Arsenic-induced protein aggregation and toxicity in *Saccharomyces cerevisiae*

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To my family

ABSTRACT

Arsenic is prevalent in the environment and this toxic metalloid poses a substantial threat to human health with 100-200 million people worldwide estimated to be at risk. Chronic exposure to arsenic is associated with neurodegenerative and age-related disorders that are characterized by the accumulation of protein aggregates, including Parkinson's and Alzheimer's disease. Despite of the undisputed toxicity of arsenic, our understanding of the underlying mechanisms and cellular responses is limited. This thesis has focused on arsenic-induced protein aggregation and toxicity in yeast, with the aim of elucidating how these aggregates are formed *in vivo*, the mechanisms by which they affect cells, how cells prevent their accumulation as well as how cells regulate the protein quality-control system to protect against toxic aggregates. The impact arsenic has on protein homeostasis may contribute to its toxicity and suspected role in protein misfolding diseases. Main findings of this thesis include the identification of novel genes whose overexpression conferred arsenic resistance. We also demonstrated the importance of accurate transcriptional and translational control for mitigating protein aggregation and toxicity during arsenite stress. In addition, we showed that the ubiquitin-proteasome system (UPS) is the main pathway that clears arsenite-induced aggregates, whilst the autophagy-vacuole pathway and the chaperone-mediated disaggregation both contribute to clearance but their roles appear less prominent than the UPS. Our findings provide novel insights into the biology of arsenic and a valuable resource for further studies on the mechanistic details of arsenic toxicity and pathogenesis.

Keywords: arsenic, protein aggregation, protein quality control, yeast.

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SAMMANFATTNING

Arsenik är ett giftigt grundämne som finns naturligt i berggrunden. Runtom i världen har arsenik i dricksvatten uppmärksammats för att orsaka allvarliga hälsoproblem. Genom dricksvattnet exponeras upp emot 100-200 miljoner människor i världen för högre arsenikhalt än vad som anses vara säkert. Långvarig arsenikexponering är förknippat med flertal sjukdomar såsom cancer, hjärtkärlsjukdomar och diabetes. Exponering för arsenik är även associerat med neurodegenerativa sjukdomar som i sin tur kännetecknas av formationen av proteinaggregat. Proteiner utgör stommen i alla cellers aktivitet och för att ett protein ska vara funktionellt behöver det veckas till en given tredimensionell struktur. Arsenik bidrar till att denna proteinveckning går fel, vilket leder till att proteinerna klumpar ihop sig och bildar aggregat som påverkar celler negativt. För att kartlägga grundläggande toxicitetsmekanismer hos arsenik samt hur celler försvarar sig mot arsenik, har vi studerat hur jästsvampen *Saccharomyces cerevisiae* reagerar på exponering eftersom många biologiska processer är jämförbara hos jästsvampen och hos djur och människor.

Vi har identifierat gener som ger resistens mot arsenik när de är överuttryckta. Vi har även visat att regleringen av transkription och translation är viktigt vid arsenikexponering. Genom att minska proteinsyntesen (tillverkningen av proteiner) bildas färre proteinaggregat vid arsenikexponering. Slutligen har vi också visat att proteasomen står för den huvudsakliga nedbrytningen av proteinaggregat som bildas i närvaro av arsenik. Sammanfattningsvis ger våra resultat nya insikter i arsenikrelaterad celltoxicitet som bidrar till en viktig grund för vidare forskning inom området.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Isik, E., C. Balkan, V. Karl, H. C. Karakaya, **S. Hua,** S. Rauch, M. J. Tamas and A. Koc (2022). "Identification of novel arsenic resistance genes in yeast." Microbiologyopen 11(3): e1284.

II. Andersson, S., A. Romero, J. I. Rodrigues, **S. Hua**, X. Hao, T. Jacobson, V. Karl, N. Becker, A. Ashouri, S. Rauch, T. Nystrom, B. Liu and M. J. Tamas (2021). "Genome-wide imaging screen uncovers molecular determinants of arsenite-induced protein aggregation and toxicity." J Cell Sci 134(11).

III. **Hua, S**., A. Klosowska, J. I. Rodrigues, G. Petelski, L. Alejo Esquembre, E. Lorentzon, L. F. Olsen, K. Liberek and M. J. Tamás (2022). “Mechanisms mediating the clearance of arsenite-induced protein aggregation in *Saccharomyces cerevisiae*.” *Manuscript under revision.*

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INTRODUCTION

METAL BIOLOGY

Metals and metalloids are natural constituents of the earth's crust and their impact on biological systems ranges from essential to non-essential and highly toxic. Some metals (e.g., copper, iron and zinc) are essential to maintain various biochemical and physiological processes in mammals, whereas other metals (e.g., arsenic and cadmium) have no established biological functions and are toxic when present in excessive amounts (Hejna et al., 2018; Waldron et al., 2009). Living organisms have always dealt with metals and utilize a variety of homeostasis mechanisms that regulate the availability of essential metals and limit the harmful effects of toxic elements. Malfunction of metal homeostatic or detoxification systems may cause serious impacts on human health including organ damage, nervous system damage and cancer. The damaging effect of many metals is undisputed but relatively little is known about their molecular modes of action (Beyersmann and Hartwig, 2008; Hejna et al., 2018; Wysocki and Tamas, 2010).

ARSENIC

The toxic metalloid arsenic is naturally present in the environment and affects the health of millions of people worldwide (Meharg, 2004; Nordstrom, 2002). It is classified as a metalloid due to the chemical characteristics that are intermediate between metals and nonmetals (Garelick et al., 2008). Arsenic occurs in several oxidation states, mainly as trivalent arsenite [As(III)] and pentavalent arsenate [As(V)], and exhibits a wide range of solubility depending on the ionic environment and pH (Sattar et al., 2016; Souri et al., 2017). The environmental presence of arsenic derives from both natural and anthropogenic sources, and this metalloid can be found in the atmosphere, soils and rocks, natural waters and organisms. Many natural processes contribute to the environmental concentrations of arsenic, including volcanic

eruptions, dust storms, geothermal/hydrothermal activity and forest fires (Garelick et al., 2008). However, the anthropogenic activities have an important additional impact through mining, the use of arsenical-containing pesticides and herbicides, and combustion of fossil fuels. Although the use of arsenical compounds, such as pesticides and herbicides, have decreased significantly in the last few decades, the impact of these compounds on the environment will remain for years.

Human exposure to arsenic occurs through several paths, including air, soil, food and water. Arsenic enters the human body via ingestion of contaminated food and drinking water, inhalation of contaminated air and dust, and also potentially through dermal contact with environmental media (Joseph et al., 2015). Among the various sources of arsenic, contamination of drinking water poses the greatest threat to human health. The concentrations of arsenic in drinking water are variable, depending on local availability of surface water (rivers, lakes, reservoirs and ponds), rain water and groundwater (aquifers), where the latter has the highest aqueous arsenic concentrations (Smedley and Kinniburgh, 2002).

More than 100 million people worldwide are estimated to be exposed to this toxic metalloid in drinking water above the WHO recommended guideline of 10μg/L (Nurchi et al., 2020). The majority lives in southern Asia (e.g., Bangladesh, China, India and Vietnam) but also populations living in other areas (e.g., Poland, Hungary, Argentina and USA) are affected (Bjorklund et al., 2018; Kumar et al., 2021). Some of the problems with arsenic-contaminated groundwater have been recognized for considerable time. The earliest cases of health affects related to this were reported from a mining area of Poland in the 1890's and in 1910's arsenic-related health issues were documented in both Argentina and Taiwan. Bangladesh is the worst affected country with a mortality rate of 20 000 annually due to arsenic poisoning, and 50 million people are at risk of severe health problems (Smith et al., 2000). One way to provide safe water is the installation of arsenic filtering devices but the maintenance of these apparatus tends to be poor and the drinking water

remains therefore contaminated. Another strategy to combat the issue is the drilling of deep wells that bypass arsenic-tainted aquifers but this has produced mixed results (Bhattacharjee, 2007).

The dual role of arsenic

Acute arsenic poisoning is associated with nausea, vomiting and abdominal pain, whilst chronic arsenic poisoning (arsenicosis) is associated with a wide range of adverse health effects, such as skin lesions, peripheral vascular diseases, reproductive toxicity and neurological effects (Bhattacharjee, 2007; Naujokas et al., 2013; Nurchi et al., 2020; Tseng, 1977; Zhou and Xi, 2018). The International Agency for Research on Cancer (IARC) has confirmed the correlation between arsenic exposure and cancer of the skin, lung and bladder, whereas reports on the association with the liver, kidney and prostate cancer are limited. Studies from countries with arsenic contaminated drinking water, such as Bangladesh, Taiwan and Argentina, have provided key epidemiological evidence regarding the carcinogenicity of arsenic (Zhou and Xi, 2018). The first documented carcinogenic effect from chronic arsenic exposure was skin cancer (Hughes et al., 2011). Skin pigmentation and lesions, including skin cancer, are characteristic of arsenicosis from drinking water (Cohen et al., 2013). Other health-related issues from prolonged arsenic exposure are diabetes, cardiovascular disease and various neurodegenerative disorders (Isik et al., 2022; Naujokas et al., 2013; Nurchi et al., 2020). Diabetes has an increased incidence in populations exposed to arsenic, which may be caused by arsenite-dependent inhibition of glucose uptake through hexose permeases (Liu et al., 2004a; Tseng et al., 2002). Chronic arsenic exposure induces oxidative stress, which may cause deteriorations in structures and functions in the cardiovascular system and thereby aggravating cardiovascular pathology (Nurchi et al., 2020). Protein misfolding and aggregation are molecular hallmarks of certain age-related and neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease (Hartl et al., 2011; Stefani and Dobson, 2003). Arsenic, in the form of As(III), causes widespread protein aggregation

in living yeast cells (Jacobson et al., 2012) and interestingly, several yeast proteins that aggregate in the presence of As(III) have human homologues that are present in aggregates linked to protein folding disorders (Ibstedt et al., 2014). Hence, the effect of arsenic may contribute to the pathology of protein misfolding disorders.

The toxicity and adverse health effects associated with arsenic are undisputed, but this toxic metalloid is also used as a therapeutic agent (Sattar et al., 2016). In the past, arsenic-based medicines were used to treat several diseases, including ulcer and abscesses (Riethmiller, 2005). In 1786, Fowler's solution was prescribed as a tonic and eventually used against various diseases such as eczema, malaria, asthma and syphilis. Another arsenic-based drug, Salvarsan (magic bullet), was introduced in 1910 and effectively used against syphilis until the breakthrough of penicillin in the 1940's (Gibaud and Jaouen, 2010; Sattar et al., 2016). Today, a revival of arsenic as a therapeutic agent can be found in the medical treatment of acute promyelocytic leukemia (APL) and human African trypanosomiasis (HAT). Arsenic is also foreseen as a potential medical agent in other hematologic diseases (Bouteille et al., 2003; Chen et al., 2011).

Due to the dual aspect of arsenic as a public health concern but also as a therapeutic agent has spurred research into the molecular mechanisms that underlie the toxicity of this metalloid and the mechanisms cells use to develop resistance.

ARSENIC TOXICITY

The toxicity of a given metal depends on its mechanisms of uptake, oxidation state and speciation. Other parameters influencing toxicity include intracellular distribution and ligand preferences (Wysocki and Tamas, 2010). Arsenic occurs in several oxidation states exhibiting different biological properties and degrees of toxicity. The toxicity of arsenate [As(V)] is mainly correlated to its structural similarity to phosphate and can therefore replace the phosphate group in several

metabolic reactions. As(V) interferes with the synthesis of ATP by binding with ADP, which results in decrease of ATPs release in the cell (Hughes, 2002; Sattar et al., 2016). Arsenite [As(III)], which is considered as the more potently toxic form, has a high reactivity with sulfhydryl groups and can bind to cysteine residues in proteins, thereby altering their folding and activity (Hughes et al., 2011; Shen et al., 2013). Arsenite-exposed cells exhibit cell cycle arrest at G_1 and G_2/M phases. In yeast, the mitogen activated protein kinase (MAPK) Hog1 is important for the exit from arsenite-induced G_1 arrest (Migdal et al., 2008). Relatively little is known about the molecular mechanisms of how arsenic exerts its toxicity, but it has been reported that arsenic may interfere with redox balance, DNA repair and functions of proteins in cells.

Oxidative stress

Cells have evolved strategies to maintain the redox balance but oxidative stress arises when toxic levels of oxygen-derived reactive species are generated (Hughes, 2002). Different forms of reactive oxygen species (ROS) are collected under the abbreviation ROS including singlet oxygen ($O^{\bullet}$), superoxide anion ($O^{\bullet}{}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). ROS are highly reactive and can damage cellular macromolecules, leading to protein oxidation, lipid peroxidation and DNA damage (Drose and Brandt, 2012; Wysocki and Tamas, 2010). As(III) induces the production of ROS in human cells within 5 min after exposure (Liu et al., 2001). In mammalian cells, the toxicity of As(III) is associated with its ability to induce the generation of ROS and oxidative stress (Ruiz-Ramos et al., 2009; Tam et al., 2020). As(III)-induced oxidative stress and lipid peroxidation were detected in yeast mutants lacking As(III) detoxification or oxidative stress defense, indicating that this metalloid affects ROS levels in yeast as well (Wysocki and Tamas, 2010).

Oxidative stress alters the redox balance in the cell and glutathione (GSH) is an essential antioxidant that could counteract this stress. It has

been proposed that exposure to As(III) depletes intracellular levels of GSH and thus cause an altered redox state in the cell (Stohs and Bagchi, 1995). In yeast, chelation to GSH is a strategy to neutralize metals and metalloids, including As(III), and this process may lead to reduced cytosolic GSH levels. Observations that argue against GSH depletion as a toxicity mechanism, at least in yeast, is that the intracellular concentration of glutathione, 1-2mM under uninduced conditions, is much higher than the toxic concentration of As(III) at ≈100μM (Wysocki and Tamas, 2010). Another observation is that GSH levels are increased in response to As(III) exposure in yeast (Thorsen et al., 2007).

Inhibition of DNA repair

Maintenance of genetic information and thus the correct sequence of nucleotides in DNA is essential for replication, gene expression and protein synthesis. Environmental agents, such as metals and UV-light, can jeopardize the genomic integrity by inducing different DNA damage, which may lead to cell cycle arrest, apoptosis, mutagenesis and cancer. To reduce adverse consequences, different DNA repair systems have evolved to maintain genetic stability (Hartwig et al., 2003). The major pathway eliminating DNA base damage is excision repair, which consists of the nucleotide excision repair (NER) and the base excision repair (BER) (de Boer and Hoeijmakers, 2000; Tam et al., 2020). As(III) targets several key players in NER and BER systems by interfering with the expression levels of DNA repair genes or catalytic activities of proteins involved in DNA repair (Tam et al., 2020).

Arsenic alone is a weak mutagen and does not damage DNA directly but it enhances the mutagenicity of other carcinogens. In the presence of other genotoxic agents, such as X-rays and UV-light, arsenic causes increased mutagenicity and tumorigenesis in mammalian cells (Andrew et al., 2006; Muenyi et al., 2015). Arsenic's role in augmenting the mutagenicity of other carcinogens may be linked to its ability to inhibit the repair of DNA damage induced by these mutagens. However, As(III) can act as a direct inducer of DNA double-stand breaks in all phases of

the cell cycle in yeast (Litwin et al., 2013) but DNA repair functions were not enriched among yeast genes sensitive to As(III) (Thorsen et al., 2009).

Impact on proteins

There is a general consensus that proteins are the prime targets of heavy metals and arsenicals. Proteins are considered to be affected by metals and metalloids through two different ways; the toxic metal ions bind to free thiols or other functional groups in proteins, or replace essential metal ions in metalloproteins (Bánfalvi et al., 2011). Only the trivalent form of arsenic interacts with proteins, and this interaction is established exclusively via cysteine residues (Kitchin and Wallace, 2005). It has been shown that As(III) interacts with many proteins (Shen et al., 2013), and that binding can lead to enzyme inhibition. As(III) binding to β-tubulin inhibits tubulin polymerization for instance (Zhang et al., 2007).

It has also been demonstrated that As(III) induces widespread protein misfolding and aggregation by targeting nascent or non-folded proteins. As(III)-induced protein aggregation appears to be concentration-dependent, and the aggregates formed are distinct from processing bodies (P-bodies) and stress granules, which are structures that are known to form in the presence of As(III). The As(III)-induced aggregates contained a variety of proteins enriched in functions related to protein synthesis, metabolism and protein folding (Jacobson et al., 2012). Proteins with high translation rates and extensive physical interactions are more susceptible to aggregation during As(III) exposure (Ibstedt et al., 2014). Moreover, As(III)-aggregated protein species may form seeds that enhance the misfolding and aggregation of other proteins (Ibstedt et al., 2014; Jacobson et al., 2012). The impact arsenic has on protein homeostasis may contribute to its suspected role in protein misfolding diseases (Andersson et al., 2021; Lorentzon et al., 2021; Tamas et al., 2014).

ARSENIC UPTAKE AND EFFLUX PATHWAYS

Arsenic is considered as an environmental pollutant and no known organism has arsenic-specific uptake systems. All uptake pathways of arsenic are via plasma membrane permeases and channels. Their physiological functions include transport of essential metals and nutrients such as phosphate, glycerol and glucose, but arsenic mimics those natural substrates structurally and can therefore share their uptake systems (Garbinski et al., 2019).

Arsenic uptake

As(V) is chemically similar to phosphate and enters into most cells through phosphate transporters (Figure 1). This was originally shown in *E. coli* that has two phosphate uptake systems; the high-affinity Pst and low-affinity Pit transporters (Garbinski et al., 2019; Rosenberg et al., 1977). In yeast cells, the entry of As(V) is mediated by two high-affinity permeases, Pho84 and Pho89, and two low-affinity permeases, Pho87 and Pho90 (Wysocki and Tamas, 2010). Deletion of *PHO84* and *PHO87* genes resulted in As(V) tolerance, suggesting that As(V) uptake is mediated by these permeases (Bun-ya et al., 1996; Yompakdee et al., 1996). Moreover, cells lacking the endoplasmatic reticulum-localized protein Pho86, which is required for the trafficking of Pho84 to the plasma membrane, exhibited increased As(V) tolerance (Bun-ya et al., 1996; Lau et al., 2000). Consistently, lack of Gtr1, a cytoplasmic GTPase regulating Pho84-dependent phosphate transport also resulted in As(V) tolerance (Bun-Ya et al., 1992).

The main form of As(III) in solution is $As(OH)_3$, which structurally resembles glycerol, and enters cells through aquaglyceroporins (Porquet and Filella, 2007; Wysocki and Tamas, 2010). Aquaglyceroporins from several organisms, including bacteria (Meng et al., 2004), plants (Bienert et al., 2008), mammals (Liu et al., 2002) and humans (Liu et al., 2004b) have been shown to mediate As(III) uptake. In yeast, the main entrance pathway of As(III) into cells is mediated by the

aquaglyceroporin Fps1 (Figure 1). Deletion of the *FPS1* gene results in reduced As(III) uptake and profoundly increased resistance to As(III). The opposite is observed in yeast cells overexpressing a hyperactive *FPS1* allele; the cells accumulated more As(III) and were hypersensitive to this metalloid (Wysocki et al., 2001).

Fps1 regulates the intracellular level of glycerol in response to changes in osmolarity and belongs to the family of major intrinsic proteins (MIP) that comprises membrane channel proteins such as aquaporins and aquaglyceroporins. Notably, aquaglyceroporins are bidirectional channels that also can mediate the efflux of As(III) (Maciaszczyk-Dziubinska et al., 2012).

Hexose permeases are also involved in the uptake of As(III) and it has been suggested that three $As(OH)_3$ molecules can form a ring-structure similar to hexose sugar, which could be recognized by these permeases. In the absence of glucose, hexose transporters mediate the major part of As(III) uptake, whereas Fps1 accounts for ≈20% of the As(III) uptake into cells (Liu et al., 2004a).

Arsenic efflux

Metalloids have a tendency to accumulate in cells and all organisms have evolved several mechanisms to minimize their toxicity in the cytosol (Ali et al., 2009). Once As(V) enters the cytoplasm through phosphate transporters (Figure 1), it undergoes a reduction to As(III) by arsenate reductases. In yeast, this reduction is mediated by the Acr2 arsenate reductase belonging to the rhodanese/Cdc25 phosphate family (Mukhopadhyay et al., 2000). Other members of the Cdc25 phosphate family have been identified to mediate As(V) reduction in plants (Duan et al., 2007) and humans (Bhattacharjee et al., 2010). The most important arsenic detoxification pathway in yeast is the export of As(III) via the plasma membrane antiporter Acr3 (Figure 1) (Ghosh et al., 1999; Wysocki et al., 1997). Acr3 is a well characterized member of the

arsenical resistance-3 (Acr3) family, which belongs to the superfamily of bile/arsenite/riboflavin transporters (BART) (Mansour et al., 2007).

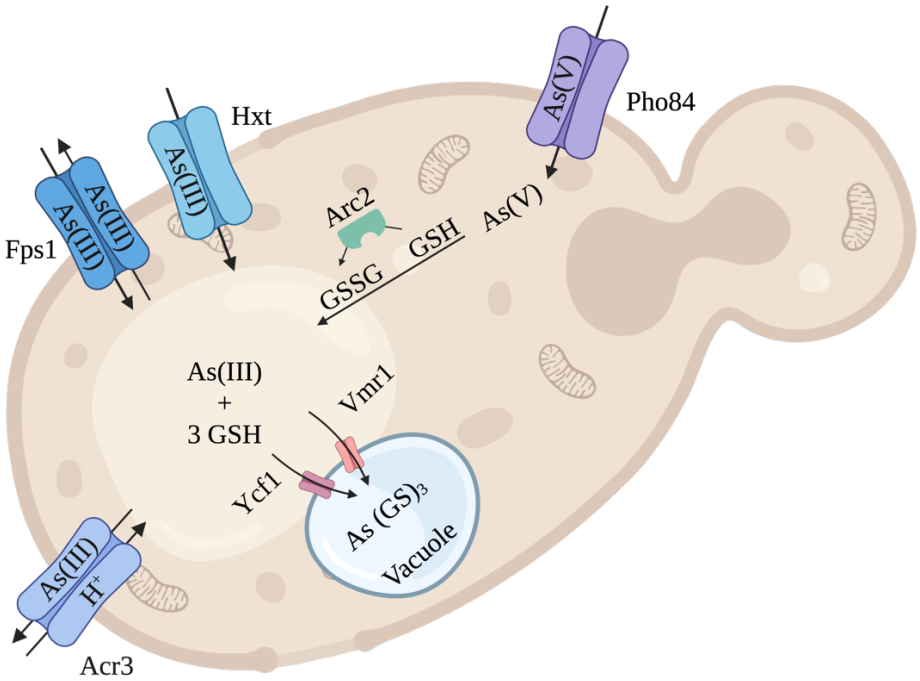

Figure 1. Arsenic uptake and efflux pathways in yeast. Uptake of As(III) is facilitated mainly through the aquaglyceroporin Fps1 but in the absence of glucose As(III) can also enter the cell via hexose permeases Hxt. As(V) enters the cell through phosphate transporters, such has Pho84, followed by reduction to As(III) by the arsenate reductase Acr2. As(III) is transported out of the cell through the antiporter Acr3 or Fps1. Moreover, intracellular GSH can bind to As(III) and form an $As(GS)_3$ complex that can be sequestered into the vacuole by the ABC-transporters Ycf1 and Vmr1. Adapted from (Maciaszczyk-Dziubinska et al., 2012). Created with BioRender.com

The *ACR3* gene was isolated from a multicopy plasmid conferring high-level As(III) tolerance when overexpressed in yeast (Bobrowicz et al., 1997). Cells lacking *ACR3* exhibited hypersensitivity to arsenic and accumulated more As(III) compared to wild type cells, whereas

overexpression of *ACR3* resulted in decreased cytosolic arsenic levels and increased tolerance (Thorsen et al., 2006; Wysocki et al., 1997). These observations established that Acr3 confers arsenic tolerance through As(III) export from the cytosol (Maciaszczyk-Dziubinska et al., 2012; Wysocki and Tamas, 2010).

ARSENIC DETOXIFICATION SYSTEMS AND THEIR REGULATION

The major pathway of arsenic detoxification is extrusion from cells through Acr3 or sequestration in intracellular compartments via transporters located on the vacuolar membrane. Another detoxification strategy involves metal chelation to specific proteins and peptides, such as GSH, and the resulting complex can be recognized by transporters for export and/or vacuolar sequestration (Rosen, 2002; Wysocki and Tamas, 2010).

Regulation of Acr2 and Acr3

During arsenic exposure, approximately 500 genes are robustly induced in yeast (Thorsen et al., 2007). The expression of proteins involved in arsenic detoxification, such Acr2 and Acr3, is tightly regulated by the transcription factor *YAP8* (also known as *ACR1/ARR1*) (Wysocki et al., 2004). *ACR2* and *ACR3* (also called *ARR2* and *ARR3*) share a common promoter and together with *YAP8* form a cluster of arsenic resistance genes (Bobrowicz et al., 1997). In the absence of As(V) or As(III), the expression of *ACR2* and *ACR3* is low, while in the presence of arsenicals both genes are up-regulated in a Yap8-dependent manner (Wysocki et al., 2004). Yap8 senses As(III) by directly binding to this metalloid *in vivo* and *in vitro*, and this physical interaction with As(III) triggers a conformational change that results in a transcriptional response (Kumar et al., 2015).

Vacuolar sequestration

Sequestration of metals into vacuoles is a common detoxification strategy in eukaryotes . In yeast, GSH forms a complex with As(III) in the ratio 3:1, annotated as the $As(GS)_3$ complex (Rai and Cooper, 2005). This complex is recognized by the ABC transporters Ycf1 and Vmr1 that mediate the vacuolar sequestration (Figure 1). How Ycf1-mediated detoxification is regulated is not well understood, but the role of Ycf1 seems to be minor when the extrusion transporter Acr3 is functional while Ycf1 becomes important in cells lacking *ACR3* (Ghosh et al., 1999; Wysocki et al., 1997)

Glutathione chelation

GSH is a key player in the defense against oxidative stress but it also contributes to metal detoxification in several ways (Grant et al., 1997). As mentioned above, GSH can form complexes with metals that are substrates for proteins involved in vacuolar sequestration, but studies have also shown that GSH can protect cells against metal-induced oxidation as well as GSH may bind to reactive sulfhydryl groups of proteins and in that way protecting them from damaging metal binding (Grant, 2001; Pompella et al., 2003). GSH is also involved in extracellular metal chelation. Yeast cells exposed to As(III) export significant amounts of GSH, which in turn can chelate As(III) and form $As(GS)_3$ complex that cannot enter the cell. Thus, beside its intracellular role, GSH serves an extracellular defense function to decrease arsenic accumulation in the cytosol (Thorsen et al., 2012). The synthesis of GSH is robustly increased upon exposure to As(III). To enable this, yeast cells appear to redirect sulfur metabolism into GSH biosynthesis instead of into protein biosynthesis (Lafaye et al., 2005; Thorsen et al., 2007).

Protein quality-control

As(III) triggers widespread protein misfolding and aggregation *in vivo* (Jacobson et al., 2012). Conserved protein quality-control mechanisms protect cells from harmful protein aggregates. These quality-control

systems include; (1) molecular chaperones that assist the folding of proteins into their native structure and (2) protein degradation pathways, such as the proteasome, and lysosomal and autophagic processes, that eliminate misfolded and aggregated proteins (Buchberger et al., 2010; Hartl et al., 2011). In response to metal exposure, expression of chaperones and proteasome-encoding genes are activated, probably to enhance the protein folding and degradation capacity respectively, whereas the expression of genes encoding aggregation-prone proteins is downregulated (Jacobson et al., 2012; Thorsen et al., 2007; Thorsen et al., 2009).

SACCHAROMYCES CEREVISIAE AS A MODEL ORGANISM

The budding yeast *Saccharomyces cerevisiae* is a well-established model organism and has proved to be a powerful tool to study the molecular details of metal action and detoxification strategies, as well as the molecular biology of protein-misfolding diseases. *S. cerevisiae* is a unicellular eukaryote, and thus it contains membrane-bound organelles, such as a nucleus, and mitochondria. Fundamental cellular mechanisms of replication, recombination, cell division and metabolism are highly conserved from yeast to mammals. Moreover, 60% of yeast genes have a homologue or share a conserved domain with a human gene. The relatively small genome size and ease of culture makes yeast suitable to high-throughput screening and the data output of this technology has led to the development of new fields of computational biology.

The combination of having a fully sequenced genome, an efficient homologous recombination system and available gene deletion libraries has given *S. cerevisiae* the most advanced selection of genetic tools available for any eukaryotic organism. This has resulted in landmark discoveries in gene regulation mechanisms and other cellular processes over the past several decades (Duina et al., 2014).

PROTEOSTASIS

The cellular proteome – the entire pool of proteins residing inside cells and in their plasma membranes – is tightly regulated to ensure that each given protein is properly synthesized, folded, transported to its correct subcellular location and degraded (Balch et al., 2008). Several cellular systems, including molecular chaperones and proteolytic machineries and their regulators, operate together to maintain protein homeostasis (proteostasis) - the stability of the proteome (Morimoto and Cuervo, 2009). These protein quality-control systems coordinate protein synthesis with polypeptide folding, maintenance of protein conformation and protein degradation (Figure 2). However, when the generation of misfolded proteins exceeds the refolding and degradation rate of the protein quality-control system, protein aggregates accumulate. This exhaustion of the protein quality-control can occur from single, severe conditions but also from the combination of different moderate events, which on their own will not overwhelm the system. Conditions that may cause perturbations in the proteostasis network include mutations in the protein quality-control system, defects in translation, environmental stress conditions and ageing (Tyedmers et al., 2010).

Sustaining proteome balance can be a challenging task when cells are exposed to external and endogenous stresses, and the collapse in protein homeostasis leads to the accumulation of damaged and misfolded proteins (Hipp et al., 2019). Misfolded and aggregated proteins are harmful to cells, not only due to loss-of-function of the non-native proteins, but mostly due to gain-of-function of protein aggregates that interfere with essential processes in the cell (Winklhofer et al., 2008). The importance of proteostasis is highlighted by the many diseases that are associated with protein misfolding, such as amyotrophic lateral sclerosis (ALS), Alzheimer's and Huntington's disease, where proteins of aberrant structure interfere with cellular systems and may cause cell death (Balch et al., 2008; Vilchez et al., 2014).

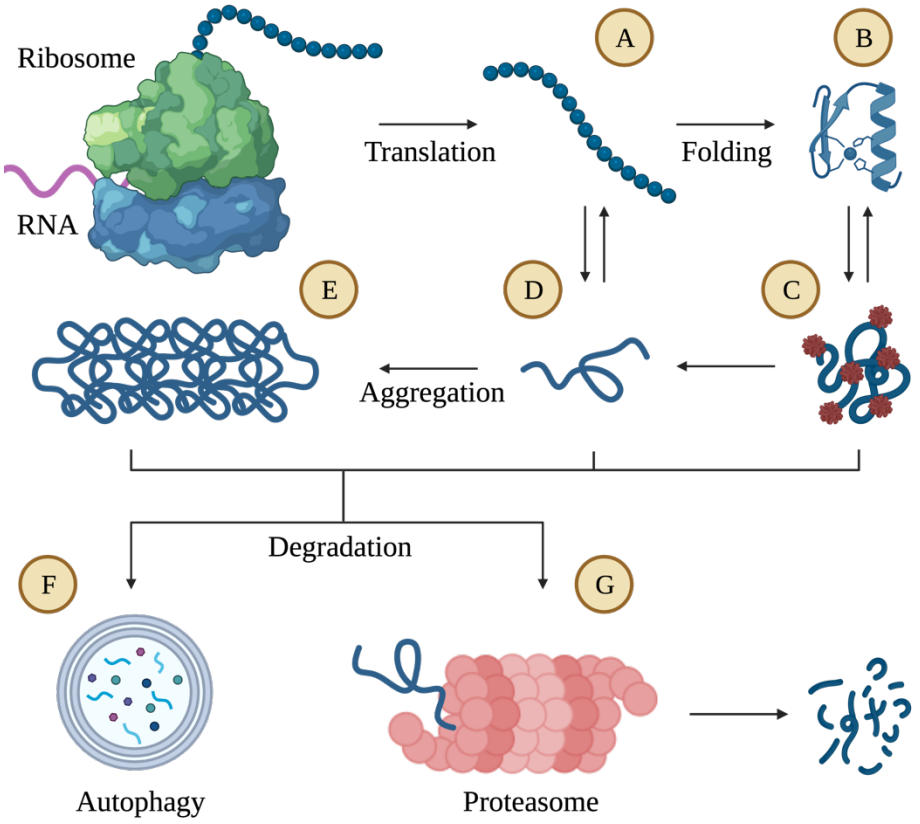

Figure 2. Protein quality-control systems. Newly synthesized polypeptides (A) are aided by molecular chaperones during and after translation to reach their native state (B). Under stress conditions, proteins can become damaged (C) or misfolded (D). These proteins can form aggregates (E) or be degraded by the autophagy pathway (F) or through the ubiquitin-proteasome system (G). Created with BioRender.com

Protein synthesis and folding

Proteins are versatile and structurally complex macromolecules that are involved in almost every biological processes. Most proteins fold into a defined three-dimensional structure, their native conformation, to gain functional activity (Hartl et al., 2011). The amino acid sequence of a protein holds the information required to reach the native structure, and

proteins can therefore, in principle, fold autonomously and attain its native state without assisting chaperones (Anfinsen, 1973; Ellis, 1997; Frydman, 2001).

The protein folding process is driven by hydrophobic forces which in a polar environment, such as the cytosol, results in non-polar amino acids being buried within the core of a protein to reach a final functional conformation that is thermodynamically stable (Hartl and Hayer-Hartl, 2002). Throughout this process, the newly synthesized polypeptide undergoes a number of intermediary structures on its way to the native state. Due to the molecular crowdedness in the cytosol, folding intermediates are at risk of harmful interactions that could trap the protein in any of these states or lead to aggregation (Hartl et al., 2011). Consequently, newly synthesized polypeptides are in need of assistance during the folding process, and a family of molecular chaperones has evolved to safeguard the nascent chains while they acquire their native structure (Feldman and Frydman, 2000).

Molecular chaperones in protein quality-control

The cellular chaperone machinery prevents the misfolding and aggregation of non-native proteins during folding and under conditions of stress, such as high temperature and oxidative stress (Hartl and Hayer-Hartl, 2002). Molecular chaperones are defined as proteins that assist other proteins to reach their functional conformation, without themselves being part of the final structure (Dobson, 2004).

Many chaperones, though constitutively expressed, are greatly increased in abundance during stress conditions and are classified as stress proteins or heat-shock proteins (Hsps) that are regulated by the heat shock transcription factor (HSF) (Wegele et al., 2006). Chaperones are classified according to their molecular weight, such as Hsp40, Hsp70, Hsp90 and Hsp100 (Hartl and Hayer-Hartl, 2009). Most of these chaperones recognize hydrophobic residues and/or unstructured backbone regions in their substrates - structural features, that normally

are buried in native proteins, typically exposed by non-native proteins. Molecular chaperones are heavy-duty machines that operate on a wide range of substrates, unlike e.g., enzymes with their precise and finely tuned active sites (Saibil, 2013). Chaperones, such as the Hsp70s and the chaperonins, promote the folding process via cycles of substrate binding and release regulated by their ATPase activity. Chaperone binding may hinder intermolecular aggregation by directly shielding the interactive hydrophobic surfaces of non-native polypeptides and it may also prevent or reverse intramolecular misfolding (Frydman, 2001; Hartl and Hayer-Hartl, 2002). Certain chaperones of the Hsp100 family have the ability to unfold proteins or disassemble protein aggregates through an ATP-dependent mechanism (Ben-Zvi and Goloubinoff, 2001).

Ribosome-associated chaperones

Ribosomes are molecular machines that synthesize polypeptides based on the genetic information delivered by mRNAs (Gautschi et al., 2002). The folding of nascent proteins is a co-translational process, which starts as soon as a sufficient length of the polypeptide has been synthesized and has exit the ribosomal tunnel. Chaperones involved in the folding of cytosolic proteins can be categorized into two groups: (1) the first group consists of chaperones that bind to both the ribosome and the emerging polypeptide, thereby safeguarding early folding during translation and (2) the second group comprises chaperones that interact with newly synthesized proteins, such as members of the Hsp40 and Hsp70 families. Both ribosome-associated and cytosolic chaperones cooperate to form a robust system for protein folding (Preissler and Deuerling, 2012).

A subset of molecular chaperones is specialized for *de novo* protein folding and installed at the ribosome where they assist protein folding, at the earliest possible time, when the nascent chains are just reaching the cytoplasm (Shen et al., 2019). The Nascent polypeptide-Associated Complex (NAC) is widely conserved from Archaea to man and has been described as the initial factor that interacts with nascent polypeptides as they emerge from ribosomes. Archaea only have a homodimeric NAC

formed by two α-subunits, whilst eukaryotes form an α-β heterodimer. It is known that the N terminus of the eukaryotic β-NAC subunit harbors a conserved ribosome-binding motif and mediates association of the complex with ribosomes. However, both NAC subunits can interact with nascent polypeptides but the substrate binding sites in the individual subunit are not yet identified (Preissler and Deuerling, 2012). The role of NAC is not completely clear, but is possibly similar to the bacterial ribosome-associated Trigger Factor (TF) that binds to nascent polypeptide chains and protects them from proteolysis (Frydman, 2001; Hartl and Hayer-Hartl, 2009; Rospert et al., 2002). Moreover, loss of NAC function in yeast results in no detectable phenotype, whereas the absence of NAC aggravates the growth defects of cells lacking ribosome-associated Hsp70 homolog Ssb and significantly enhances aggregation of newly synthesized proteins. This suggest that NAC might be a component of the chaperone network that is dedicated to support folding processes (Koplin et al., 2010).

The Hsp70 proteins in bacteria and higher eukaryotes can act both co – and post-translationally, whereas yeast has cytosolic Hsp70 homologs, Ssb1 and Ssb2, that are specialized in nascent polypeptide binding. This function of Ssb1 and Ssb2 appears to be mediated by another Hsp70, Ssz1, that forms a ribosome-associated complex (RAC) together with zuotin (Zuo1), a Hsp40 partner of the Ssb proteins. The Ssb proteins and RAC act together in stabilizing nascent chains (Hartl and Hayer-Hartl, 2002; Rospert et al., 2002). The emerging polypeptide chain from the ribosome can interact with either NAC or Ssb-RAC, or both (Koplin et al., 2010).

The Hsp70/40 system

Once released from the ribosome, the newly synthesized polypeptide can interact with cytosolic Hsp70s, which assist the folding process. Hsp70 is the most abundant chaperone and coordinates cellular functions by directing substrates for unfolding, refolding, disaggregation or degradation (Saibil, 2013). The substrates of Hsp70

are usually partially unfolded proteins with exposed hydrophobic residues, and the binding of Hsp70 to these non-native proteins facilitates correct folding and prevents formation of dysfunctional aggregates (Rosenzweig et al., 2019).

The Hsp70 proteins consist of an ATPase domain, a substrate-binding domain and a C-terminal domain and together with co-chaperones from the Hsp40 family function by binding and releasing in an ATP-dependent manner. The Hsp40 chaperones are a diverse group of proteins that aids and provides specificity to Hsp70 folding and refolding activities (Kampinga and Craig, 2010). Hsp40 interacts directly with unfolded polypeptides and recruits them to an ATP-bound Hsp70, and stimulates a high-affinity binding between the polypeptide and the Hsp70 through ATP hydrolysis. Sis1 is an essential yeast Hsp40 that regulates the activity of both cytosolic and ribosome-bound Hsp70. The hydrolysis of ATP to ADP results in a conformational change in Hsp70 that closes the substrate-binding domain and traps the polypeptide. In yeast, Sse1 and Sse2 are Hsp110 chaperones and function as nucleotide exchange factors (NEFs) that release the trapped substrate from Hsp70 through the exchange of ADP to ATP (Figure 3) (Hartl and Hayer-Hartl, 2009; Rosenzweig et al., 2019). This cycle can be repeated several times until folding is completed (Mayer and Bukau, 2005).

In yeast, there are fourteen Hsp70 proteins that share a sequence similarity of 50-90%. The major cytosolic Hsp70 family consists of four Ssa proteins (Ssa1-Ssa4). These are functionally redundant to some degree as expression of at least one Ssa isoform is essential for cell viability (Hasin et al., 2014; Werner-Washburne et al., 1987). Although other cytosolic Hsp70s cannot replace the function of Ssa proteins, the four isoforms can compensate for each other (Frydman, 2001). Constitutively expressed Ssa1 and Ssa2 are 97% identical, whereas the stress-inducible Ssa3 and Ssa4 are 87% identical to each other and share an identity of 80% with Ssa1-2. There are functional differences between the constitutive and the inducible Ssa isoforms, where the latter

forms confer increased resistance to heat stress and have an increased capacity to propagate prions (Hasin et al., 2014).

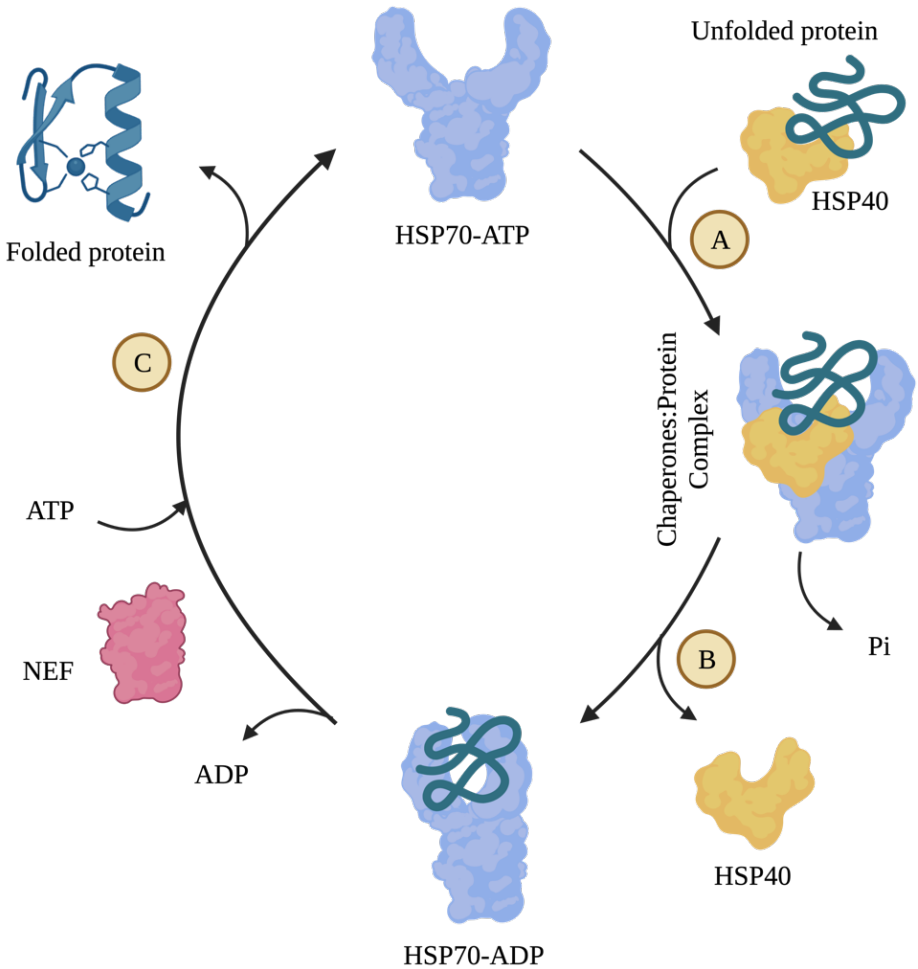

Figure 3. The Hsp70/40 system. Hsp40 (Sis1) interacts directly with unfolded polypeptides and recruits them to an ATP-bound Hsp70, and stimulates a high-affinity binding between the polypeptide and the Hsp70 through ATP hydrolysis (A). The hydrolysis of ATP to ADP results in a conformational change in Hsp70 that closes the substrate-binding domain and traps the polypeptide (B). In yeast, Sse1 and Sse2 are Hsp110 chaperones and function as nucleotide exchange factors (NEFs) that release the trapped substrate from Hsp70 through the exchange of ADP to ATP (C). Created with BioRender.com

Hsp90

Another abundant and ubiquitous chaperone is the Hsp90 protein that has diverse biological roles but its mechanism of action has remained elusive. It is a highly dynamic protein and has several hundred client proteins, which makes Hsp90 a central modulator of many cell processes including stress regulation, protein folding, DNA repair, neuronal signaling and the immune response (Schopf et al., 2017). Like other heat-shock proteins, Hsp90 can bind to non-native proteins and thereby prevent misfolding and/or aggregation. Hsp90 appears to work downstream of Hsp70 and interacts with partially folded intermediates or even fully folded proteins, thus providing stabilization and aid in the final maturation of the substrate. Yeast possesses two cytoplasmic Hsp90s, the constitutive Hsc82 and the stress-inducible Hsp82 (Girstmair et al., 2019). Although being essential, it has been shown that Hsp90 is not required for the folding of the majority of yeast proteins but it is needed for a specific subset of proteins that have difficulty reaching their native structures (McClellan et al., 2007; Nathan et al., 1997).

Chaperonins

The chaperonins are a family of barrel shaped multi-subunit molecular chaperones with a central cavity where folding, of substrate proteins up to ≈ 60 kDa, occurs in an ATP-dependent manner (Bukau and Horwich, 1998). They are present in every kingdom of life and all chaperonins share the same structural architecture, containing an apical, intermediate and equatorial domain. The chaperonins are categorized into two groups based on their sequence homology. Group I is composed of the bacterial chaperonin GroEL and its co-chaperonin GroES, as well as the chaperonins from endosymbiotic organelles with their Hsp10 co-chaperonins. Group II includes the archael thermosome and eukaryotic CCT (chaperonin containing TCP1; also known as TriC) and these chaperonins are independent of co-chaperonins (Hartl, 1996; Saibil, 2013). The major CCT folding substrates are the abundant cytoskeletal

proteins actin and tubulin, which are dependent on CCT to reach their native structure (Spiess et al., 2004; Sternlicht et al., 1993).

Arsenic has been shown to interfere with CCT both *in vivo* and *in vitro* in yeast, thus affecting the folding of actin and tubulin, resulting in disruptions in the cytoskeleton (Pan et al., 2010; Thorsen et al., 2009).

Small heat-shock proteins

The small heat-shock proteins (sHsps) are a family of molecular chaperones that lack an ATPase domain. They are characterized as small proteins, with a molecular weight between 12 and 43 kDa, containing a conserved C-terminal α-crystallin domain. (de Jong et al., 1998; Treweek et al., 2015). sHsps interacts with non-native proteins through exposed hydrophobic residues in order to stabilize the polypeptide and prevent further misfolding and/or aggregation (Sharma et al., 1997). Therefore, sHsps act early in the chaperone processing of non-native proteins, often prior to the other ATP-dependent chaperone complexes (Bakthisaran et al., 2015; Haslbeck and Vierling, 2015). In yeast, Hsp26 and Hsp42 are the main cytosolic sHsps (Haslbeck et al., 2020). Hsp26 functions predominantly at heat shock temperatures, whereas Hsp42 has a ubiquitous activity with and without stress (Haslbeck et al., 2004).

PROTEIN AGGREGATION

Cells of all kingdoms of life have evolved an elaborate protein quality-control system that prevents the accumulation of misfolded and/or aggregated proteins (Figure 2). The balance between the speed of translation, the rate of protein folding and the stability of that fold determines the amount of proteins that reaches the final native structure, which is critical as only correctly folded proteins function properly (Bollen et al., 2021). When this balance is perturbed, proteins can unfold and/or misfold, leading to exposed hydrophobic residues that trigger protein aggregation. Through hydrophobic interactions un- and misfolded proteins can associate in the crowded intracellular

environment, resulting in the formation of protein assemblies called protein aggregates (Balchin et al., 2016). These aggregates often begin as small soluble oligomers and can grow into large insoluble structures, manifesting the final result of disturbances of proteostasis. Protein aggregation generally impairs protein activity or cause other proteins to aggregate and can thus impair critical cellular functions, such as growth and survival, that eventually may lead to cell death (Cox et al., 2020; Stefani and Dobson, 2003). Cells have evolved several different systems to deal with protein aggregates, which include disaggregation followed by either refolding or proteasomal degradation, autophagic clearance (Mogk et al., 2018) and secretion of aggregates as exophers into the extracellular environment (Melentijevic et al., 2017). As an intermediate to these processes, cells can sequester non-native proteins into different intracellular deposition sites (Kaganovich et al., 2008).

Protein aggregates and cellular toxicity

It has been suggested that aggregation is an intrinsic attribute of all proteins and that proteins are only soluble at concentration levels corresponding to their expression levels in the cell. In line with this, predicted aggregation-prone yeast proteins are kept at concentrations below a critical threshold that would prevent their aggregation. The expression of the proteins that were found to aggregate in yeast during arsenic stress was downregulated in the presence of the metalloid (Ibstedt et al., 2014). Downregulation of these proteins might protect the cell from misfolding and aggregation, but this remains to be demonstrated (Tamas et al., 2014).

The toxic nature of protein aggregates has been a matter of debate and it has been suggested that aggregate toxicity depends on certain aspects including localization, compartmentalization, the reactive surface and the stability of the aggregate (Mogk et al., 2018). The mechanisms of aggregate toxicity comprise physical damage to membranes (Lashuel et al., 2002), impairment of transport processes (De Vos et al., 2008) and nucleo-cytoplasmic trafficking (Grima et al., 2017), translation

impairment (Smith and Mallucci, 2016) and co-aggregation of essential proteins including transcription factors (Chu et al., 2007). Moreover, trapping of components of the protein quality-control network, such as chaperones, may lead to proteostasis collapse and that in turn can be a contributor to the self-propagating nature of aggregate toxicity (Hipp et al., 2014). It has been shown that several chaperones co-sedimented with arsenite-induced protein aggregates in yeast, indicating that arsenite promotes protein misfolding *in vivo*. In addition, arsenite-induced aggregates can act as seeds recruiting other labile proteins to misfold and aggregate (Jacobson et al., 2012).

Protein aggregates can adopt different structural conformations and sizes, and were initially classified as either insoluble protein aggregates that remain amorphous or highly structured prefibrillar aggregates, which can mature into amyloid fibrils and act as seeds for other proteins to adopt an amyloid configuration (Tyedmers et al., 2010). This broad grouping has now expanded to also include: soluble oligomers, protofibrils and other superstructural variants including spherulites and particulates. The amyloid fibril structure, with β-sheets running perpendicular to the axis of the fibril, is a classical signature of several neurodegenerative diseases, which forms through a nucleation-dependent mechanism (Cox et al., 2020). Those disorders associated with amyloid-like aggregates include a range of sporadic, familial or transmissible degenerative diseases, some of them affect the brain and the central nervous system (e.g. Alzheimer's and Parkinson's diseases), whilst others affect peripheral tissues and organs (e.g. systemic amyloidosis) (Dobson, 2001). The presence of amyloids in the tissues and organs is the hallmark of all these disorders, suggesting a causative correlation between aggregate formation and pathological symptoms (Kelly, 1998; Reilly, 1998). The causative link between amyloid formation and disease is widely accepted due to the large number of biochemical and genetic studies that support the hypothesis (Stefani and Dobson, 2003). It is very likely that the impairment in cellular functions,

detected in neurodegenerative diseases, follows directly from the interaction of aggregated proteins with cellular components.

In addition to the undisputed detrimental effects of amyloids, it has also been suggested that the precursors of amyloid fibrils can be toxic to cells. Experimental data suggest that the pre-fibrillar aggregates are highly toxic to cells rather than the mature fibrils into which they often develop. A number of reports concerning Aβ peptides and α synuclein indicate that these early aggregates are the most toxic species (Bhatia et al., 2000; Conway et al., 2000; Nilsberth et al., 2001) and this may provide an explanation for the lack of correlation between the density of fibrillar plaques in the brains of victims of Alzheimer's disease and the severity of symptoms (Stefani and Dobson, 2003).

Amyloids have strongly been associated with cell dysfunction and toxicity, as stated above, but that is not always the case. In contrast to the non-functional amyloid aggregates, the endogenous amyloids in bacteria have been linked to functional protein assemblies. For instance, the intracellular replication initiation factor RepA has been demonstrated to form amyloid oligomers that inhibits plasmid replication. Moreover, extracellular bacterial amyloid fibrils appear to serve as structural elements and adhesion factors of the extracellular matrix and are involved in host colonization (Schramm et al., 2020). Functional amyloids have also been described in yeast, the RNA-binding protein Rim4 forms amyloid aggregates that repress translation of *CLB3*, a cell cycle progression gene, and other mRNAs. The amyloid form of Rim4 sequesters mRNAs that are toxic if translated early in meiosis but are essential for late meiotic events. Regulated phosphorylation of the intrinsically disordered C-terminal domain of Rim4 results in amyloid disassembly and subsequently the release of late meiotic mRNA and degradation of Rim4 (Berchowitz et al., 2015; Carpenter et al., 2018; Fassler et al., 2021). Another example of regulated functional amyloid is the human melanocyte protein Pmel17, whose amyloid-forming conformation is pH-dependent and requires the mildly acidic environment found in the melanosome lumen. Pmel17

fibrils disassociate at neutral pH to regenerate monomers. By means of this aggregation/disaggregation cycle cells can prevent Pmel17 fibrils in the cytosol, thus circumventing their toxicity (Fassler et al., 2021; McGlinchey and Lee, 2018).

STRESS GRANULES AND P-BODIES

Cells are constantly endangered by various environmental stresses, including high temperature, metals and oxidative stress, that can affect proteostasis (Hartl and Hayer-Hartl, 2009; Sharma et al., 2008; Tamas et al., 2014; Tyedmers et al., 2010).

Cells can counteract proteotoxic stress by repressing bulk translation, mediated through phosphorylation of the eukaryotic initiation factor 2 alpha (elF2α), and thereby reducing the load of endangered protein, resulting in translational arrest, polysome disassembly and formation of stress granules (SGs) (Aulas et al., 2017; Wolozin and Ivanov, 2019). SGs are dynamic non-membranous cytoplasmic organelles consisting of messenger ribonucleoprotein complexes (mRNPs), RNA-binding proteins (RBPs), mRNA-associated translation initiation components, and several additional proteins including misfolded proteins (Protter and Parker, 2016). These granules are formed transiently to reprogram RNA translation under stress conditions by affecting mRNA function and localization, however an excessive formation and persistence of SGs has been implicated in the pathogenesis of a number of human diseases including cancer and neurodegenerative disorders (Anderson and Kedersha, 2006; Anderson et al., 2015; Buchan, 2014). SGs can interact with P-bodies, another dynamic cytoplasmic granule consisting of non-translating mRNAs, translation repressors and proteins involved in mRNA degradation, such as the decapping protein Dcp2 (Balagopal and Parker, 2009; Swisher and Parker, 2010). In yeast, it has been shown that both SGs and P-bodies are formed upon As(III) exposure (Jacobson et al., 2012).

CLEARANCE AND DEGRADATION OF PROTEIN AGGREGATES

Protein quality-control systems monitor the functionality of the proteome and protect cells against protein aggregates during stress conditions and the ageing process. When the activity of chaperones is insufficient to maintain proteome stability, misfolded proteins and aggregates may accumulate and impair cell functions. Damaged, misfolded and aggregated proteins can be degraded by the ubiquitin proteosome system (UPS) or via the autophagy-lysosome pathway (Figure 2) (Tyedmers et al., 2010). In addition, existing protein aggregates can be resolubilized (Neuwald et al., 1999) as well as sequestered into intracellular deposit sites (Kaganovich et al., 2008).

IPOD and JUNQ

The principles of aggregate deposition appear evolutionarily conserved from bacteria to mammalian cells. Cells can sequester aggregated proteins to specific deposit sites, seemingly to protect the intracellular environment from potentially toxic protein species (Rothe et al., 2018; Tyedmers et al., 2010). Upon stress, misfolded and aggregated proteins can be found at several sites throughout the yeast cytosol, termed stress foci, Q-bodies or CytoQ (Escusa-Toret et al., 2013; Spokoini et al., 2012). These aggregated proteins have been shown to fuse and accumulate at two distinct locations in the cell under prolonged stress (Spokoini et al., 2012). One inclusion was detected in proximity to the nucleus and is called the juxta-nuclear-quality-control (JUNQ). Cells can relocate misfolded proteins that are ubiquitylated to JUNQ, where they are processed for degradation, whereas damaged proteins and amyloid fibrils are associated with the peripheral insoluble-protein-deposit (IPOD) (Kaganovich et al., 2008). This compartment was later shown to be located to the surface of the vacuolar membrane (Spokoini et al., 2012). The localization of JUNQ has also been questioned and studies have suggested that this quality-control site might actually reside *inside* the nucleus, therefore the name IntraNuclear quality-control

(INQ) was suggested (Miller et al., 2015). It is still under debate whether the JUNQ and the INQ represent identical or independent structures (Rothe et al., 2018).

The regulation of protein sorting to quality-control compartments is still not fully known. It was first suggested that JUNQ harbored proteins that had the potential to be refolded or degraded, whilst the IPOD contained terminally damaged proteins and amyloids (Kaganovich et al., 2008). The clearance of the proteins in IPOD has been shown to be dependent on the disaggregase activity of Hsp104, which argues against the notion that the proteins in this quality-control compartment are terminally misfolded (Miller et al., 2015). It has also been suggested that the ubiquitination status of a misfolded protein functions as a sorting signal, an increased ubiquitination enhanced the direction of proteins towards the JUNQ (Kaganovich et al., 2008). The amyloidogenic yeast protein Rnq1, that exclusively localizes to the IPOD, could partly be redirected to JUNQ by the addition of a ubiquitination signal. Whether metal-induced protein aggregates are sequestered to specific deposit sites remains to be investigated.

Hsp104

Studies on the Hsp104 disaggregase in yeast have demonstrated the importance of protein disaggregation. Hsp104 does not prevent the formation of protein aggregates or target substrate proteins for proteolysis, but functions as a disaggregase that mediates the resolubilization of proteins from existing aggregates (Parsell et al., 1994). Because of its localization to protein aggregates, Hsp104 has been used as a marker to study cellular aggregation and disaggregation (Glover and Lindquist, 1998). Cells lacking functional Hsp104 are unable to develop thermotolerance (Sanchez and Lindquist, 1990) and inversely, the upregulation of Hsp104 alone is sufficient to induce tolerance to severe heat (Lindquist and Kim, 1996). This thermotolerance has been shown to be dependent on Hsp104's ability to disassemble aggregated proteins (Parsell et al., 1994). Similarly, the

bacterial homolog ClpB is required for heat resistance (Squires et al., 1991).

Both ClpB and Hsp104 are Hsp100 members of the AAA+ (ATPases associated with diverse cellular activities) ATPase family and form large hexameric ring-structures. They can unfold bound substrates or remodel and dissociate protein complexed through ATP hydrolysis (Mogk et al., 2018). Conserved AAA domains mediate ATP binding and hydrolysis, as well as oligomerization, which is needed to form the active hexameric structure (Katikaridis et al., 2021). ClpB/Hsp104 disaggregate aggregated proteins by threading them through the central pore of the hexamer (Haslberger et al., 2008). The N-terminal domain of Hsp104 is required for initial substrate interaction (Rosenzweig et al., 2015), whereas the M-domain is essential in regulating disaggregation activity (Heuck et al., 2016; Oguchi et al., 2012).

Co-operation with the Hsp70/40 system (Ssa1 and Ydj1) is necessary in order to return the substrate proteins to their native conformations (Glover and Lindquist, 1998; Shorter and Lindquist, 2008). The interaction with Hsp70 is mediated through the M-domain of Hsp104, only substrate-bound Hsp70 can bind and activate the disaggregase. Next, Hsp104 recognizes hydrophobic regions of the aggregated substrate and can actively displace the substrate from Hsp70 by applying a pulling force (Haslberger et al., 2007; Rosenzweig et al., 2013). It has been suggested that ClpB switches back to a repressing conformation after substrate transfer, thereby obscuring the Hsp70 binding site, which results in Hsp70 dissociation (Deville et al., 2017).

Hsp104 homologs are absent in mammalian cells, instead Hsp70 appears to be responsible for the disaggregation activities. It has been suggested that a specific set of Hsp70 co-chaperones, Hsp110 and Hsp40, empowers Hsp70 to exhibit potent disaggregation activity (Mogk et al., 2018). Hsp110 is of particular interest as it can boost Hsp70 disaggregation activity *in vitro* and it has also been demonstrated that Hsp110 is crucial for protein disaggregation in nematodes (Rampelt

et al., 2012; Shorter, 2011). The lack of Hsp110 in *C. elegans* displayed compromised dissolution of heat-induced aggregates and shortened lifespan after heat shock (Rampelt et al., 2012).

Proteasomal degradation

The quantity of a certain protein in the cell is regulated not only by its gene expression level and synthesis rate, but also by its degradation rate. The protein turnover varies greatly within the proteome, ranging from minutes to days (Belle et al., 2006). In addition, misfolded proteins that cannot be rescued by the chaperone system need to be eliminated in order to prevent cytotoxic damage. Cells have therefore evolved an intricate system for the specific recognition, tagging and degradation of proteins – the ubiquitin-proteasome system (UPS) (Figure 4). The UPS is a key component of the proteostasis network to eliminate damaged and misfolded proteins (Goldberg, 2003; Tyedmers et al., 2010; Vilchez et al., 2014).

Proteins destined for UPS-mediated degradation are attached to a chain of ubiquitin (Ub), a highly conserved 76 amino-acid residue polypeptide, through a series of steps involving the E1-E4 enzymes (Figure 4) (Finley, 2009). Ubiquitin is activated by the E1 ubiquitin-activating enzyme and then transferred to one of several E2 ubiquitin-conjugating enzymes. The E2 enzyme conjugates with an E3 ubiquitin-ligase that can transfer the activated ubiquitin from E2 to the substrate protein. When the first ubiquitin has been attached to the substrate protein, subsequent additions of ubiquitin molecules to the first one results in the formation of a polyubiquitin chain that acts as a signaling agent. This is achieved through cooperation of E2 and E3 with an E4 enzyme (Haas and Rose, 1982; Hwang et al., 2010). Ubiquitin has 7 lysine residues that can be conjugated to a second ubiquitin molecule, resulting in the formation of distinct polyubiquitin chains and a diversity of signaling modes. For instance, K48-linked chains target the substrate protein for proteasomal degradation, whereas K63-linked chains are involved in DNA repair and the trafficking of membrane proteins

(Finley et al., 2012). The specificity of substrate recognition and polyubiquitin chain variability in the ubiquitination process is conferred through the range of different E3 ligases.

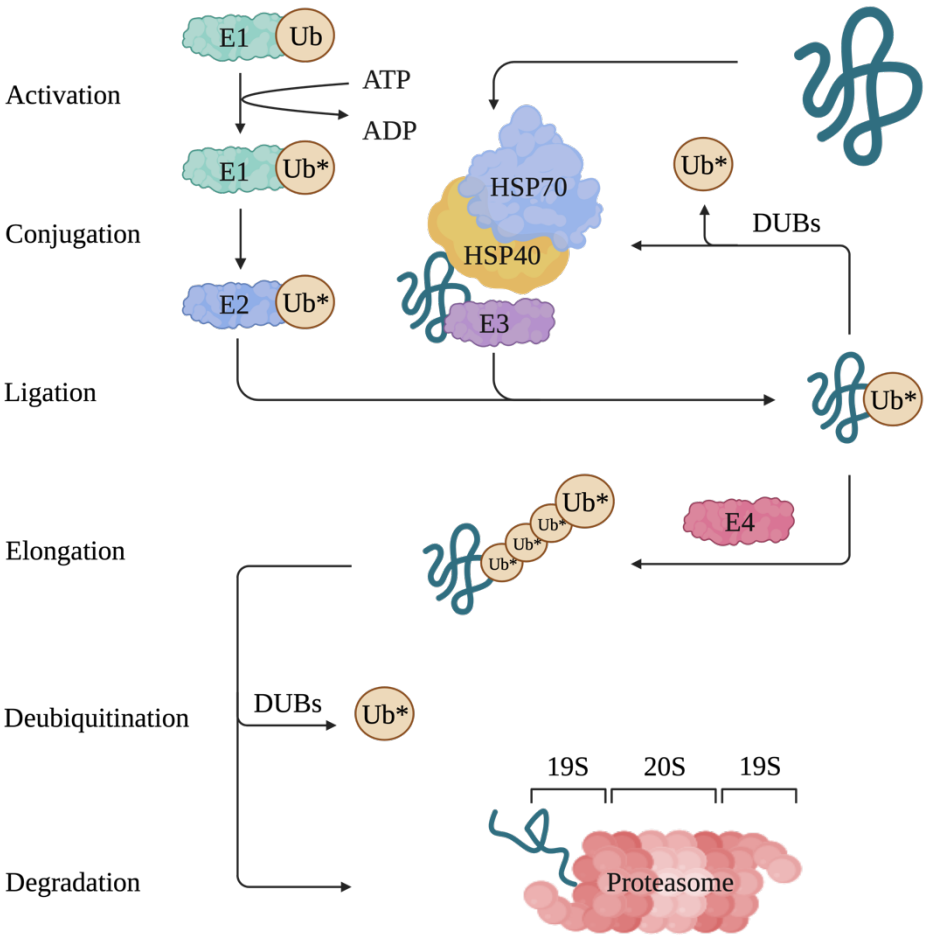

Figure 4. The ubiquitin-proteasome system. Ubiquitin (Ub) is activated by E1 in an ATP-driven process and the activated ubiquitin (Ub*) is transferred to the conjugating E2. Ub is then attached to the misfolded substrate that is bound by the E3 ligase and chaperones. Polyubiquitination of the substrate is mediated through cooperation with an E4 enzyme, and is necessary for the recognition by the 19S lid of the proteasome. Deubiquitinases (DUBs) remove the Ub chain before the substrate enters the 20S for degradation. DUBs can also act earlier in the process and rescue proteins from degradation.

In yeast, only one E1 activating enzyme has been identified, whilst 11 E2s and 42 E3s have been described (Lee et al., 2008). Specific E3 ligases (Ubr1, Rsp5, Hul5 and San1 in yeast) can mediate the labeling of cytosolic misfolded proteins for degradation (Eisele and Wolf, 2008; Fang et al., 2014; Fang et al., 2011; Heck et al., 2010). E3 ligases can also act in concert with cytoplasmic chaperones, such Ssa1, Ssa2 and Ydj1, for recognition and ubiquitination of substrate proteins (Fang et al., 2014; Heck et al., 2010; Park et al., 2007).

The proteasome can recognize and degrade proteins that are marked for degradation via the attached chain of, at least four, ubiquitin molecules (Thrower et al., 2000). The 26S proteasome is a large multimeric complex, consisting of the catalytic 20S core and one or two 19S lids (**Figure *4***). The 19S lids are responsible for recognition of poly-ubiquitinated substrates, removal of the ubiquitin tag and unfolding of the substrate protein prior to its entry into the 20S core that encompasses the proteolytic activity (Finley, 2009; Goldberg, 2003; Lam et al., 2002). A number of deubiquitinases (DUBs), which are not part of the proteasomal lids, catalyze the removal of ubiquitin from substrate proteins (**Figure *4***). This deubiquitination enables recycling of ubiquitin, but also an additional regulation where proteins can be rescued from degradation (Amerik and Hochstrasser, 2004).

Autophagy

Another pathway for protein degradation is mediated through autophagy and vacuolar/lysosomal proteases. In macroautophagy, cytoplasmic components are engulfed by a double membrane structure, called autophagosome, that fuses with the vacuole/lysosome where the degradation takes place (Nakatogawa et al., 2009). Autophagy is an essential process, allowing the cell to adapt to environmental changes but has also been suggested to be involved in the trafficking, recycling and degradation of various proteins (Reggiori and Klionsky, 2013).

The formation of autophagosome starts at a site, called the phagophore assembly site (PAS), located next to the vacuole in yeast. The PAS generates an expanding membrane, through the addition of phospholipids, that encapsules the cytosolic cargo. Once the autophagosome is completely formed, it will be transported to the vacuole via the vesicle trafficking machinery of the cell (Nakatogawa et al., 2009).

In mammalian cells, autophagy has been suggested to be a major contributor in the clearance of misfolded and aggregated proteins (Cuervo and Wong, 2014; Iwata et al., 2005). This system seems to be interconnected with the UPS, since a decline in the efficiency of the autophagy-lysosome pathway leads to compensatory responses by the UPS and vice versa (Korolchuk et al., 2009; Korolchuk et al., 2010). Perturbations of the UPS in the fly *D. melanogaster* enhances protein degradation activity through autophagy (Pandey et al., 2007). In yeast, it has been reported that autophagy is the main degradation pathway for alpha synuclein aggregates but the involvement of this process in the degradation of endogenous misfolded and aggregated proteins remains elusive (Petroi et al., 2012).

AIM AND MAIN FINDINGS OF PAPERS

PAPER I

Arsenic is a toxic metalloid that is associated with numerous diseases and therefore poses a public health concern. Yeast has been extensively utilized as a eukaryotic model organism to elucidate the molecular mechanisms underlying arsenic toxicity and resistance. In this study, a high-copy genomic DNA library screening was performed to identify yeast genes that provide arsenic resistance.

- The genomic library screen identified novel genes including *PHO86*, *VBA3* and *UGP1*, that provide arsenic resistance when overexpressed.
- Overexpression of *PHO86* resulted in higher intracellular arsenic levels but no increased protein aggregation.
- Overexpression of *VBA3* conferred arsenic resistance despite higher cellular arsenic and protein aggregation levels, while overexpression of *UGP1* resulted in lower intracellular arsenic and protein aggregation level.

In conclusion, the identified genes appear to provide arsenic resistance through distinct mechanism. Dedicated follow-up experiments will be required to elucidate the underlying mechanisms.

PAPER II

Arsenic triggers widespread protein aggregation, but how these aggregates are formed *in vivo* and how cells prevent their accumulation is not fully understood. A genome-wide imaging screen was performed in yeast to find components involved in these processes.

- Many genes and cellular systems contribute to protein homeostasis during arsenic stress.

- Protein aggregation is correlated with intracellular arsenic levels and arsenic toxicity.
- Appropriate transcriptional control is important for proteostasis and arsenic resistance.
- Translational control is crucial to alleviate arsenic-induced proteotoxicity.

In conclusion, this study highlights the importance of transcriptional and translational control for mitigating protein aggregation and toxicity during arsenic stress.

PAPER III

Evolutionarily conserved protein quality-control systems protect cells against proteotoxic stress. In this study, we assessed the contribution of the ubiquitin-proteasome system, the autophagy-vacuole pathway and the chaperone-mediated disaggregation to the clearance of arsenite-induced protein aggregation in yeast.

- The ubiquitin-proteasome system is the main pathway that clears arsenite-induced aggregates.
- The autophagy-vacuole pathway and chaperone-mediated disaggregation both contribute to clearance but appear less prominent than the ubiquitin-proteasome system.
- *In vitro* assays showed that Hsp104 and Hsp70 activity was not affected by arsenite, but chaperone-binding to the aggregates formed in the presence of this metalloid is impaired.

In conclusion, our study demonstrated the differential contributions of protein quality-control systems to aggregate clearance during arsenite exposure.

CONCLUDING REMARKS

Arsenic is a toxic metalloid that poses a substantial threat to human health with 100-200 million people worldwide estimated to be at risk (Podgorski and Berg, 2020). Exposure to arsenic can cause numerous diseases, such as cancers of the lung, liver and kidney as well as cardiovascular, respiratory and neurological disorders (Naujokas et al., 2013). At the cellular level, arsenic interferes with redox metabolism and induces oxidative stress, impairs DNA repair mechanisms and inhibits protein function and activity (Wysocki and Tamas, 2010). In addition, arsenic targets newly synthesized polypeptides and triggers protein aggregation (Jacobson et al., 2012). Despite of the undisputed toxicity of arsenic, our understanding of the underlying mechanisms and cellular responses is limited.

This thesis has focused on arsenic-induced protein aggregation and toxicity in yeast, with the aim of elucidating how these aggregates are formed *in vivo*, the mechanisms by which they affect cells, how cells prevent their accumulation as well as how cells regulate the protein quality-control system to protect against toxic aggregates. The impact arsenic has on protein homeostasis may contribute to its toxicity and suspected role in protein misfolding diseases. The three papers included in this thesis provide novel insights into the role of cellular machineries and systems in the prevention and clearance of arsenic-induced aggregates and toxicity. These findings collectively contribute to the understanding of arsenic toxicity and pathogenesis.

In **paper I-II**, two different screening approaches were applied to identify genes that provide arsenic resistance when overexpressed (**paper I**) and to uncover molecular determinants of arsenite-induced protein aggregation and toxicity (**paper II**). The genomic DNA overexpression screen revealed novel genes, including *PHO86*, *VBA3*, *UGP1* and *TUL1*, that conferred arsenic resistance. Overexpression of *PHO86* resulted in higher cellular arsenic levels but no additional effect

on protein aggregation, suggesting that these cells efficiently protect the proteome from intracellular arsenic and thereby preventing its toxic effects on protein folding and activity. We hypothesized that overexpression of *PHO86* confers As(V) resistance through increased uptake of phosphate and/or iron. Further experimental data, such as intracellular measurement of phosphate and/or iron levels, is needed to assess the mechanism by which *PHO86* overexpression results in improved As(V) resistance. *VBA3* overexpression caused resistance despite higher intracellular arsenic and protein aggregation levels, while overexpression of *UGP1* resulted in lower intracellular arsenic and protein aggregation. *TUL1* overexpression had no impact on intracellular arsenic or protein aggregation levels. Thus, the identified genes appear to confer arsenic resistance through distinct mechanisms and dedicated follow-up experiments will be required to elucidate the molecular details.

The genome-wide imaging screen in **paper II** uncovered novel genes and processes that may act specifically during As(III)-induced protein aggregation or as general proteotoxic stress factors. Several systems identified were already known or anticipated to influence protein quality-control, validating the screening approach. For instance, a set of hits with reduced levels of protein aggregation are associated with protein biosynthesis, supporting the notion that As(III) targets nascent proteins and triggers aggregation. A set of hits with enhanced levels of protein aggregation are linked to protein folding, supporting the previous findings that As(III) interferes with protein folding processes *in vivo*. In addition, the hit list from the current screen is also interesting in the context of human pathogenesis as several homologs to proteins related to human diseases were identified. Here, we selected several hits and demonstrated the importance of accurate transcriptional and translational control for mitigating protein aggregation and toxicity during arsenite stress. The broad network of cellular systems identified in the screen provides a valuable resource for further studies on the mechanistic details of arsenic toxicity and pathogenesis.

Evolutionarily conserved protein quality-control systems protect cells against the proteotoxic effects caused by metals, and we have therefore, in **paper III**, systematically assessed the contribution of several quality-control systems to the clearance of As(III)-induced protein aggregates. We showed that the UPS is the main pathway that clears these aggregates and that cells depend on this pathway for optimal growth, whilst the autophagy-vacuole pathway and the chaperone-mediated disaggregation both contribute to clearance but their roles appear less prominent than the UPS. *In vitro* assays demonstrated that chaperone binding to aggregates formed in the presence of As(III) is impaired, whereas the activity of Hsp104 and Hsp70 was not affected by this metalloid, suggesting that As(III) influences aggregate structure making them less accessible for chaperone-mediated disaggregation. The findings extend our understanding of how the protein quality-control systems operate during As(III) stress and this may contribute to the development of strategies for treatment of arsenic poisoning.

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