tens of millions of people that are exposed to arsenic through the alimentary chain. At the same time, a potential for the use of arsenical compounds as a pharmaceutical tool can be seen.

2 Metals and toxicity

Metals and metalloids have been known for long time and are used in a lot of tools and technology applications in our daily life. They can be stored in the body over time and exposure from food and drinking water is the main source of metal intoxication. Metalloids (also known as half-metals) are chemical elements with properties of both metals and non-metals. There is no precise definition for what elements should classify as metalloids but the elements most commonly recognized as metalloids are arsenic, antimony, silicon, germanium, tellurium and boron.

2.1 *Saccharomyces cerevisiae* as a model organism

To better understand metal toxicity, we have studied how arsenic and cadmium affects living cells. Fundamental cellular mechanisms of replication, recombination, cell division and metabolism are highly conserved from yeast to higher eukaryotes, including mammals. The budding yeast *Saccharomyces cerevisiae* is commonly used in brewing, baking and wine fermentation, in biotechnology and in research as a model organism. We use *S. cerevisiae* in our studies since yeast cells grow rapidly, exist in both a haploid and a diploid version, have a fully sequenced genome and are easy to manipulate genetically. They are easy to grow, store and are harmless to humans. *S. cerevisiae* lives naturally on sugar-containing substrates as flowers and fruits and are very resistant to variations in pH, temperature and osmolarity. All organisms have evolved systems to react and adapt to changes in their environment, for microorganisms these adaptations are extremely important since their mobility is limited or absent.

2.2 Arsenic: occurrence and sources of exposure

Arsenic is a toxic element, a metalloid with semi-metallic properties naturally occurring as a component in a wide variety of minerals in the Earth´s crust. Arsenic can be found in many oxidation states in nature, depending on the pH and redox state of the surrounding environment. Two abundantly occurring forms are the trivalent arsenite and the pentavalent arsenate, of which the earlier is the more reactive form. Arsenic can leak into the groundwater through different geochemical and microbial processes (Garelick *et al.*, 2008). Raise of geothermal fluids is one

source of arsenic contamination, and rocks of volcanic origin often contain higher amounts of arsenic compared to sedimental rocks. In regions where the arsenic load in the bedrock is high, arsenic-contaminated drinking water from tube-wells is a huge problem. More than 40 millions of people worldwide are estimated to be exposed to arsenic contaminated drinking water. Bangladesh is the worst affected country, West Bengal in India and parts of China are other regions where drinking water contaminated with arsenic from natural sources is affecting human health of tens of millions of people (Meharg, 2004). Technology to filter arsenic from drinking water exist, but the maintenance of the filtering devices is often lacking and many water sources therefore provides contaminated water (Bhattacharjee, 2007). Also a rice-based diet can contribute to arsenic poisoning. There are differences in arsenic uptake into the rice grain depending on the rice variety and on the cultivation technique; anyhow rice can assimilate up to ten times more arsenic compared to other crops, due to the anaerobic microenvironment created by the flooded growth conditions. The arsenic content in the rice grain can be reduced if boiling the rice in an abundant volume of clean water (Raab *et al.*, 2009). The tendency of some plants to hyper-accumulate arsenic through the roots can also be used to clean polluted soils; this is known as phytoremediation (Hettick *et al.*, 2015)

Anthropogenic sources of arsenic surface-pollution arise mainly from mining, since coal and minerals with precious metals often contain arsenic as well. Arsenic is also enriched in areas where industrial or agricultural activities have left polluted areas (e.g. pesticides, wood-preservative) (Garelick *et al.*, 2008). Chronic exposure to arsenic leads to arsenic poisoning (arsenicosis) where skin pigmentation and lesions are early signs. Cancer in mainly skin, lung, kidney and bladder are among many diseases associated with long-term chronic arsenic exposure (Bhattacharjee, 2007). Also diabetes has an increased incidence in populations exposed to arsenic (Tseng *et al.*, 2002), this is possibly caused by arsenite-dependent inhibition of glucose uptake through hexose permeases (Liu *et al.*, 2004).

2.3 Arsenic as a pharmaceutical tool

A variety of arsenic-containing mixtures such as Fowler´s solution has historically been used to treat diseases like asthma, cholera, syphilis, skin diseases and cancer both in the western world and in Chinese traditional medicine. Today a revival of arsenic as a drug can be seen; the areas of arsenic use in medical treatment are

mainly relapsed APL (acute promyelocytic leukemia) and HAT (humane African trypanosomiasis), and is foreseen as a potential treatment in other hematologic diseases (Bouteille *et al.*, 2003; Chen *et al.*, 2011).

Hence, arsenic is a human carcinogen, but is also used as a drug against certain variants of cancer due to its toxicity. This dual aspect of the cellular responses to arsenic makes it a very interesting area of research. Better understanding of the mechanisms of toxicity could provide knowledge on how to design more efficient arsenic-based drugs with reduced negative side-effects. Resistance-development is another issue in treatments with arsenic-containing compounds (Gourbal *et al.*, 2004). Hence, a better understanding of cellular defense functions that increases the tolerance for arsenic would potentially enable a more efficient drug design, for treatments of both cancer and protozoan infections.

The two inorganic forms that are most relevant for living systems are the pentavalent arsenate and the trivalent arsenite. In my research, I have solely used the trivalent form since it is the more toxic form and also the form of arsenic used in pharmaceutical drugs; it is hence the more interesting form to gain more knowledge about.

2.4 Arsenite toxicity

A broad range of toxicity mechanisms have been identified for arsenic, where individual chemical species of arsenic have specific modes of action. Depending on the oxidation state of arsenic, it has different preferential binding partners in biomolecules; oxygen in its higher oxidation state and sulphur in its lower oxidation state (Summers, 2009).

The toxicity of the pentavalent arsenate is mainly correlated to its structural similarity to phosphate and therefore ability to mimic phosphate in different cellular mechanisms (Del Razo *et al.*, 2001). Arsenate interferes with DNA synthesis and inhibits oxidative phosphorylation, e.g. uncouples the ATP production hence disturbing the energy production in cells (Wysocki & Tamás, 2011).

Trivalent arsenite binds to proteins and can hence interfere with their activity and function (Aposhian & Aposhian, 2006; Hughes *et al.*, 2011; Kitchin & Wallace, 2008). Arsenite also has damaging effects on DNA (Shi *et al.*, 2004). Another

effect observed for arsenite is its influence on the cell cycle progression; arsenite-exposed cells arrest in all phases of the cell cycle. It has been shown that the mitogen activated protein kinase (MAPK) Hog1 in yeast is important for the exit from arsenite-induced G_1 arrest (Migdal *et al.*, 2008) and that Hog1 is phosphorylated upon arsenite exposure (Thorsen *et al.*, 2006).

We have identified a novel toxicity mechanism for arsenite in the interference with proper protein folding by targeting nascent polypeptides and chaperones *in vivo* (**paper III**).

2.5 Cadmium: applications and sources of exposure

Cadmium is a toxic element that is ubiquitously present as an environmental pollutant. Cadmium is a xenobiotic transition metal that has no role in biology, the only documented case of cadmium use in biological systems is from the marine diatome *Thalassiosira weissflogii*, where it could replace zinc in carbonic anhydrases (Lane & Morel, 2000). The content of cadmium in the earth´s crust is between 0.1-0.5 ppm, which is low and cadmium can be considered as a rare metal, but the metal becomes steadily more used in chemical and technological industry and hence the anthropogenic availability of cadmium increases. Historically cadmium was used as an anticorrosive agent and as a coloring agent in paint. The largest use of cadmium today is in rechargeable batteries, in solar cells and in different alloys (Godt *et al.*, 2006). Cadmium has neurotoxic effects (Wang & Du, 2013) and is classified as a human carcinogen by the International Agency for Research on Cancer (http://www.iarc.fr) and occupational cadmium exposure is associated with cancers in several organs (Byrne *et al.*, 2013; Hartwig, 2010; Khlifi & Hamza-Chaffai, 2010). The anthropogenic exposure poses a big threat to human health and the most common uptake route is through the food chain and cigarette smoke (Hecht *et al.*, 2013; Satarug & Moore, 2004; Thevenod & Lee, 2013) . The concentration of cadmium is generally lower in tobacco than in food, but the lungs absorb cadmium very efficiently. Cadmium-contaminated drinking water is a common source of exposure, especially in developing countries. As is the case with arsenic, rice plants efficiently accumulate cadmium from the soil. The use of fertilizers from sewage sludge and commercial fertilizers results in accumulation of cadmium in crop fields and hence in food and feed (Maret & Moulis, 2013).

2.6 Cadmium toxicity

The toxic effects of cadmium are linked to more than one molecular mechanism. Divalent cadmium ions are similar to calcium- and zinc ions in terms of size and charge (Choong *et al.*, 2014; Maret & Li, 2009; Maret & Moulis, 2013; Zhou *et al.*, 2015) and can therefore displace these ions from metalloproteins (Faller *et al.*, 2005; Kozlowski *et al.*, 2014). Cadmium has a high affinity for thiol groups and can interfere with protein function through binding to cysteine residues (Helbig *et al.*, 2008; Maret & Li, 2009; Martin, 1987; Stohs & Bagchi, 1995). Cadmium exposure leads to indirect induction of reactive oxygen species (ROS) (Hartwig, 2013). A mutagenic effect is observed for cadmium which is not due to direct interaction with DNA but rather to inhibition of DNA repair systems (Jin *et al.*, 2003; Serero *et al.*, 2008), reviewed in (Bertin & Averbeck, 2006; Giaginis *et al.*, 2006; Hartwig, 2013). In this thesis, we demonstrate that cadmium induces widespread protein aggregation in yeast cells (**paper IV**).

3 Metal uptake and transport

3.1 Arsenite uptake

Toxicity of arsenite is linked to uptake into the cell. The entry routes for arsenic into the cells depend on the oxidation state of the metalloid. The pentavalent form arsenate can enter most cells, including yeast cells and mammalian cells, through phosphate transporters due to structural similarity between the arsenate oxyanion and inorganic phosphate (Wysocki & Tamás, 2010). In yeast, pentavalent arsenate can use the high affinity transporter of inorganic phosphate Pho84 to enter the cell (Bun-ya *et al.*, 1996) (Fig. 2). Trivalent arsenite in the form of As(OH)3 enters the yeast cell through the aquaglyceroporin Fps1 (Fig. 2) (Wysocki *et al*., 2001) due to structural mimicking of glycerol (Fig. 1). Aquaglyceroporins from several organisms, including bacteria (Meng *et al*., 2004), *Leishmania* (Gourbal *et al*., 2004), plants (Bienert *et al*., 2008), mammals (Liu *et al*., 2002), and humans (Gourbal *et al*., 2004) have been shown to mediate arsenite uptake. As the aquaglyceroporins are membrane channels, the transport is bidirectional, and driven by differences in the concentration gradient (Maciaszczyk-Dziubinska *et al*., 2010). In the absence of glucose, Fps1 accounts for only about 20% of the arsenite uptake into the yeast cell, while hexose permeases mediates the major part of the uptake. It has been proposed that three arsenite ($As(OH)_3$) molecules together can form a ring-structure which is recognized as a substrate by the hexose transporters (Fig. 1). At high concentrations, arsenite can work as a competitive inhibitor of glucose and reduce glucose uptake into yeast cells. In the case of human hexose permeases it is likely that arsenite causes an irreversible inhibition of glucose uptake through binding (Liu *et al*., 2004).

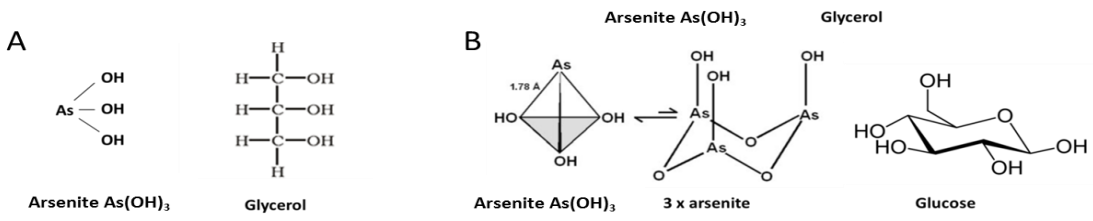

Figure 1. Arsenite mimics glycerol and hexose molecules. A) Arsenite can enter the yeast cell through aquaglyceroporins by mimicking glycerol. B) Three arsenite molecules can form a ring-structure that resembles a glucose molecule, and hence enter the yeast cell through hexose transporters. Figure modified from (Liu *et al.*, 2004).

3.2 Cellular strategies to decrease cytosolic arsenite

Diminished uptake of toxic compounds is a defense strategy often used by microorganisms. Upon arsenite exposure, the main arsenite entrance protein Fps1 (Fig.2) is phosphorylated and this phosphorylation down-regulates the transport through the protein. This down-regulation of the arsenite influx through Fps1 is linked to the presence of the mitogen activated protein kinase (MAPK) Hog1 that phosphorylates Fps1. Hog1 is itself phosphorylated and activated in response to arsenite. Deletion of *HOG1* decreases the phosphorylation of Fps1 and hence increases its transport activity (**paper II**), (Thorsen *et al.*, 2006).

The main arsenite detoxification mechanism in yeast is extrusion from the cytoplasm via the plasma membrane antiporter Acr3 (Fig. 2) (Ghosh *et al.*, 1999; Wysocki *et al.*, 1997). Acr3 transports arsenic in the form of $As(OH)_2O^-$ (Maciaszczyk-Dziubinska *et al.*, 2011). There is no pump for extrusion of arsenate, but the resistance through extrusion is extended to include also the pentavalent form through the arsenate reductase Acr2 (Bobrowicz *et al.*, 1997). Acr2 reduces arsenate to arsenite that can then be extruded via Acr3 (Fig. 2).

In yeast, about 500 genes in total are up-regulated upon arsenic exposure (Thorsen *et al.*, 2007). The expression of the proteins involved in arsenic detoxification in *S. cerevisiae* is regulated by various transcription factors. Two AP-1–like transcription factors, Yap1 and Yap8, regulate tolerance by activating expression of separate subsets of detoxification genes (Wysocki *et al.*, 2004). Yap8 transcribes the two resistance genes *ACR2* and *ACR3* (also known as *ARR2* and *ARR3*) encoding the arsenite extrusion pump Acr3 and the arsenate reductase Acr2 from the same promoter but in opposite directions. Yap8 has been shown to be associated to the promoter both upon arsenite exposure and in unstressed growth conditions (Wysocki *et al.*, 2004). The activation of Yap8 upon arsenic exposure is mediated by a conformational change due to direct binding between arsenite and Yap8 (Kumar *et al.*, 2015). Over expression of *ACR3* confers stronger resistance to both arsenite and arsenate, this indicate that Acr3, and not Acr2 or Yap8, is rate-limiting for arsenic resistance (Ghosh *et al.*, 1999).

Another pathway for cells to decrease the cytosolic arsenite is through chelation with glutathione and vacuolar sequestration via the Ycf1 transporter (discussed in chapter 4.4). In **paper I** we identify export of glutathione and extracellular chelation of arsenite as a novel detoxification mechanism.

In **paper II** we investigate the role and contribution of different transporters and their regulation upon arsenite exposure. We show that Fps1 is regulated in both a Hog1-dependent and a Hog1-independent way, and that the vacuolar sequestration via Ycf1 is of major importance only when Acr3 is absent.

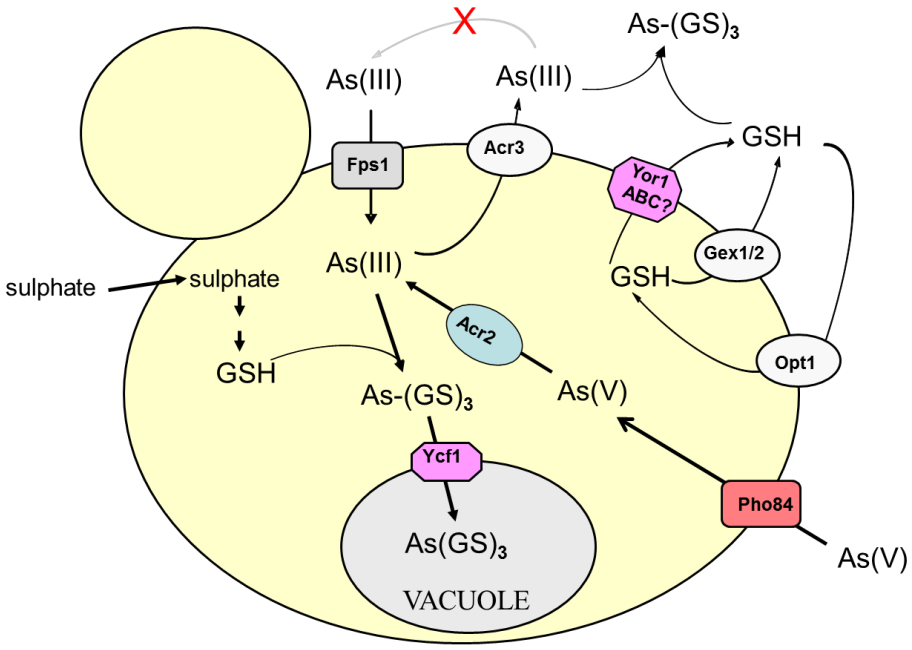

Figure 2. Arsenic uptake and detoxification pathways. Pentavalent arsenate enters the cell through phosphate transporters. Trivalent arsenite can enter the yeast cell through the aquaglyceroporin Fps1. Intracellular GSH can bind to arsenite and form an $As(GS)_3$ complex, that can be sequestered into the vacuole via the ABC-transporter Ycf1. GSH can be secreted through plasma membrane ABC-transporters like Yor1, and through Gex1/2. Opt1 mediates reuptake of extracellular GSH during unstressed conditions. Upon arsenite exposure, the biosynthesis of GSH increases and GSH is accumulated in the extracellular environment. Acr3 is an arsenite extrusion pump, and extracellular arsenite can form the $As(GS)_3$ complex with exported GSH, which blocks the (re)entrance of the metalloid.

3.3 Cadmium uptake

The uptake of cadmium ions into yeast cells is mediated by the same transporters as for essential cations like Zn^{2+}, Ca^{2+}, Fe^{2+}. Zrt1is a high affinity zinc ion transporter (Eng *et al.*, 1998; Zhao & Eide, 1996) responsible for a major part of the cadmium uptake into yeast cells (Gitan *et al.*, 2003; Gomes *et al.*, 2002). Zrt1 is ubiquitylated upon exposure to high levels of cadmium and zinc and hence removed from the

plasma membrane by endocytosis to limit the entrance (Gitan & Eide, 2000; Gitan *et al.*, 1998; Gitan *et al.*, 2003).

An alternative entry route for cadmium in yeast cells is through the plasma membrane transporter Smf1 and the related Smf2. These transporters have high affinity for manganese but also a broad specificity for other metal ions, including cadmium (Chen *et al.*, 1999; Liu *et al.*, 1997; Sacher *et al.*, 2001; Supek *et al.*, 1996). The over-expression of *SMF1* or *SMF2* results in increased cadmium sensitivity (Ruotolo *et al.*, 2008).
The low affinity Fe^{2+}-transporter Fet4 can also mediate uptake of zinc (Dix *et al.*, 1997; Waters & Eide, 2002). The transport activity of Fet4 is inhibited by cadmium (Dix *et al.*, 1994) and mutations resulting in higher *FET4* mRNA levels also mediates cadmium sensitivity (Jensen & Culotta, 2002). Hence, it is possible that Fet4 is involved in cadmium uptake into yeast cells.

Since cadmium is similar to calcium regarding size and charge, it is plausible that cadmium could enter cells through calcium channels. In fact, it has been observed in certain mammalian cell lines that cadmium tolerance can be increased by the addition of different inhibitors of calcium channels (Choong *et al.*, 2014). Mid1 is a Ca^{2+} channel in yeast that is stretch-activated, and has been shown to constitute yet another entry pathway for cadmium (Gardarin *et al.*, 2010).

3.4 Cadmium export

Pca1 is the main efflux transporter for cadmium in *S. cerevisiae*, but in most laboratory strains a Gly970Arg mutation in a conserved ATP-binding pocket renders the Pca1 nonfunctional (Adle *et al.*, 2007). The plasma membrane multidrug exporter Yor1 is probably involved in cadmium export since the *YOR1* deletion increases cadmium sensitivity (Cui *et al.*, 1996; Nagy *et al.*, 2006). The cytosolic levels of cadmium can also be decreased by vacuolar sequestration of glutathione-conjugated cadmium via the Ycf1 transport protein (discussed in chapter 4.4).

4 Cellular responses to metals

4.1 Redox balance and oxidative stress

The cytosol is normally a reducing environment and cells have evolved strategies to maintain the redox balance. Different forms of reactive oxygen species are collected under the abbreviation ROS. ROS are highly reactive and could potentially create extensive damage to proteins, lipids and other macromolecules in the cell. They are produced during normal aerobic metabolism and small variations in cellular redox balance can modify the activity of signaling molecules that affects processes such as differentiation, proliferation and apoptosis. Since ROS are formed during basal metabolism and growth, all cells have developed systems for their detoxification. The superoxide dismutase catalyses the reaction from superoxide anion (O_2^-) into hydrogen peroxide and oxygen (Drose & Brandt, 2012). The cytoplasmic superoxide dismutase is encoded by the *SOD1* gene, and the mitochondrial superoxide dismutase is encoded by the *SOD2* gene. The *CTA1* and the *CTT1* genes encode catalase, that catalyses the degradation of hydrogen peroxide (H_2O_2) to water and oxygen (Jamieson, 1998). When the efficacy of the detoxification systems is not sufficient compared to the amount of ROS generated, the cell will suffer so-called oxidative stress. The oxidative stress condition alters the redox balance in the cell, and glutathione is an important antioxidant that could counteract this stress. Glutathione can also chelate toxic compounds, bind to broken disulphide bridges on proteins (known as S-glutathionylation) and protect those from irreversible oxidative damage (Wysocki & Tamás, 2010).

4.2 Glutathione

Glutathione (GSH) is an essential tripeptide that plays an important role in redox regulation, the cellular response to oxidative stress and in metal detoxification. Glutathione is formed by three amino acids; glutamic acid, cysteine and glycine. Glutathione is synthesized in two steps involving different ATP-dependent enzymes. *GSH1* encodes the gamma glutamylcysteine synthetase (Grant *et al.*, 1997). Gsh1 catalyzes the formation of an unusual peptide bond between the amino group on the cysteine and the carboxy group on the carbon in position gamma (and

not on alpha as in an ordinary peptide bond) in the side chain of the glutamic acid (Fig. 3).

The next step is catalyzed by the glutathione synthetase, encoded by the *GSH2* gene, and consists of an ATP-dependent formation of a regular peptide bond between the cysteine in the formed dipeptide and glycine. The unusual peptide bond in the glutathione peptide makes the molecule more resistant to peptidase activity in the cell. The glutathione has a sulfhydryl group (-SH) on the cysteinyl residue, which is the part of the molecule with strong electron-donating properties and therefore makes the molecule act as a reductant (Fig. 3) (Snoke *et al.*, 1953).

Glutathione is essential for yeast cells and *gsh1Δ* cells that are unable to synthesize glutathione cannot proliferate unless glutathione is provided exogenously (Grant *et al.*, 1996b). Cells unable to synthesize glutathione are sensitized to arsenite exposure (Preveral *et al.*, 2006).

Availability of the three composing amino acids is important for the rate of glutathione synthesis, and it has been observed that over expression of the enzymes encoded by the *CYS3* and *CYS4* genes, involved in the cysteine biosynthesis pathway, can increase the amount of produced glutathione (Orumets *et al.*, 2012).

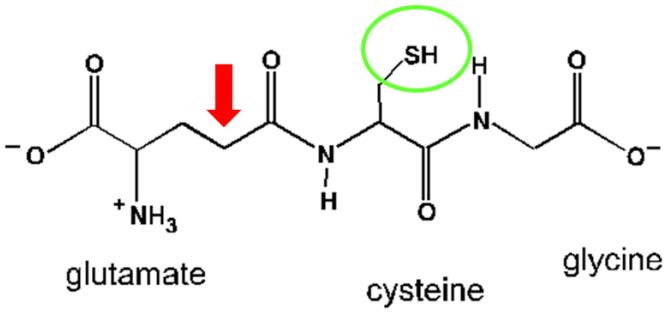

Figure 3. Structure of glutathione. Glutathione (GSH) is formed with an unusual peptide bond between the amino-group on the cysteine and the carbon in position gamma on the glutamic acid (arrow). The sulfhydryl group on the cysteinyl residue (circle) confers the electron-donating properties to the molecule.

Glutathione-S transferases are enzymes that catalyze the conjugation between glutathione and different substrates (Fig. 4). Some glutathione-S transferases also possesses peroxidase activity, and can reduce hydroperoxides such as hydrogen peroxide and lipid hydroperoxides in a GSH dependent manner (Pompella *et al.*,

2003). When reducing other molecules, glutathione itself becomes oxidized, and two molecules will form a glutathiol (GSSG), with a disulfide bond between the oxidized sulfur groups on each cysteinyl residue. The GSH/GSSG redox couple is an important system for the redox balance in the yeast cell (Grant, 2001). Oxidized GSSG can be reduced to GSH by the glutathione reductase enzyme Glr1 in a NADPH-dependent reaction (Fig. 4) (Grant *et al.*, 1996a; Pompella *et al.*, 2003) Glutaredoxins (Fig. 4) and thioredoxins are small oxidoreductases involved in the defense against oxidative stress through reduction and repair of damaged proteins (Grant, 2001). Yeast contains two genes encoding for thioredoxins (*TRX1*, *TRX2*) and two genes encoding for glutaredoxins (*GRX1*, *GRX2*). Thioredoxins and glutaredoxins contain two functional cysteine residues in their active site (Holmgren, 1989). While reducing other proteins, thioredoxins and glutaredoxins become oxidized and form an intramolecular disulphide. Glutaredoxin is reduced directly by glutathione while thioredoxin is reduced by thioredoxin reductase in an NADPH-dependent reaction (Grant, 2001).

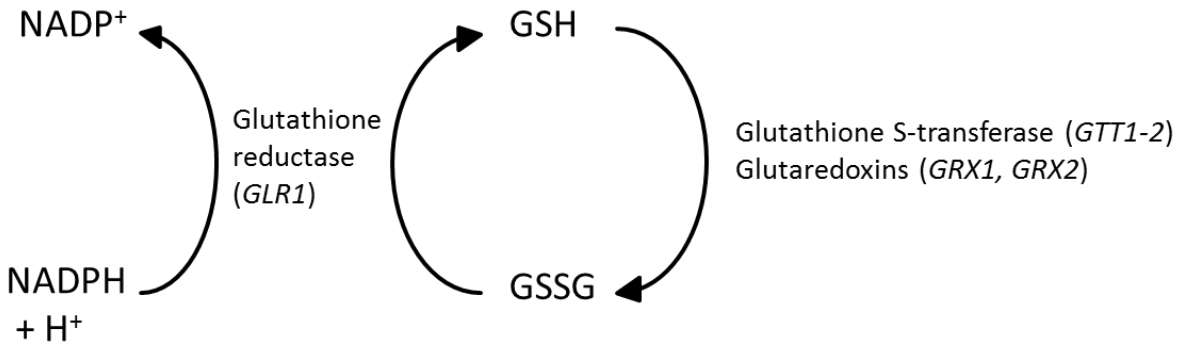

Fig. 4. The redox balance in the cell affects the pools of oxidized and reduced glutathione.

4.3 Redox regulation and glutathione upon metal exposure

Yap1 transcription factor regulates expression of genes involved in the oxidative stress response (Kuge & Jones, 1994; Schnell *et al.*, 1992; Wysocki & Tamás, 2010). Yap1 transcription factor up-regulates the expression of about 70 genes upon arsenite exposure; these genes encode antioxidant defense functions and functions in sulfur metabolism and glutathione biosynthesis. Arsenite and cadmium activates the Yap1 transcription factor, and the *yap1Δ* mutant is sensitive to both

metal(oid)s (Hirata *et al.*, 1994; Thorsen *et al.*, 2007). Expression of the *GSH1* gene is controlled by both Yap1 and Met4 transcription factors (Stephen *et al.*, 1995; Thorsen *et al.*, 2007; Wheeler *et al.*, 2003; Wysocki *et al.*, 2004). Yap1 also regulates many ABC transporters, including the *YCF1* gene (see below).

Yeast cells strongly increase the synthesis of glutathione upon exposure to both arsenite and cadmium. To enable this strong increase in glutathione synthesis, the yeast cells channel their sulphur metabolism mainly into this pathway instead of into protein biosynthesis (Lafaye *et al.*, 2005; Thorsen *et al.*, 2007). Upon cadmium exposure, cells also trigger the sulphur-sparing response (Fauchon *et al.*, 2002), where sulphur-rich enzymes involved in carbohydrate metabolism are repressed and isoenzymes with lower content of sulphur-containing amino acids are induced. Met4 transcription factor regulates genes involved in the sulfur metabolism and glutathione synthesis and regulates this isoenzyme switch in the sulphur-sparing program (Fauchon *et al.*, 2002; Lagniel *et al.*, 2002).

4.4 Glutathione chelation and vacuolar sequestration

Glutathione can chelate toxic compounds and can form a complex with trivalent arsenite in the ratio 3:1, annotated as the $As(GS)_3$ complex. Glutathione can also bind to the divalent cadmium ion in the ratio 2:1, forming $Cd(GS)_2$. The formation of these complexes occurs spontaneously in the cytosol and does not require any glutathione S-transferase or glutathione-dependent oxidoreductase (Rai & Cooper, 2005). The complexes are substrates for the glutathione S-conjugate transporter Ycf1 which is localized in the vacuolar membrane (Fig. 2). Ycf1p is a member of the ATP binding cassette (ABC) transporter family, responsible for vacuolar sequestration of these complexes and hence part of the cellular defense to arsenite (Ghosh *et al.*, 1999) and cadmium (Li *et al.*, 1997). *In vitro*, the $As(GS)_3$ complex is more stable at acidic pH compared to neutral pH (Cánovas *et al.*, 2004; Rey *et al.*, 2004). It is therefore believed that the acidic environment of the vacuole stabilizes this complex. On the contrary, the $Cd(GS)_2$ complex has shown to be destabilized at acidic pH (Delalande *et al.*, 2010; Leverrier *et al.*, 2007).

Transport of $As(GS)_3$ into the vacuole was totally inhibited by equimolar amounts of cadmium or antimony with glutathione in excess, indicating that the chelated metal ions are competitive substrates for Ycf1 (Ghosh *et al.*, 1999). When the arsenite extrusion pump Acr3 is functional, the role of Ycf1 seems to be minor in the arsenite detoxification, while Ycf1 becomes important if *ACR3* is deleted

(**paper II**) (Ghosh *et al.*, 1999; Wysocki *et al.*, 1997). Deletion of the *YCF1* gene renders the cell very sensitive to cadmium (Szczypka *et al.*, 1994) and moderately sensitive to arsenite. Bpt1 and Vmr1 are two other vacuolar transporters that contribute to $Cd(GS)_2$ sequestration to a lesser extent (Sharma *et al.*, 2002; Wysocki & Tamás, 2010).

4.5 Glutathione depletion?

In mammalian cells, the toxicity of arsenite is highly linked to its ability to promote the formation of reactive oxygen species and hence to induce oxidative damage, (Liu *et al.*, 2003), but this connection has not been established for yeast cells.

Cadmium is a redox inactive metal that cannot directly generate reactive oxygen species, but it can cause oxidative stress indirectly and has also shown to induce lipid peroxidation in yeast (Beyersmann & Hartwig, 2008; Brennan & Schiestl, 1996; Howlett & Avery, 1997; Skipper *et al.*, 2016; Stohs & Bagchi, 1995). The toxicity of cadmium is closely linked to its capacity to indirectly induce oxidative damage; addition of N-acetylcysteine, a scavenger of hydroxyl radicals, increases survival of cadmium exposed yeast cells significantly (Brennan & Schiestl, 1996).

It has been proposed that exposure to arsenite and cadmium could deplete intracellular levels of GSH and hence cause an altered redox state in the cell (Stohs & Bagchi, 1995). That a depletion of glutathione actually occurs in yeast cells and is a reason for the toxicity is unlikely, especially since the intracellular concentrations of glutathione (1-2mM under *uninduced* conditions) are several orders of magnitude higher than toxic concentrations of arsenite (~100µM) and cadmium (~10µM) (Lafaye *et al.*, 2005; Wysocki & Tamás, 2010). Moreover, arsenite and cadmium strongly stimulates glutathione synthesis and accumulation.

4.6 Extracellular glutathione

Glutathione is continuously "leaking" out from yeast cells, but during unstressed conditions almost all of the secreted glutathione is re-uptaken by yeast via the plasma membrane proton coupled oligopeptide transporter Opt1 (also known as Hgt1) (Fig. 2) (Bourbouloux *et al.*, 2000; Perrone *et al.*, 2005). One possible reason for this cycling of glutathione could be regulation of the redox status in the periplasmic space and hence a regulation of membrane proteins with extracellular

disulfide bridges. Export of glutathione as an extracellular redox regulator has been reported in bacteria (Pittman *et al.*, 2005).

In **paper I**, we found that accumulation of glutathione in the extracellular environment is induced upon arsenite exposure as part of an extracellular defense function. This accumulation can logically have two reasons, which is increased secretion or diminished reuptake. We show that this extracellular glutathione can chelate the arsenite and form $As(GS)_3$ complex, as occurs inside the cell. The formed complex cannot enter the cell, so this is an example of extracellular chelation as a defense mechanism (Fig. 2). This extracellular defense mechanism seems to be specific for arsenite since cadmium-exposed cells do not accumulate glutathione extracellularly even though the increase in glutathione synthesis is similar upon arsenite- and cadmium exposure. Furthermore, addition of extracellular glutathione to cadmium-exposed cells did not suppress growth inhibition, whilst it suppressed arsenite sensitivity. The protective role of the increased amounts of glutathione produced upon cadmium exposure is hence solely on an intracellular level.

Then, how is the glutathione exported? Yor1 is an ABC transporter in the plasma membrane (Fig. 2), regulated by the transcription factor Pdr1. *YOR1* gene and other ABC-transporter encoding genes are upregulated upon arsenic exposure (Thorsen *et al.*, 2007). We found that overexpression of *YOR1* leads to increased extracellular glutathione levels. We also found that Yor1 is not the only ABC transporter that can mediate glutathione transport, since expression of a constitutively active allele of the Pdr1 transcription factor increases extracellular glutathione levels also in a *yor1Δ* mutant (**paper I**). It has been shown that plasma membrane ABC transporters mediate the export of glutathione also in bacteria and mammals (Hammond *et al.*, 2007; Pittman *et al.*, 2005).

Gex1 and Gex2 were identified in yeast as GSH/H^+ antiporters (Fig. 2), found mainly in the plasma membrane of iron-depleted early-log phase cells (Dhaoui *et al.*, 2011). Thus, several transporters independently contribute to GSH export in yeast.

4.7 Mathematical modeling of arsenic fluxes and localization

In **paper II** we investigated the role and contribution of different transport proteins upon arsenic exposure, and the intracellular distribution of arsenic once entered the cell. We performed arsenic transport assays with a series of strains and measured intracellular arsenic concentrations. It is hard to experimentally distinguish between different arsenic species, and also hard to experimentally determine the intracellular distribution of arsenic in different compartments. Therefore, we designed a mathematical model that could describe what we saw and that could be used as a predictive tool to generate new hypotheses.

A series of different mutant strains lacking transport proteins or regulators thereof were used and all strains were pre-incubated for 24h with a low concentration of arsenite in order induce the expression of *ACR3* (Ghosh *et al.*, 1999; Thorsen *et al.*, 2006). Cells were then stressed for 1h with 1mM arsenite before the cells were washed and resuspended in arsenite-free media. We measured the intracellular content of total arsenic in samples taken after the 24h pre-incubation, during the hour of stress and during recovery in arsenite-free media.

The model indicated that the prevalent arsenic species in the cell after 24h incubation with low arsenite was glutathione-bound ($As(GS)_3$), either cytosolic and/or vacuolar, depending on the strain. When 1mM arsenite was added after these 24h of low arsenite incubation, the model predicted that the increase in intracellular arsenic concentration was coupled to an increase of protein bound arsenic. Model simulations indicated that glutathione binding is important for long-term binding of arsenite after chronic exposure, while protein binding is the result of acute exposure.

Further, the model suggests that the glutathione availability is rate-limiting for the $As(GS)_3$ formation and that the basal levels of Ycf1 is sufficient for vacuolar sequestration of the intracellularly formed $As(GS)_3$. The only strain where the model suggests an upregulation of the Ycf1 protein levels is in the *acr3Δ* strain that lacks the plasma membrane extrusion pump (**paper II**). It has been shown that over expression of *YCF1* increases the tolerance for cadmium, but not for arsenite (Preveral *et al.*, 2006), and this is in line with the model predictions for Ycf1 upon arsenic exposure.

Since the model identifies protein-bound arsenite as the most abundant species during short term (acute) exposure, I focused my further studies on the relation between metals and proteins.

5 Metals and protein interactions

A classical view of arsenite cytotoxicity involves interactions with vicinal thiols/suphydryl groups on proteins and hence the inhibition of enzymes (Aposhian & Aposhian, 2006). Arsenite binding has been identified in a number of proteins (Shen *et al.*, 2013) and our model predictions suggest that a large fraction of the intracellular arsenite may indeed be protein-bound (**paper II**). Anyhow, this thiol-mediated interaction model for toxicity has not been unequivocally shown (Taylor, 2010). Arsenite-induced inhibition of pyruvate dehydrogenase has been observed *in vitro*. Pyruvate dehydrogenase catalyzes the formation of acetyl-coenzyme A through oxidative decarboxylation of pyruvate and a potential mitochondrial toxic effect for arsenite is therefore identified. Since enzymatic inhibition of pyruvate dehydrogenase seems regulated by reactive oxygen species rather than by arsenite binding, a link between arsenite-induced toxicity and oxidative stress as discussed above is potentially seen (Samikkannu *et al.*, 2003). Hence, it is not clear whether pyruvate dehydrogenase inhibition is mediated via direct arsenite binding or via oxidative modification.

5.1 Metallothioneines

Metallothioneins (MTs) are small cysteine-rich proteins implicated in the detoxification of many toxic metals, scavenging of free radicals (Sato & Bremner, 1993), and metal transport (Yanagiya *et al.*, 2000).

Metallothioneines are capable of binding a high number of metal ions and have evolved for keeping the homeostasis of essential metal ions like zinc, selenium and copper. In yeast there are two genes encoding metallothioneines, *CUP1* (*CUP1-1* and the *CUP1-2* paralog), and *CRS5*. Cup1 was identified as a cytosolic copper-binding protein, also capable of binding cadmium and zinc (Winge *et al.*, 1985). The main role of Cup1 is in copper homeostasis, but it also seem to protect cells from cadmium toxicity since high *CUP1* levels results in increased cadmium tolerance (Ecker *et al.*, 1986). Crs5 is more cysteine-rich than Cup1 and binds to copper, cadmium and zinc (Culotta *et al.*, 1994; Pagani *et al.*, 2007).

Cadmium has a higher affinity for thiol groups than zinc, and cadmium can therefore replace zinc in metallothioneines (Maret & Moulis, 2013). The expression of metallothioneines is upregulated by cadmium, probably as an indirect effect of cadmium exposure. It has been shown in *S. cerevisiae* that the cadmium-induced upregulation of metallothioneine expression is mediated by oxidative stress since it can only be observed under aerobic conditions. Cultivation under anaerobic conditions or additions of N-acetylcysteine decreases the cadmium-mediated induction of metallothioneines (Liu *et al.*, 2005). Cadmium has been observed to upregulate metallothioneines also in human cells, but not through a direct interaction with the MTF-1 transcription factor. Instead cadmium replaces zinc in the already existing metallothioneines, releasing zinc ions which alter the levels of free zinc ions in the cytoplasm, these will bind to and activate the MTF-1 transcription factor and hence initiate transcription (Zhang *et al.*, 2003).

Arsenite has been observed to interact *in vitro* with rabbit metallothionein II (G. Jiang *et al.*, 2003) and human metallothioneine-2 (Toyama *et al.*, 2002), but the relevance of this interaction *in vivo* remains unknown .

5.2 Cadmium and zinc

Zinc is a structural component of many proteins and the potential zinc-proteome ranges between 4-10% of the total proteome in different organisms. In yeast cells about 8% of all proteins are zinc-binding proteins (Andreini *et al.*, 2006). Zinc can have both catalytic functions and structural functions in proteins (Coleman, 1992). Zinc can use oxygen, nitrogen and sulphur as ligands (Summers, 2009) and is often coordinated by thiol groups. As mentioned above, cadmium has a higher affinity for thiol groups than zinc, and can therefore displace zinc in different proteins. In zinc finger proteins one zinc ion coordinates four cysteines and/or histidines (Fig. 5). Zinc finger structures can mediate binding to DNA or other proteins and are often found in e.g. transcription factors. *In vitro* titration showed that a CCHH (cys-cys-his-his) zinc finger motive preferentially binds zinc rather than cadmium, a CCHC motive provides equal binding affinities for both metals, and a CCCC motive binds cadmium with a clearly stronger affinity than for zinc (Hartwig, 2001). Anyhow, the presence of nanomolar concentrations of cadmium has been observed to inhibit activity *in vivo* for the TFIIIA zinc finger transcription factor with a CCHH motive (Hanas & Gunn, 1996), potentially depending on cadmium interacting with other thiols in this cysteine-rich protein.

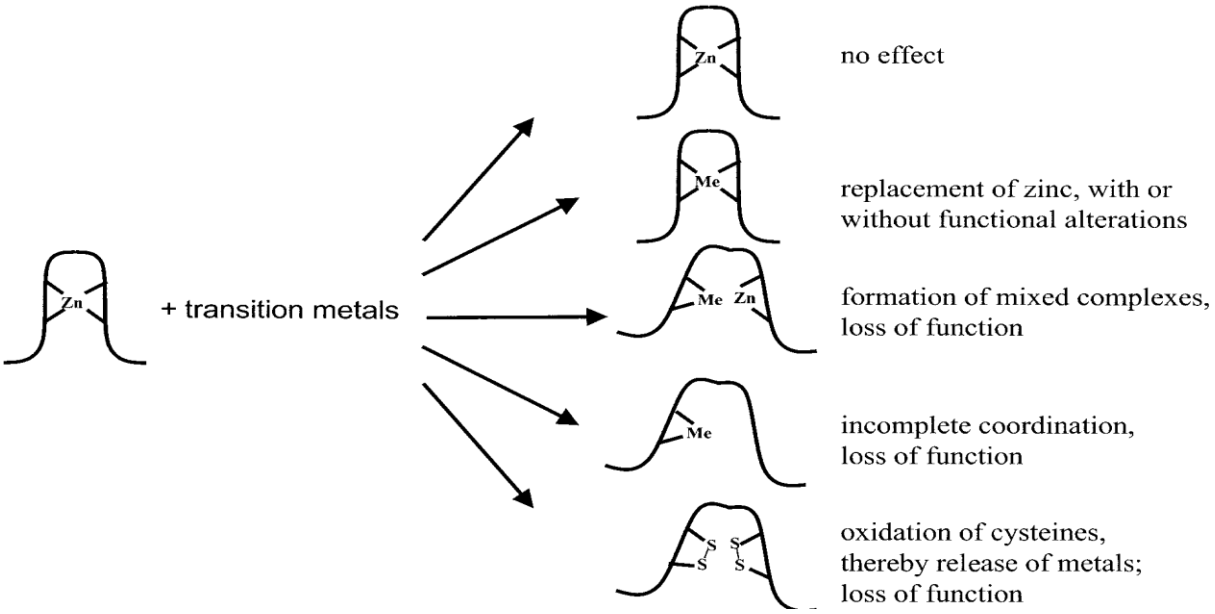

Figure 5. **Potential effects on zinc finger structures by toxic transition metals like cadmium.** Figure from (Hartwig, 2001).

Hence, the interference of cadmium with zinc binding proteins is linked to toxic effects of cadmium. Another study that links cadmium toxicity and zinc levels was recently presented; urinary cadmium concentrations and cancer mortality was found to be linked in a study of more than 5000 americans, and interestingly, the cadmium-linked mortality was found to be inversely related to dietary zinc intake (Lin *et al.*, 2013).

5.3 Cadmium and calcium

Some chemical properties of cadmium resemble those of calcium, such as charge (2+) and similar ionic radius (Choong *et al.*, 2014). *In vitro* studies have shown that cadmium can displace calcium in a number of proteins including calmodulin (Chao *et al.*, 1984; Choong *et al.*, 2014). Calmodulin regulates intracellular calcium levels; it binds to calcium and changes conformation upon binding and can thereby interact with a series of receptor enzymes (Choong *et al.*, 2014). Calcium functions as a second messenger and is actively kept away from the cytoplasm by Ca^{2+}-ATPase pumps at the plasma membrane and at the endoplasmic reticulum. Exposure to cadmium results in a disruption of calcium homeostasis. An initial increase of cytoplasmic calcium will result from inhibition of Ca^{2+}-ATPase activity (Akerman *et al.*, 1985) and cadmium binding to proteins involved in calcium

sequestration (Choong *et al.*, 2014). The release of intracellular calcium has been connected to induction of apoptosis (Olofsson *et al.*, 2008). Addition of calcium improves growth of yeast cells exposed to cadmium (Gardarin *et al.*, 2010).

5.4 Endoplasmic reticulum (ER) and the unfolded protein response (UPR)

About one-third of the proteome is translocated into the ER lumen. A majority of these proteins are secretory proteins and membrane proteins and obtain their native fold in the ER before they are directed to their final localization. While the cytoplasm generally presents a reducing environment, the ER lumen is more oxidizing and favors therefore the formation of disulphide bond formation which stabilizes proteins aimed for the extracellular environment. Accumulation of unfolded proteins in the ER can occur upon exposure to a range of environmental stimuli (Kaufman, 1999). Cadmium, but not arsenite, induces ER stress both in yeast (Gardarin *et al.*, 2010) and mammalian cells (Hiramatsu *et al.*, 2007; Liu *et al.*, 2006). This ER stress has shown to lead to a perturbation of the cellular calcium homeostasis (Biagioli *et al.*, 2008; Gardarin *et al.*, 2010). The response to accumulation of unfolded proteins in eukaryotic cells involves the onset of transcriptional response, translational attenuation and protein degradation, collectively called the unfolded protein response (UPR)(Mori, 2000). In *S. cerevisiae*, the Ire1 kinase senses ER stress through direct interaction with unfolded proteins (Kimata *et al.*, 2007). Ire1 clusters upon the ER stress (Korennykh *et al.*, 2009) and hence assume endonuclease activity, which induces an alternative splicing of the *HAC1* mRNA (Ruegsegger *et al.*, 2001). The formed Hac1 protein initiates the transcription of a series of genes encoding chaperones and other proteins aimed to mitigate the accumulation of unfolded proteins in the ER (Kaufman, 1999). Ire1 is a conserved protein that can also be found in mammalian cells, where a failure to increase the protein folding capacity enough upon ER stresses will result in the induction of apoptosis (Li *et al.*, 2010). ER stress in higher eukaryotes can therefore result in tissue damage and diseases (Hetz *et al.*, 2015; Oakes & Papa, 2015; Schonthal, 2012).

6 Protein synthesis and folding

Every protein synthesized in the cell has to fold into its right three-dimensional structure in order to be functional. Since chemically denatured proteins are able to refold spontaneously *in vitro*, all the information about the native structure is intrinsic in the amino acid sequence, and the polypeptide chain is potentially able to assume the correct fold without assistance (Ellis, 1997; Frydman, 2001; Hartl & Hayer-Hartl, 2009). The polypeptide will fold into the conformation with the lowest free energy with respect to positively and negatively charged amino acid residues, and to hydrophobic/hydrophilic interactions. The native state of a protein has been described as "the structure that is most stable under physiological conditions" (Dobson, 2004). A protein has to assume its correct fold for biologic activity; a misfolded protein can result in loss of activity or aberrant interactions with other proteins. In **paper III** and **paper IV** we demonstrate that arsenite and cadmium causes protein misfolding and hence aggregation *in vivo*.

6.1 The role of molecular chaperones

In the cytosol where the protein synthesis takes place, the concentration of proteins and other macromolecules is about 300-400mg/ml (Dobson, 2004). Hence, the nascent polypeptide chain will be subjected to both numerous interactions from the surrounding molecules, and to a restricted space. Therefore, a significant fraction of all proteins that are synthesized in the eukaryote cell needs help and protection of other proteins to assume the correct folding. Proteins that are not (yet) folded into their native conformation risk to form unspecific interactions with other (unfolded) proteins and form protein aggregates since hydrophobic residues can be exposed instead of hidden inside the protein. Molecular chaperones are proteins that assist cellular proteins to find the correct fold by cycles of binding and release. Chaperones efficiently recognize and bind to proteins that did not assume their correct fold, like partially folded intermediates of proteins and nascent polypeptide chains. The newly synthesized polypeptide is hence being protected from aggregation and from aberrant interactions with the surrounding environment until the correct fold has been assumed, but the chaperones do not add any information

on how the folded protein should be structured (Dobson, 2004; Hartl & Hayer-Hartl, 2009).
The folding of a protein can occur post-translationally, but the N-terminal part may be folded into domains co-translationally to prevent unwanted interactions between N- and C-termini. The majority of proteins synthesized in eukaryotic cells are assisted by chaperones and folding is initiated before the entire polypeptide has been synthesized (Frydman, 2001). The nascent polypeptide chain emerges from the large ribosome subunit through a channel which is long enough to cover 40-60 amino acid residues. A series of chaperones interact with the emerging polypeptide chain in order to allow complete folding units to exit the ribosome before the folding process takes place. These interactions also prevent the nascent chain from aggregating with adjacent macromolecules. The fact that 40-60 amino acids are covered by the ribosome means that the C-terminal end of the protein will not be able to participate in the folding process until the translation is terminated (Ellis, 1997; Hartl & Hayer-Hartl, 2009).

6.2 Ribosome-associated chaperones

The Nascent polypeptide-Associated Complex (NAC) is a heterodimer which consists of Egd2 (NACα in mammalian cells) and Egd1 or Btt1 (NACβ in mammalian cells). NAC is located close to the site on the ribosome where the nascent polypeptide chain emerges, and binds to the polypeptide during the synthesis (Fig. 6). The role of NAC is not completely clear, but is possibly similar to the bacterial ribosome-associated Trigger Factor, that binds and protects the nascent polypeptide chain. The binding of ribosome-associated chaperones to the emerging polypeptide chain could protect from premature folding and aggregation until enough polypeptide has emerged in order to assume a functional fold (Frydman, 2001; Hartl & Hayer-Hartl, 2009; Rospert *et al.*, 2002).

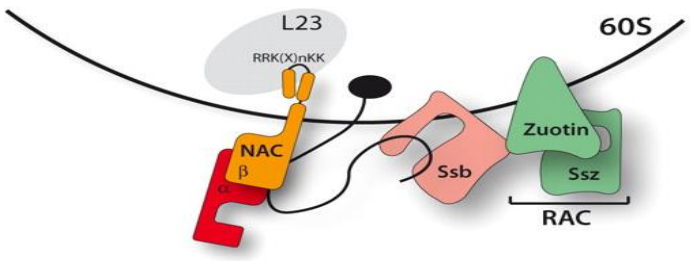

Figure 6. The nascent polypeptide is bound by NAC and/or SSB-RAC.
Figure from (Koplin et al. 2010)

The Ribosome-Associated Complex (RAC) is another ribosome-anchored chaperone complex, consisting of a stable heterodimer built up by the Hsp70-homologe Ssz1 (also called Pdr13) and the Hsp40-homolog protein zuotin encoded by the *ZUO1* gene (Rospert *et al.*, 2002). RAC together with "Stress70 B" Hsp70 proteins Ssb1 or Ssb2 forms the SSB-RAC complex (Fig. 6). Ssb1 and Ssb2 are reversibly bound to the ribosome, and can also be found in the cytoplasm (Frydman, 2001). The nascent polypeptide chain that emerges from the ribosome can interact with either NAC or SSB-RAC, or both (Koplin *et al.*, 2010).

6.3 Heat shock proteins 70 and 40

After NAC/RAC, the nascent polypeptide can interact with cytosolic Hsp70s; these can assist post-translationally but also in the co-translational folding (Hartl & Hayer-Hartl, 2009). Hsp70s and chaperonins are two classes of chaperones that recognize exposed hydrophobic residues and bind to those. The binding of these chaperones to unstructured proteins will help the latter to assume their native conformation, but as importantly, will also prevent formation of dysfunctional aggregates as they hide the hydrophobic residues (Hartl & Hayer-Hartl, 2009). Hsp70s are central in the overall regulation of the network of chaperone activity since they can direct proteins to chaperonins or Hsp90s for further action. Yeast contains four cytosolic Hsp70s, Ssa1-Ssa4, and the three Hsp70s bound to the ribosome, Ssb1, Ssb2 and Ssz1. Although Ssb1 and Ssb2 are reversibly bound and can be found in the cytoplasm, they cannot replace the function of the essential Ssa-proteins (Frydman, 2001). The Hsp40 chaperones or sHSP (small heat shock protein) deliver proteins to ATP-bound Hsp70. Hsp40 stimulates a high-affinity binding between the peptide and the Hsp70 by stimulation of ATP hydrolysis. Sis1 is an Hsp40 protein that regulates the activity of both cytosolic and ribosome-bound Hsp70. The ATP hydrolysis mediates the "closure" of an α-helical lid over the β-sandwich where the binding occurs, and hence a tighter binding between the Hsp70 chaperone and the substrate. Sse1 is an Hsp110 chaperone and functions as a nucleotide exchange factor (NEF) and will displace the ADP that remains bound to the Hsp70 protein after the ATP hydrolysis, consequently this NEF can be replaced by a new ATP molecule. The ATP binding opens the lid of the Hsp70 and allows the substrate to diffuse from the chaperone (Fig. 7) (Hartl & Hayer-Hartl, 2009).

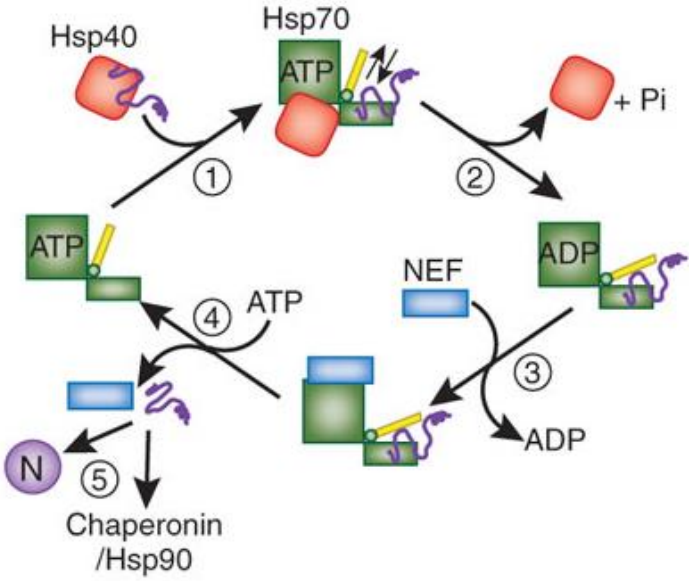

Figure 7. Hsp70 assists folding through cycles of binding and release. 1) Hsp40 delivers a (partially) unfolded polypeptide to the ATP-bound Hsp70. 2) Hsp40 also stimulates the hydrolysis of ATP, which mediates a tight binding of the Hsp70 chaperone to the substrate; the α-helical "lid" closes over the β-sandwich where the substrate is bound. 3) The ADP molecule that remains bound to the Hsp70 chaperone after the ATP hydrolysis is released by a nucleotide exchange factor (NEF). 4) The NEF is then replaced by a new ATP molecule, the ATP binding again opens the α-helical "lid" and 5) the substrate can leave the chaperone. Figure from (Hartl & Hayer-Hartl, 2009).

6.4 Chaperonins and folding of cytoskeletal proteins

The chaperonin-containing T complex (CCT complex, also called TRiC) is an 8-subunit chaperon complex consisting of Cct1-Cct8. Chaperonins are big protein complexes with a cylindrical cavity and an α-helical extension that works as a lid. Chaperonins provides a protected environment for a protein where it can assume the correct fold without interactions from other macromolecules (Frydman, 2001). In mammalian cells, CCT participates in the folding of 10-15% of the cytosolic proteins (Thulasiraman *et al.*, 1999). Folding of actin and tubulin depends on assistance of the CCT complex (Spiess *et al.*, 2004), together with the Hsp70-like GimC chaperone complex, also known as prefoldin (Vainberg *et al.*, 1998). Arsenic has been shown to interfere with CCT both *in vivo* in yeast and *in vitro*, and hence disturbs folding of both actin and tubulin, which results in disruptions in the cytoskeleton (Pan *et al.*, 2010; Thorsen *et al.*, 2009).

Arsenic can bind directly to tubulin and interfere with its function in mammalian cells (Li & Broome, 1999) but probably not in yeast cells; the yeast *TUB2* gene encoding β-tubulin does not contain the Cys12 residue that has been identified as critical for arsenite binding in the human ortholog (Pan *et al.*, 2010; Zhang *et al.*, 2007). Arsenic has been observed to interfere with actin organization in mammalian cell lines (Li & Chou, 1992).

6.5 Redox regulated chaperones

Heat shock regulated chaperones (Hsp90, Hsp70 and Hsp60) require ATP binding and hydrolysis for their activity and can be categorized as foldases. These are inactivated upon oxidative stress due to a rapid decrease in intracellular ATP concentrations. Hsp33 in *Escherichia coli* is transcriptionally regulated as a heat shock protein, but is post-translationally activated only upon oxidative stress conditions by formation of a disulphide bond (Jakob *et al.*, 1999). Hsp33 is a holdase that binds to substrate proteins to prevent their irreversible aggregation (Winter *et al.*, 2005). The release of the bound substrate protein does not occur until the cytoplasm is again restored to a reducing environment (Hoffmann *et al.*, 2004).

In *S. cerevisiae* the Get3 protein (also known as Arr4) has been identified to be similarly activated upon oxidative conditions. Under non-stressed conditions, Get3 guides tail-anchored proteins to receptors on the endoplasmic reticulum (Schuldiner *et al.*, 2008). Get3 is a zinc binding protein with four conserved cysteines (Metz *et al.*, 2006). Upon oxidative conditions, zinc is released and disulphide bonds are formed following conformational rearrangements. Get3 tetrameres can thereby be assembled and these tetramers can bind to unfolding proteins and hence prevent their aggregation (Voth *et al.*, 2014). *In vitro* studies have established that Get3 demonstrates ATP-independent chaperone activity, and *in vivo* experiments have confirmed that Get3 co-localizes with unfolded proteins and chaperones under ATP-depleting conditions (Powis *et al.*, 2013).

7 Formation and clearance of protein aggregates

7.1 Protein aggregation

All organisms contain chaperones that help nascent proteins to assume the correct fold; the activity of the chaperones can prevent misfolding. The cellular responses to damaged proteins consist of increased chaperone activity to refold denatured proteins and degradation of damaged proteins. When the capacity of these quality control mechanisms are not enough, misfolded/denatured proteins will accumulate in the cytosol and proteins with exposed hydrophobic residues and unstructured backbone can interact and form dysfunctional aggregates (Hartl & Hayer-Hartl, 2009). This process normally occurs at a low rate in all cells.

(Tyedmers *et al.*, 2010) identified four classes of events that could result in protein aggregation:

- Mutations that either lead to a tendency of the affected protein to misfold and aggregate, or mutations of protein quality control system affecting the folding status of client proteins.
- Defects in translation and formation of protein complexes.
- Environmental stress conditions.
- Exhaustions of the quality control systems due to ageing.

Several metals induce protein aggregation, but the mode of aggregate induction differs between different metals (Fig. 8). Metal ions can form monodentate (binds to one ligand in the protein) or pluridentate (binds more than one ligand on the protein) complexes with proteins, interacting primarily with S, N and O-groups. Non-native proteins are more susceptible to binding of metals not only because of exposure of side chains normally hidden inside the native fold, but also because they are more flexible and hence more easily can form pluridentate complexes with the metal, which makes the interaction stronger (Sharma *et al.*, 2011; Tamas *et al.*, 2014). Metals can also affect the proteome by interfering with the folding of proteins, chaperone activity and proteolytic degradation (**paper III**). Spontaneous refolding of chemically denatured proteins *in vitro* is inhibited by addition of cadmium (Sharma *et al.*, 2008, 2011) and arsenite (**paper III**).

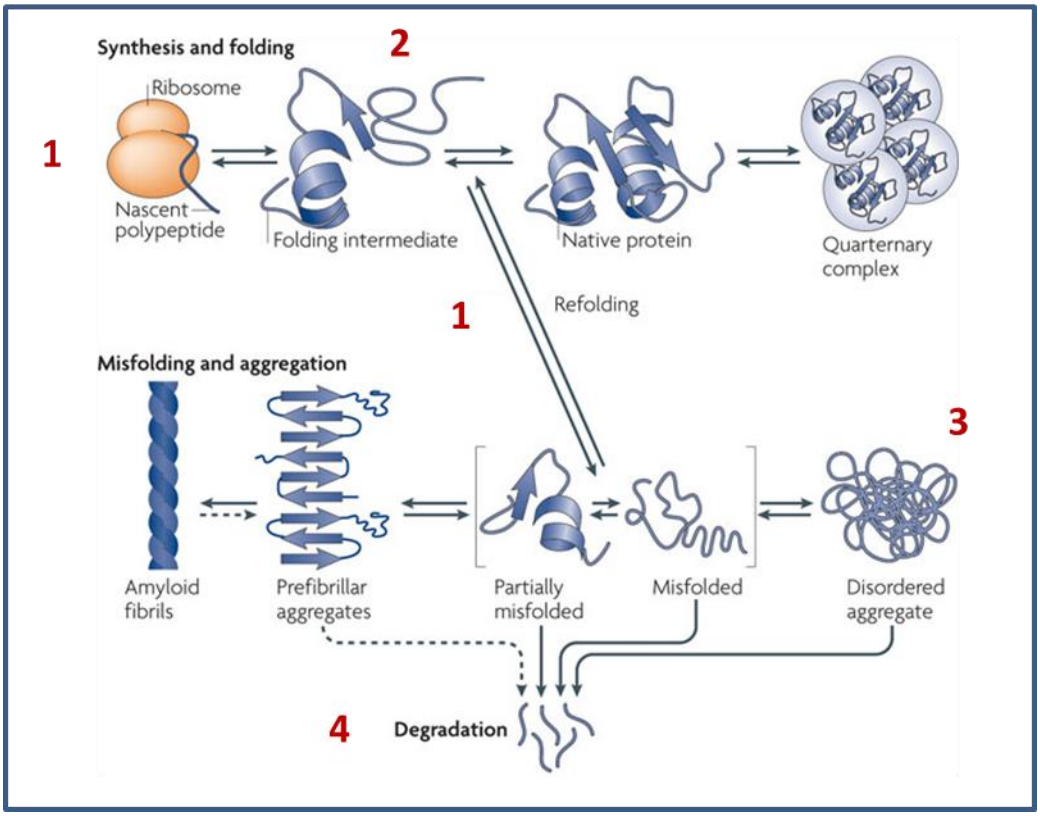

Fig. 8. Mechanisms by which metals may trigger protein aggregation; 1) Inhibition of cytosolic/ribosome associated chaperones or quality control-systems 2) Interaction with nascent polypeptides/folding intermediates 3) Primary aggregates may act as seeds committing other proteins to aggregate 4) Interference with aggregate degradation pathways. Figure adapted from (Tyedmers *et al.*, 2010).

Cycloheximide (CHX) is known to bind to ribosomes in eukaryotes and thereby inhibit protein synthesis (Obrig *et al.*, 1971). We showed that arsenite- and cadmium-induced protein aggregation is completely inhibited by addition of CHX, while heat-induced protein aggregation could not be inhibited the same way (**paper III and IV**). This indicates that the mechanism of protein aggregation upon heat shock is different from the mechanism of protein aggregation upon arsenite and cadmium exposure.

It has been observed that arsenite has a DNA damaging effect that promotes mutations, and that this mutagenicity is linked to the generation of oxidative damage both in yeast and in mammalian cells (Hei & Filipic, 2004; Litwin *et al.*, 2013). This effect could potentially lead to aggregation of mutated proteins due to misfolding, but this is not a relevant mechanism in the time-frame in which we monitor the arsenite induced protein aggregation.

There might be a link between the arsenite-induced production of ROS and the formation of aggregates upon arsenite exposure; also hydrogen peroxide induces protein aggregation, although the size and morphology of the arsenite-induced and the hydrogen peroxide-induced aggregates are dissimilar (**paper III**; Jacobson and Tamás, unpublished data). *In vitro* studies indicate that arsenite can inhibit oxidative folding of proteins (Ramadan *et al.*, 2009).

7.2 Neurodegenerative diseases and protein aggregation

Accumulation of misfolded and aggregated proteins is harmful to cells, less because of the loss-of-function of the non-native protein, but more because of a gain-of-function; the new properties obtained by proteins in misfolded and aggregated states (Winklhofer *et al.*, 2008). Partially folded or misfolded proteins can end up in amorphous aggregates or highly structured prefibrillar aggregates, which can mature into amyloid fibrils. In several human neurodegenerative diseases, aggregation of proteins into unstructured aggregates or into highly structured amyloid aggregates is coupled to the loss of normal function of neural cells (Buchberger *et al.*, 2010; Goldberg, 2003).

Mutations that result in increased tendency for specific proteins to aggregate are linked to the familial (inherited) form of neurodegenerative diseases like of Huntington´s, Parkinson's and Alzheimer's diseases (Tyedmers *et al.*, 2010). Misfolded proteins with a β-sheet conformation build up a particular form of fiber-like aggregates known as amyloids. Mutations resulting in polyglutamine (CAG) stretches increase the propensity for amyloid formation, and the length of the stretch is relative to the tendency for aggregate formation (Ross & Poirier, 2004). A late onset of these diseases are instead believed to be related to an exhaustion of the cellular protein quality control mechanisms during ageing (Tyedmers *et al.*, 2010).
There is accumulating evidence that metals interfere with several proteins involved in formation of aggregation in neurodegenerative diseases (Tamas *et al.*, 2014). A series of mechanisms for arsenic described in the literature have been used to set the "arsenic exposure hypothesis" for Alzheimer´s disease (Gong & O'Bryant, 2010). Cadmium has been observed to interfere with the microtubule stabilizing protein tau, which is coupled to Alzheimer´s disease and is hence a possible etiological factor for neurodegenerative diseases (Jiang *et al.*, 2007).

7.3 Hsp104 and disaggregation

When the aggregation of proteins is a fact, there is still a possibility that they could return to their native state. Hsp104 is a molecular chaperone identified in yeast and an orthologue to the bacterial ClpB. Hsp104 is not essential for viability under unstressed conditions and it is expressed at low concentrations. Yeast cells strongly upregulate the expression of Hsp104 upon heat stress and this chaperone is essential for the heat tolerance (Lindquist & Kim, 1996; Sanchez & Lindquist, 1990). Hsp104 does not prevent the formation of protein aggregates, or target the client protein for proteolysis but functions as a disaggregase mediating the resolubilization of proteins from already formed aggregates (Parsell *et al.*, 1994b).

In our work we use the fact that the Hsp104 chaperone co-localizes with aggregated proteins and we monitor the formation and clearance of protein aggregates by monitoring the localization of a GFP tagged version of the chaperone. Hsp104-GFP foci have shown to correspond to sites of protein aggregation (Glover & Lindquist, 1998; Kawai *et al.*, 1999; Lum *et al.*, 2004).

Co-operation with the Hsp70 system (Hsp70/Ssa1 and Hsp40/Ydj1) is required in order to fully return the client proteins into their native conformations (Glover & Lindquist, 1998; Shorter & Lindquist, 2008). Hsp104 is dependent on the co-chaperones in order to localize aggregates and disaggregate proteins (Tyedmers *et al.*, 2010). An *ydj1Δ* strain has more Hsp104-GFP aggregates after 3h of arsenite exposure than after 1h (Navarrete and Tamás, unpublished data), to be compared with a WT strain that can clear the cytosol from arsenite-induced aggregates in 3h (**paper III**), indicating a delay in finding the aggregates when Ydj1 is missing. Hsp104 alone without Hsp70 has no disaggregation activity. The binding of Ssa1 (Hsp70) and Ydj1 (Hsp40) to the protein aggregates, will not only guide Hsp104 but will also protect the aggregated proteins from protease activity (Tyedmers *et al.*, 2010).

Hsp104 is a member of the AAA+ ATPase family (ATPases associated with diverse cellular activities), that form large hexameric ring-structures (Parsell *et al.*, 1994a). Binding to ATP/ADP mediates the assembly of hexameres, which are the functional form of the chaperones (Bösl *et al.*, 2006). Hsp104 has a peptide-binding region and a nucleotide-binding domain (NBD2) in the C-terminal part of the protein. Peptide binding and subsequent ATP hydrolysis mediates a conformational change in the middle linker region (M-domain) which mediates ATP hydrolysis in the N-terminal ATPase domain (NBD1) (Cashikar *et al.*, 2002).

The C-teminal α-helical sensor and substrate discrimination domain (SSD) is necessary for the hexamerization (Smith *et al.*, 1999). The M-domains of Hsp104 are localized on the outside of the ring upon hexamerization (Lee *et al.*, 2010) and mediates the specificity in the interaction with Hsp70 and Hsp40 (Sielaff & Tsai, 2010).

The molecular mechanisms behind the disaggregating properties of Hsp104 are yet to be understood. It has been proposed that the flexible Hsp104 M-domain linker region might function as a molecular crowbar pulling apart proteins in aggregates, exerting a mechanical force due to a conformational change upon ATP hydrolysis, (Lee *et al.*, 2004). It has also been proposed that Hsp104 performs its disaggregating function by threading aggregated proteins one by one out from the aggregate, probably in an ATP-dependent process (Bösl *et al.*, 2006).

7.4 IPOD and JUNQ

Cells can also sequester aggregated proteins into distinct locations, probably in order to diminish the damage to the rest of the proteome. A partition of aggregated proteins into two distinct quality-control compartments has been reported in yeast. Insoluble terminally aggregated proteins accumulate in a perivacuolar location, called IPOD (Insoluble PrOtein Deposit), and misfolded, ubiquitylated proteins that are still soluble accumulate in a juxtanuclear quality-control compartment (JUNQ) (Kaganovich *et al.*, 2008). Ubiquitylation of the proteins in the JUNQ compartment suggests that these proteins are destined for proteasomal degradation. The targeting of proteins into these two compartments is dependent on a functional cytoskeleton (Tyedmers *et al.,* 2010). Except from keeping the aggregates away from the cytosol, this sequestration also renders the clearance of the aggregates easier to handle. Whether arsenite- or cadmium-induced protein aggregates co-localizes with any of these two compartments remains to be investigated. This type of partition of aggregates is a conserved strategy that can be observed from bacteria to mammalian cells (Tyedmers *et al.*, 2010).

7.5 P-bodies and stress granules

Another kind of cytoplasmic structures are the mRNA-containing processing bodies (P-bodies) and stress granules. These are cytoplasmic structures where mRNAs have shown to accumulate upon stress conditions, and in mammalian cells these are known to be induced upon arsenic exposure. P-bodies contain mRNAs and proteins involved in mRNA degradation, as the decapping protein Dcp2 (Balagopal & Parker, 2009; Swisher & Parker, 2010). Stress granules are dynamic cytoplasmic foci that can be detected under glucose starvation and other environmental stresses. Stress granules contain translationally inactive mRNA´s and numerous components of the translational machinery; the large ribosomal subunit and several proteins, including the poly-A binding protein Pab1 (Balagopal & Parker, 2009; Swisher & Parker, 2010). We show that both stress granules and P-bodies are formed upon arsenite exposure in yeast cells. Our data show that arsenite-induced Hsp104-containing aggregates do not co-localize with Dcp2 and Pab1, hence arsenite-induced protein aggregates are different from P-bodies and stress granules (**paper III**).

7.6 Protein degradation

Degradation of proteins occurs at all times, and the turn-over of proteins together with their expression regulates the concentration of different proteins in the cell under different conditions. The half-life of different proteins varies largely from minutes to days. Accumulation of misfolded proteins and protein aggregates is negative for the cell and in addition to increasing the folding capacity of the cell by inducing and activating chaperones, the cell will also increase its capacity of protein degradation upon proteotoxic stress.

7.7 Proteasomal degradation

The proteasome system enhances the cellular tolerance for protein-damaging agents by preventing the accumulation of non-functional, potentially toxic proteins in the cytosol (Goldberg, 2003). The proteasome is a large protein complex that degrades proteins and most proteins in eukaryotes that are destined for degradation are ubiquitylated. The ubiquitin-proteasome system (UPS) works as a part of the

cellular proteome control, both for the normal turnover of proteins, and is important for removal of damaged proteins from the cytosol when misfolding of proteins overruns the capacity of the chaperone system (Goldberg, 2003; Tyedmers *et al.*, 2010).
Protein degradation is an energy requiring process; ATP is required for the activation of ubiquitin and this process is catalyzed by an E1 enzyme. Thereafter one of several E2 ubiquitin-conjugating enzymes delivers the ubiquitin molecule to the target protein, which will be recognized with the help of an E3 ubiquitin-ligase enzyme. The ubiquitin ligase provides the specificity for the substrate protein and will catalyze the binding of the ubiquitin. This process can be repeated by E2 and E3 by cooperation with an E4 enzyme until an extended polyubiquitin chain attached to the target protein is recognized by the proteasome (Haas & Rose, 1982; Hwang *et al.*, 2010; Thrower *et al.*, 2000). There are several E2 and E3 enzymes in yeast (11 and 42), but only one E1 activating enzyme has been identified (Lee *et al.*, 2008).

The eukaryotic proteasome is built up by one 20S core and one or two 19S lids, to form a 26S complex. The 20S catalytic core consists of four rings; two alpha and two beta subunits, with a central cavity. The hydrolysis of client proteins is exclusively performed inside the central cavity of the 20S core, and this structural solution protects cytosolic proteins from the proteolytic activity. The 19S lid contains at least 19 different subunits, divided into base and lid. The base of the 19S particle consists of 6 ATPases that regulates the opening of the 20S cavity, the unfolding of the substrate protein and its translocation into the catalytic core. The 19S lids are responsible for recognition of poly-ubiquinated substrates, recycling of ubiquitin and for protein unfolding prior to degradation (Beck *et al.*, 2012; Benaroudj *et al.*, 2003; Finley, 2009; Goldberg, 2003). It is unclear whether the 26S proteasome can degrade proteins that are not tagged by ubiquitin in eukaryote cells. Ubiquitin-independent 26S degradation can be seen *in vitro* (Tarcsa *et al.*, 2000) as well as in archaea and bacteria (Goldberg, 2003).

In vitro experiments have shown that purified 26S proteasomes are unable to degrade aggregated proteins, and the classical view that aggregated proteins are degraded through the 26S proteasome is hence being questioned (Venkatraman *et al.*, 2004; Verhoef *et al.*, 2002). A newer view is that the increased levels of aggregates found in strains deficient in proteasome activity is due to an accumulation of misfolded proteins since the control of the proteome is defective in those strains (Tyedmers *et al.*, 2010). As always, *in vitro* experiments do not

necessarily reflect the events *in vivo,* and one could speculate that the Hsp104 disaggregating system is necessary in order to render aggregated proteins available for proteasomal degradation.

The transcription factor Rpn4 regulates the expression of proteasomal components (Medicherla & Goldberg, 2008; Xie & Varshavsky, 2001) and the Rpn4-dependent transcription is induced upon arsenite treatment (Haugen *et al.*, 2004; Thorsen *et al.*, 2007). It has been shown that absence of Rpn4-induced expression of proteasome components negatively affects growth under various stress conditions (Wang *et al.*, 2008), and in our experiments differences in viability between the *rpn4Δ* mutant and the corresponding wild type can be seen upon exposure to arsenite or cadmium. Rpn4 is important for tolerance to both arsenite and cadmium; an *rpn4Δ* mutant is more sensitive to arsenite and cadmium compared to a WT strain and proteasomal function is important for arsenite tolerance as well as for aggregation clearance (**paper III and IV**), (Medicherla & Goldberg, 2008; Thorsen *et al.*, 2009). We observed that arsenite treated cells have a substantial increase in proteasomal activity, while the increase of proteasomal components was only marginal. The upregulation of proteasomal activity is hence not only depending on an increase of proteasomal components; another notion in the same direction is the fact that also the *rpn4Δ* mutant was able to increase the proteasomal activity upon arsenite exposure, even if to a lower extent than the corresponding wild type (**paper III**).

How are chaperones and proteasomes cooperating in the elimination of damaged proteins? A model has been proposed where chaperones directly recruit proteases or ubiquitination enzymes whenever they fail to guide their client proteins into their native fold, in order to avoid cytosolic accumulation of non-native proteins (Goldberg, 2003). Chaperones can maintain client proteins in an unfolded state in order to ease the activity of proteases (Kandror *et al.*, 1999; Wickner *et al.*, 1999). In mammalian cells, there is an E3 ubiquitin ligase (CHIP) that needs to be guided by Hsp70 or Hsp90 to specifically ubiquitylate its substrate proteins (Murata *et al.*, 2001). Hence, Hsp70 and Hsp90 do not only guide proteins during folding, but are also able to play a role in selective degradation and protect the cytoplasm from proteins that fail to find their correct fold and are therefore potentially toxic. In yeast, the Hsp70 chaperone Ssa1 and its Hsp40 co-chaperone Ydj1 are required for protein degradation through the ubiquitin-proteasome pathway (Park *et al.*, 2007).

7.8 Autophagy

Autophagy is another pathway for protein degradation that destines proteins for hydrolysis in lysosomes and vacuoles. In macroautophagy, a double membrane structure will form around cytoplasmic components which will be engulfed and released into the acidic lumen of lysosomes or vacuoles (He & Klionsky, 2009; Nakatogawa *et al.*, 2009).

There is an interplay between the degradation pathways; although ubiquitin is a typical hallmark for proteasomal degradation, the 26S-dependent degradation is not efficient for aggregated proteins as discussed above. Instead, autophagy receptors have been identified that can bind to both ubiquitin and autophagy-specific ubiquitin-like modifiers, and that provides a link between the two mechanisms of protein degradation (Kirkin *et al.*, 2009). A decline in the efficiency of the autophagy-lysosome pathway also affects the degradation through the ubiquitin-proteasome pathway (Korolchuk *et al.*, 2009). Impairment of the ubiquitin-proteasome pathway in the fly model *Drosophila melanogaster* induces compensatory protein degradation through the autophagy-lysosome pathway (Pandey *et al.*, 2007).

In mammalian cells, autophagy has been observed to play a major role in the degradation of misfolded and aggregated proteins (Cuervo & Wong, 2014; Iwata *et al.*, 2005). Autophagy has also been observed to be the main degradation pathway for alpha-synuclein aggregates formed in *S. cerevisiae* (Petroi *et al.*, 2012), but whether autophagy is actually involved in degradation of endogenous aggregated proteins in yeast is not known.

8 Aim of the thesis and main findings

8.1 Main findings and conclusions; Paper I

We present a novel extracellular detoxification mechanism to arsenite that involves glutathione chelation.

- Yeast cells export and accumulate glutathione extracellularly upon arsenite exposure.
- The extracellular glutathione binds to arsenite forming the $As(GS)_3$ complex which cannot efficiently enter the yeast cells.
- Increased levels of extracellular glutathione is beneficial for cells during arsenite exposure, while decreased glutathione levels is negative for cell growth.

This defense mechanism seems specific for arsenite since cadmium does not induce a similar accumulation of extracellular glutathione, nor is the cadmium-induced growth inhibition relieved by addition of glutathione.

8.2 Main findings and conclusions; Paper II

Experimental data and mathematical modelling indicates that:

- Protein-bound arsenite is the predominant arsenic species upon short-term (acute) exposure, whilst glutathione-bound arsenite is the predominant species upon long-term (chronic) exposure.
- The arsenite flux through Fps1 is controlled in both a Hog1-dependent and a Hog1-independent manner.
- Ycf1 protein levels increases upon arsenite exposure only in *acr3Δ* cells.
- The $As(GS)_3$ complex is not stably retained in the vacuole.

Arsenite is primarily affecting proteins, production of glutathione protects the proteome from arsenite-mediated damage.

8.3 Main findings and conclusions; Paper III

We present a novel toxicity mechanism for arsenite, as a potent inducer of protein aggregation.

- Arsenite targets newly synthesized proteins and induces aggregation.
- Arsenite targets chaperone activity both *in vivo* and *in vitro*.
- The proteins that aggregate upon arsenite exposure belong to different functional categories including UPR, protein folding and stabilization, protein synthesis, proteins with binding function or cofactor requirements, and metabolism.
- Proteasomal activity is involved in the clearance of arsenite-induced protein aggregates.
- Arsenite-induced protein aggregates interferes with folding of other proteins *in vitro*.

Arsenite inhibits chaperone activity and interferes with proteins during synthesis inducing protein aggregation.

8.4 Main findings and conclusions; Paper IV

Cadmium induces protein aggregation and targets newly synthesized proteins.

- Cadmium targets newly synthesized proteins and induce aggregation, and proteasomal activity is involved in the clearance of these aggregates.
- Cadmium does not interfere with chaperone activity *in vivo*.
- Addition of zinc can prevent the formation of cadmium-induced aggregates.

Contrary to arsenite, cadmium has no effect on chaperone activity *in vivo*, but might target a specific group of proteins. We hypothesize that cadmium replaces zinc in folding proteins and hence induces protein aggregation. In turn these aggregates might affect other labile proteins to misfold and aggregate.

9 Conclusions and perspectives

In this thesis I have investigated how two toxic metal(oid)s, arsenic and cadmium, affect cells using budding yeast as a model system. I have identified metal-induced toxicity mechanisms and also responses that are part of the cellular defense.

A novel mechanism of metal detoxification in yeast, the extracellular glutathione chelation of arsenite that keeps the arsenite from entering the cell has been described. We have investigated the role and contribution of different proteins in the response and defense to arsenic, and have concluded that glutathione is important for the protection of the proteome upon chronic arsenite exposure. We have also shown a novel mechanism linking the toxicity of both arsenic and cadmium to the induction of protein aggregation in yeast. A similarity between the modes of action of arsenite and cadmium is the requirement of active protein synthesis for the induction of protein aggregates. The precise molecular mechanisms by which arsenite and cadmium induces aggregates remains to be investigated, but the way the two metals targets proteins for aggregation is partly dissimilar. Arsenite interferes with the folding of nascent polypeptides and interferes with chaperone activity *in vivo*. Even though cadmium has been observed to interfere with chaperone activity *in vitro* (Sharma *et al.*, 2008), we do not see any inhibitory effect on chaperones *in vivo*. Instead we suggest a mechanism where cadmium replaces zinc in newly synthesized proteins and hence induces misfolding and aggregation. Indeed, high concentrations of zinc suppress the cadmium-induced protein aggregation. Another divalent ion is calcium, and the cellular metabolism and protein binding of calcium is affected by the presence of cadmium. However, addition of calcium does not influence on the aggregation levels upon cadmium exposure.

We do not know if the metals cause aggregation of proteins due to direct binding, or if it occurs via a secondary indirect mechanism mediated by another cellular response. A technical tricky experiment that would provide interesting information is to investigate if there is arsenic/cadmium *in* the aggregates, or if the aggregates are just a response of the cellular *presence* of the metals.

We have analyzed the proteins that end up in arsenite-induced aggregates and have identified that they belong to different categories, but with the common feature to

be highly translated. A very interesting comparison would of course be to run the same kind of analysis on aggregates isolated from cadmium-treated cells. A recent study indicate that three different stress conditions that induce protein aggregation through different mechanisms, result in aggregation of similar types of proteins (Weids *et al.*, 2016).

We would also like to identify genes that regulate the formation and clearance of the protein aggregates, and compare potential differences in this regulation between arsenite- and cadmium induced aggregation. We have seen that proteasomal degradation is important for the cell in order to clear the aggregates. A forthcoming task for the group would be to investigate the contribution of autophagy for the clearance of metal-induced aggregates.

Heat-induced aggregates have been observed to re-localize into the two quality control compartments IPOD and JUNQ. This redistribution has been observed to depend on a functional cytoskeleton. An interesting comparison would be to see whether metal-induced Hsp104-aggregates co-localizes with these compartments. On one hand, we do see a reduction of the number of aggregates over time before the aggregates are completely cleared from the cytoplasm (a shift from ≥3 aggregates/cell to 1-2 aggregates/cell). On the other hand, both arsenite and cadmium affects the cytoskeleton and we have performed preliminary experiments indicating that actin cables are affected differently upon treatment with arsenite and with cadmium.

Further, it would be of great interest to expand these studies and investigate how far our observations are conserved in higher eukaryotes.

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