The Impact of Arsenic on Protein Homeostasis and Aggregation in *Saccharomyces cerevisiae*

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Cover illustration “A study of Arsenic” by Emma Lorentzon

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" What is there that is not poison?
All things are poison,
and nothing is without poison.
Solely the dose determines it
either a poison or a remedy."
(Paracelsus, 1538)

Abstract

Arsenic and cadmium are two toxic heavy metals that occur naturally in bedrock. Arsenic is found in high concentrations in certain areas and can contaminate groundwater, leading to exposure through drinking water and crop irrigation for the local population. Cadmium is primarily dispersed in the environment with fertilizers and as a byproduct of the electronics industry, and it is absorbed by the body through food and cigarette smoke. Long-term exposure to these heavy metals is associated with cardiovascular diseases, cancer, diabetes and neurodegenerative diseases. One of the primary reasons for their toxicity is their ability to interact with proteins in cells, essential for normal cellular function. This leads to protein misfolding and aggregation, and disruption of cellular processes. We used the yeast *Saccharomyces cerevisiae* to better understand how these toxic substances affect cells. The first article demonstrates how yeast cells mobilize specific control pathways to varying degrees to manage protein homeostasis and eliminate arsenic stress. The second article focuses on the roles of chaperones and ubiquitin ligases in maintaining protein balance under arsenic stress. We show that the ubiquitin-proteasome pathway is the major player in preventing and eliminating arsenite-induced protein aggregates. The third article shows that arsenite and cadmium alter the formation and structure of alpha-synuclein amyloid fibers, as well as cause changes in the protein's cellular localization. The final study provides a proteome-wide analysis of arsenic-binding proteins and demonstrates that nuclear transport is a direct target of arsenite-induced proteotoxicity. Together, these studies offer a comprehensive insight into the mechanisms by which arsenite disrupts protein homeostasis - from interactions with proteins to aggregate management mechanisms. This dissertation aims to deepen our understanding of cellular responses to heavy metal exposure, hopefully with implications for future therapeutic strategies against metal-related diseases.

Sammanfattning

Arsenik och kadmium är två giftiga tungmetaller som finns naturligt i berggrunden. Arsenik förekommer i höga koncentrationer på vissa platser, och kan förorena grundvattnet, vilket leder till exponering via dricksvatten och bevattning av grödor för lokalbefolkningen. Kadmium sprids i naturen främst genom konstgödsling och biprodukter från elektronikindustrier, och tas upp av kroppen via föda och cigarettrök. Långtidsexponering av tungmetallerna är förknippat med hjärtkärlsjukdomar, cancer, diabetes och har även associerats med neurodegenerativa sjukdomar. En av huvudorsakerna till deras toxicitet är deras förmåga att interagera med proteiner som är livsviktiga för normal cellulär funktion. Detta orsakar felveckning av proteiner och leder till att proteinerna klumpar ihop sig och bildar aggregat som kan skapa problem för cellen. För att bättre förstå hur dessa toxiska ämnen påverkar celler, har vi använt oss av bagerijästen *Saccharomyces cerevisiae*. I den första artikeln visar vi hur jästceller mobiliserar specifika kontrollvägar i olika grad för att hantera arsenik-inducerade proteinaggregat. Den andra artikeln fokuserar på chaperonernas och ubiquitinligasernas roller i att upprätthålla proteinbalans under arsenikstress. Vi visar att dessa system är avgörande för att förhindra och eliminera arsenik-inducerade proteinaggregat. I den tredje artikeln undersöks effekterna av arsenik och kadmium på α-synuklein – ett protein involverat i uppkomsten av Parkinsons sjukdom. Resultaten visar hur dessa toxiska metaller förändrar uppkomsten och formen av amyloida fibrer, samt var proteinet och dess aggregat befinner sig i cellen. Den sista studien ger en omfattande analys av arsenikbindande proteiner och visar att nukleär transport är ett direkt mål för arsenik-inducerad toxicitet. Sammantaget ger dessa studier en omfattande inblick i de mekanismer genom vilka arsenik stör protein-balans: från interaktioner med specifika proteiner till mekanismer för aggregathantering. Denna avhandling ämnar fördjupa vår förståelse av cellernas svar på arsenikexponering, med förhoppningar för terapeutiska strategier mot arsenikrelaterade sjukdomar i framtiden.

List of papers

This thesis is based on the following studies:

I. **Differential contributions of the proteasome, autophagy, and chaperones to the clearance of arsenite-induced protein aggregates in yeast**
Hua, S., Kłosowska, A., Rodrigues, JI., Petelski, G., Esquembre, LA., Lorentzon, E., Olsen, LF., Liberek, K., and Tamás, MJ. *Journal of Biological Chemistry*, 12, 298: 102680 (2022).

II. **Yeast chaperones and ubiquitin ligases contribute to proteostasis during arsenite stress by preventing or clearing protein aggregates**
Rodrigues, JI., Lorentzon, E., Hua, S, Boucher, A. and Tamás, MJ., *FEBS letters*, 13, 597: 1733-1747 (2023).

III. **Effects of the toxic metals arsenite and cadmium on α-synuclein aggregation *in vitro* and in cells**
Lorentzon, E., Horvath, I., Kumar, R., Rodrigues, JI., Tamás, MJ.*, Wittung-Stafshede, P.*. *Int. J. Mol. Sci*. 22, 1 (2021).

IV. **Proteome-wide identification of arsenite-binding proteins reveals that nuclear transport is a direct target of arsenite-induced proteotoxicity**
Lorentzon, E., Lee, J., Masaryk, J., Karlsson, N., Keuenhof, K., Galipaude, C., Madsen, R., Höög, JL., Levin, DE. and Tamás, MJ., *Manuscript,* (2024).

The following are not included in this thesis:

Etp1 confers arsenite resistance by affecting ACR3 expression
Romero, AM., Maciaszczyk-Dziubinska, E., Mombeinipour, M., Lorentzon, E., Aspholm, E., Wysocki, R., Tamás MJ., *FEMS Yeast Research*, 22, 1–9 (2022)

Differential effects of Cu^{2+} and Fe^{3+} ions on *in vitro* amyloid formation of biologically-relevant α-synuclein variants
Lorentzon, E., Horvath, I., Kumar, R., Wittung-Stafshede, P., *BioMetals*, 33(2-3), 97–106 (2020)

Author contribution

Paper I

I performed growth assays and fluorescence microscopy, quantified fluorescence images, and participated in editing the manuscript.

Paper II

I performed growth assays and the global translation assay, as well as acquiring and preparing samples for internal arsenic concentration measurements. I also helped with analyzing results and preparing figures.

Paper III

I performed the *in vitro* protein aggregation assays, AFM imaging, fluorescence microscopy and quantifications, ITC measurements, and most growth assays. I prepared the fiber samples for MS analysis. I participated in the discussion and formal analysis of all the results. I wrote most of the first draft and contributed to all subsequent revisions and figures.

Paper IV

I performed fluorescence microscopy and the subsequent quantifications and analyses. I performed the direct interaction study and aggregate isolations. I did statistics, bioinformatics, and 3D structural analysis. I stained, imaged, and quantified the EM samples. I participated in the discussion and formal analysis of the results. I contributed to all revisions of the manuscript and figures.

List of abbreviations

αSyn	Alpha-synuclein
ATP	Adenosine Triphosphate
CHX	Cycloheximide
EDC	Electron Dense Content
ER	Endoplasmic Reticulum
GSH	Glutathion
GTP	Guanosine Triphosphate
HSP	Heat-Shock Protein
INQ	Intra Nuclear Quality control compartment
IPOD	Insoluble Protein Deposit
JUNQ	Juxta Nuclear Quality control compartment
KAP	Karyopherin
NAC	Nacent polypeptide-Associated Complex
NCT	Nucleo-Cytoplasmic Transport
NE	Nuclear Envelope
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NTR	Nuclear Transport Receptors
NPC	Nuclear Pore Complex
PD	Parkinson's Disease
PQC	Protein Quality Control
RAC	Ribosome-Associated Complex
ROS	Reactive Oxygen Species
RQC	Ribosomal Quality Control
SUMO	Small Ubiquitin-related Modifier
UPS	Ubiquitin Proteasome System
UTR	Untranslated Region

Contents

Introduction to the field

1. Yeast as a model system

The budding yeast *Saccharomyces cerevisiae* is a well-established model organism that has proven to be a powerful tool for studying the molecular details of metal action, detoxification strategies, and protein-misfolding diseases. Since many fundamental cellular mechanisms — such as replication, recombination, cell division, and metabolism—are highly conserved from yeast to mammals, insights gained from yeast can be directly applied to human biology (Duina et al., 2014). In fact, about 60% of yeast genes have a homologue or share a conserved domain with human genes, making it an ideal model for understanding cellular processes in higher organisms (Botstein et al., 1997). Studying a simpler, single-celled species like yeast allows us to explore complex cellular processes that occur in human cells in a more manageable context. These discoveries can then be applied to animal or human cell models, advancing our understanding of how these processes function in more complex organisms. The relatively small genome size of yeast and its ease of culture make it well-suited for high-throughput screening, a technology that has propelled the development of new fields in computational biology (Norcliffe et al., 2013).

Additionally, yeast has a fully sequenced genome, an efficient homologous recombination system, and readily available gene deletion libraries, thus having a wide selection of genetic tools for any experiment. These advantages have led to significant breakthroughs in gene regulation mechanisms and other cellular processes over the past several decades (Duina et al., 2014).

2. Metals in nature

Many metals are essential for numerous processes in living organisms. In most organisms, more than one-third of the proteome involves metals playing structural and chemical roles as cofactors. This mobilization of metals is a common mechanism for signaling to regulate cellular activity (Jomova et al., 2022). Metals function as structural elements and stabilizers of biological structures, and they are critical components of control mechanisms in nerves and muscles. They also act as activators or integral parts of redox systems (Nordberg et al., 2007). Today, the list of metals considered to be essential for humans includes Na, K, Mg, Ca, Mn, Fe, Co, Cu, Zn, and Mo (Zoroddu et al., 2019). Cells and organisms must maintain a delicate balance in cellular metal concentrations as excess can be toxic while deficiency may result in poor performance or disease.

2.1. Essential metals

Transition metals, in particular, are critical components of the active centers in many enzymes, such as copper-zinc superoxide dismutase (Cu,Zn-SOD), manganese **s**uper**o**xide **d**ismutase (Mn-SOD), and catalase (Jomova et al., 2022). These enzymes play critical roles in protecting cells from oxidative stress by catalyzing the conversion of reactive oxygen species into less harmful molecules (Zoroddu et al., 2019). In addition to their roles in enzymatic activity, metals such as iron, zinc, and copper are involved in various physiological processes.

Iron (Fe) is a component in at least a hundred enzymatic reactions; from DNA synthesis, electron transport, and oxygen transport and utilization (Crichton, 2016, Aisen et al., 2001). In the human body, iron is stored in heme proteins such as hemoglobin and myoglobin, and non-heme complexes like transferrin and ferritin (Aisen et al., 2001,

Zoroddu et al., 2019). Disruptions in iron homeostasis, resulting in for example elevated iron levels in the brain, have been linked to the onset of several neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease (Snyder et al., 2010, Kim et al., 2018a, Crichton et al., 2012). These disorders are multifactorial but are characterized by an accumulation of misfolded proteins in different parts of the brain (see chapter *neurodegenerative diseases*). On one hand, iron deficiency can result in anemia, while excess iron can cause conditions like hemochromatosis (Crichton, 2016).

Copper (Cu) is vital for the heart and immune system's proper functioning. It acts as an essential component or cofactor for many enzymes involved in electron transfer reactions (Valko et al., 2005). These enzymes are crucial in key biological processes such as cellular respiration and antioxidant defense. For instance, copper-dependent enzymes like cytochrome c oxidase are involved in the mitochondrial electron transport chain, which is essential for energy production. Additionally, Cu is necessary for enzymes such as lysyl oxidase, which are important for the formation and stabilization of connective tissues, and ceruloplasmin which is involved in iron homeostasis (Jomova et al., 2022). Cu imbalance is associated with disorders such as Wilson's disease and Menkes disease, demonstrating that the correct regulation and activity of copper-dependent enzymes are crucial (Jomova et al., 2022).

Zinc (Zn) plays a crucial role in DNA synthesis, immune function, and wound healing. Zinc finger motifs (ZnFs) coordinate zinc atoms with cysteine and/or histidine residues (Klug, 2010). When the oxidation threshold of ZnFs is exceeded, the zinc ion is released, functioning as a redox switch in signaling and regulation. Zinc finger proteins are abundant and participate not only in redox processes but also as

components of proteases, ubiquitin ligases, and transcription factors (Cassandri et al., 2017). Zn ions are one of the primary regulatory ions, sharing signaling capacity with calcium, and can act as messengers of both intracellular and extracellular information (Maret, 2017). High levels of zinc ions in cells lead to the production of metallothionein, a metal-binding protein that can protect against the harmful effects of excessive copper, as seen in Wilson's disease mentioned above (Horn et al., 2019, Avan et al., 2022). However, zinc deficiency can impair immune function and growth, while excessive zinc can interfere with the absorption of other essential minerals (Zoroddu et al., 2019).

Cells rely on the coordinated action of various metal homeostasis factors to ensure an adequate metal supply for proper cellular function while avoiding overaccumulation and its related toxic effects. These factors include acquisition and storage proteins, transporters, metallo-chaperones, and metal-sensing transcriptional regulators. While essential metals play crucial roles, certain heavy metals, such as arsenic and cadmium, disrupt these vital processes, particularly in relation to protein homeostasis. This disruption forms the foundation of my thesis.

2.2. Nonessential heavy metals

The term "heavy metal" lacks a precise definition and is often the subject of debate. However, chemists generally define a heavy metal as any metal that exhibits toxicity, regardless of its atomic mass or density. In essence, a metal or metalloid is considered a "contaminant" if it is present in an environment where it is not wanted or in a form or concentration that has harmful effects on humans or the environment. Common examples of heavy metals and metalloids include lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), chromium (Cr), copper (Cu), selenium (Se), nickel (Ni), silver (Ag), and zinc (Zn) (Jaishankar

et al., 2014). Among these, arsenic and cadmium are two heavy metals of particular interest to me due to their toxic effects and impact on the global disease burden.

2.3. Cadmium

Cadmium (Cd) is chemically similar to zinc and often occurs in zinc or lead ores at relatively high concentrations. Elevated levels of cadmium can be found in air, water, and soil near nonferrous mining and metal refining industries. Due to its ability to protect iron products from rusting, cadmium has been, and in some countries still is, used as an anti-corrosive coating for steel through electroplating (Nordberg et al., 2022). Additionally, cadmium compounds are used in pigments such as cadmium sulfide and cadmium sulfoselenide, which are still used to a limited extent in plastics and various paints. One of the most important current uses of cadmium, however, is as an electrode component in alkaline batteries. Cadmium telluride, in particular, is increasingly used in solar panels. Moreover, cadmium is present in most foodstuffs, and its level varies greatly depending on dietary habits. Smoking tobacco is a significant route of cadmium exposure for the general population (Carl and Järup, 1996, Munisamy et al., 2013).

Acute cadmium poisoning is thankfully rare today but was more common when industrial safety practices were less stringent. Inhalation of workroom air or cigarettes was the primary route of exposure (Hecht et al., 2016, Nordberg et al., 2022). Skin exposure is of minor importance due to the low degree of absorption through the skin. Oral exposure to cadmium is also possible, such as through the use of utensils containing cadmium. Cadmium ions in gastric juice strongly stimulate the gastrointestinal mucosa, causing nausea, vomiting, abdominal pain, and diarrhea within minutes of ingestion, with severe cases potentially leading to shock (Nordberg et al., 2022).

Acute symptoms include irritation, dryness of the nose and throat, coughing, headaches, chills, fevers, and chest pains. These symptoms can progress to pulmonary edema or chemical pneumonitis, sometimes resulting in death (Zang and Bolger, 2014). Cadmium also disrupts the metabolism of iron, copper, and zinc in humans and other mammals (Rajakumar et al., 2019, Bhagavan, 2002).

Long-term low-level exposure to cadmium results in considerable age-related accumulation in the body, as only a small portion of the absorbed cadmium is excreted (Nordberg et al., 1985). Chronic exposure to cadmium can lead to a range of adverse health effects. One of the primary targets of cadmium toxicity is the kidneys (Nordberg et al., 2022). Chronic cadmium exposure can cause kidney damage that can progress to severe renal dysfunction and potentially chronic kidney disease. Additionally, cadmium can affect bone health. Prolonged exposure can lead to the weakening of bones, known as osteomalacia, and an increased risk of fractures (Moy and Todd, 2014). This is partly due to cadmium interfering with calcium metabolism and vitamin D function, which are crucial for maintaining bone strength and density. One well-recognized severe form of bone disease has been manifested in Japan as the itai-itai disease (literally translated to "ouch-ouch" disease, due to the severe pain in the spine and joints it creates) (Moy and Todd, 2014). Cadmium exposure has also been linked to cardiovascular problems. Studies have suggested an association between chronic cadmium exposure and hypertension, as well as an increased risk of cardiovascular diseases such as stroke and myocardial infarction (Dutta and Sharma, 2019, Nordberg et al., 2022). Chronic inhalation exposure can lead to **c**hronic **o**bstructive **p**ulmonary **d**isease (COPD) and emphysema, characterized by long-term breathing problems and poor airflow (Nordberg et al., 2022). Moreover, cadmium is classified as a human carcinogen by the **I**nternational **A**gency for **R**esearch on **C**ancer (IARC). Long-term

exposure to cadmium has been associated with an increased risk of lung cancer, as well as potential links to prostate and kidney cancers (Chen et al., 2019).

2.3-1. Cadmium uptake and resistance

Cadmium shares similar properties as zinc and can thus be taken up by cells via zinc transporters (Nordberg et al., 2022). In *S. cerevisiae*, Zn uptake is primarily mediated by the high-affinity Zrt1 and low-affinity Zrt2 zinc transporters, members of the ZIP (**Z**RT, **I**RT-like **P**rotein) family of metal transporters (Zhao and Eide, 1996). Studies in yeast show that cells accumulate less Cd when ZRT1 is deleted compared to wild-type cells (Gomes et al., 2002) and Zrt1-mediated zinc uptake is strongly inhibited in the presence of cadmium (Gitan et al., 2003). Gitan et al showed that in the presence of Cd, Zrt1 becomes ubiquitinated and is removed from the cell surface. This inactivation may be an important mechanism to protect zinc-limited cells from cadmium toxicity (Gitan et al., 2003, Tamás et al., 2005). Cadmium can also enter cells via the Smf1 protein, a yeast member of the Nramp (**n**eutral **r**esistance-**a**ssociated **m**acrophage **p**rotein) family of metal transporters found in bacteria, fungus, plants, and mammals (Cellier et al., 1995). The Nramp transporters, like the ZIP family proteins, can recognize a wide range of substrates such as copper, cadmium, manganese, and iron (Forbes and Gros, 2001). The **A**TP-**b**inding **c**assette (ABC) transporter Ycf1 in yeast also helps to transport cadmium from the cytosol into the vacuole. When the YCF1 gene is over expressed, the cells exhibit elevated cadmium resistance while the ycf1-deletion mutant is hypersensitive to Cd (Szczypka et al., 1994).

2.4. Arsenic

Arsenicals have been used throughout the ages, having its golden age during the Victorian era (19th century) in England, Western Europe and the United States. It was in *everything*, wallpapers, clothing dyes, cosmetics and household cleaning products. Arsenis-containing soaps and pills were advertised in catalogues and newspapers promising perfect skin and healthy complexion – free of freckles and imperfections (Figure 1). Scheele's green, often referred to as Paris Green, was a popular wallpaper hue that had a remarkably high concentration of arsenic—"no less than one third of its whole weight consisted of Arsenite of copper" (Bentley and Chasteen, 2002). Scheele's green was also alarmingly enough used in candy and desserts as a food dye, until children all over started showing signs of mild arsenic poisoning (Doyle, 2009).

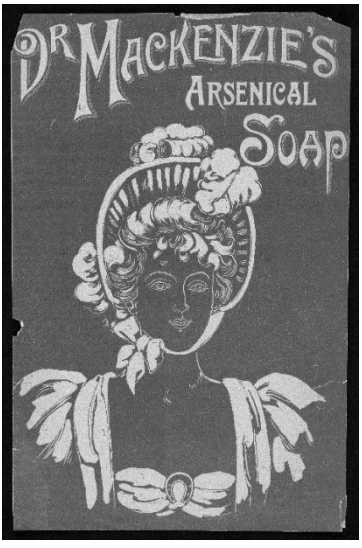

Figure 1 - Dr Mackenzies arsenical soap from the 1800s, which claims to cure spots, pimples and produces a lovely complexion. The ingredients included zinc and arsenic. Public domain image from the history picture archive.

Arsenic has also been the preferred poison of "homicidal practitioners" (Ferguson and Gavis, 1972, Whorton, 2011). It was especially appreciated by ruling families of Europe as a way to get rid of competition, thus dubbed "inheritance powder" or "Poison of Kings" (Watson, 2020). As little as 1-2.5 mg/kg of arsenic trioxide can be lethal, with early symptoms of acute poisoning ranging from 30 minutes to several hours, including lip burning, pharyngeal constriction, severe stomach pain, and nausea (World Health

Organization, 2022). At the time it was easily accessible in high concentrations in a pharmacy under the guise of buying cleaning products or rat poison; and with the delay in reaction coupled with it being hard to detect (odorless and tasteless), made it easy to get away with (Watson, 2020). Later, following the development of the Marsh test, an accurate forensic method for measuring arsenic created in 1836 by the English scientist James Marsh, as well as restrictions in pharmacies regarding who's buying arsenic in the 20th century, the prevalence of arsenic poisonings decreased (Gorby, 1988). With emerging knowledge on the highly unpleasant side effects and incidents of sudden death that followed the use of arsenic, it made a career in literature instead; with writers such as Agatha Christie and Gustave Flaubert making arsenic one of the most well-known murder weapons in popular crime fiction.

2.4-1. Arsenic in medicine

Despite its toxic properties, or perhaps because of them, arsenic has been used in medicine throughout the ages. Ancient civilizations such as Greece, China, Egypt, and India utilized arsenic for medicinal purposes for thousands of years to treat a variety of ailments (Doyle, 2009). In Chinese traditional medicine, forms of arsenic have been used for over 3000 years and continue to be employed under strict regulations today to treat conditions like dyspnea (shortness of breath) and asthma (Chinese Medicine Ordinance, 1999). Paul Ehrlich was awarded the Nobel Prize in 1908 for his work on immunity and serum therapy. In that same year, he developed the first effective drug for syphilis, an arsenic-based compound known as 'compound 606' (arsphenamine) (Doyle, 2009). Despite its numerous side effects, arsphenamine remained the only useful treatment for syphilis until the discovery and development of penicillin in the 1950s.

Fowler's solution (solution of 1% potassium arsenite) was another arsenic-containing compound that gained popularity with the general public. Although it was never very effective, it was widely recommended by eminent doctors and surgeons to treat a variety of conditions including diabetes, snake bites, pregnancy-related vomiting, malaria, typhoid fever, epithelioma, and various skin conditions during the 1800s. In 1865, however, physicians in Berlin reported success using Fowler's solution to treat chronic myelocytic leukemia (Lissauer, 1865).

This treatment was later "rediscovered" in the early 1900s and continues to be used today for **a**cute **p**romyelocytic **l**eukemia (APL) (Khairul et al., 2017, Wysocki et al., 2023). Even though As exhibits anticancer activity *in vitro*, it has not been shown to be effective in treating any other cancer types than APL in clinical trials (Fang and Zhang, 2020). However, its efficacy in anticancer therapy may potentially be increased when combined with other anticancer drugs (Jiang et al., 2023). At present, the organic arsenical melarsoprol is used as treatment for African trypanosomiasis or "sleeping sickness"; a tropical disease caused by *Trypanosoma brucei*, a parasitic haematoprotozoa causing fatigue, high fever, headaches, and muscle aches and if not treated, can be deadly (Barrett and Barrett, 2000).

2.4-2. Arsenic in the environment

Today, most homicidal arsenic cases stay in books. Instead, we have a much slower, but very real, exposure to arsenic through groundwater and crops. This is a rather recent discovery through epidemiological studies, that exposure to low levels of arsenic in the general environment greatly contribute to the global emergence of certain diseases (Prüss-Ustün et al., 2011). Arsenic is currently classified as one

of the most hazardous chemicals of major public health concerns, according to the WHO (Podgorski and Berg, 2020). Several continents have reported levels well above the recommended WHO standard for daily intake of inorganic arsenic in drinking water (10µg/L (World Health Organization, 2022), with over 200 million people being at risk of exposure (Garelick and Jones, 2008), see Figure 2. Some of the most severe and well documented cases of contaminated groundwater can be found in Asia (Bangladesh, Nepal, China, India) and South America (e.g. Mexico and Argentina) reaching levels up to 3000µg/L (Naujokas et al., 2013).

Moreover, the water is utilized not just for drinking but also for agriculture irrigation and food preparation, which can cause arsenic to build up in crops or soils that are then ingested. For example, a sizable portion of the Asian population consumes a diet high in rice. Because rice in these regions is frequently irrigated with water contaminated with arsenic, in conjunction with arsenic already in the soil, results in elevated concentrations of arsenic in the rice grains (Meharg, 2004). Due to their high susceptibility to metals, infants and young children may experience toxic consequences at exposure levels significantly lower than those that have a negative impact on adult workers. The potential lifetime exposure and the relationship between aging and cumulative exposure make elderly people a particularly susceptible target group as well (Fowler et al., 2022). Long term exposure to arsenic can cause changes in skin pigmentation and skin lesions, cardiovascular diseases, neurological disorders, diabetes and cancer of bladder, lung or kidneys (Beyersmann and Hartwig, 2008, Martinez et al., 2011, IARC, 2012).

Figure 2 – Map of arsenic affected countries of the world with severity shown in darker color. Note that South American and South Asian countries are the most affected. *Image recreated from Halem et al. 2009 and E Shaji et al. 2021, with Biorender.*

Arsenic occurs naturally in the earth's crust in its inorganic form, especially abundant in ores and minerals containing copper and lead. The metalloid has many inorganic and organic forms, however, the inorganic arsenic trioxide "white arsenic" (arsenite) is considered to be the most toxic and prevalent form (CDC, 2014). Arsenic trioxide is usually a by-product during the smelting and processing of ores which contain arsenic compounds. Soils underlying sulfide ore deposits frequently contain arsenic at several hundred parts per million; the documented highest is 8,000 ppm (National Research Council Committee on Medical and Biological Effects of Environmental, 1977). Arsenic is mobilized by human activity in the form of mining, mineral processing, and pesticide, herbicide, and wood preservative usage (Bowell et al., 2014).

Today, a significant portion of semi-conductor production involves arsenic. Arsenic and phosphorus contribute additional negative charges to silicon, whereas indium, gallium, and phosphorus increase electrical charges to silicon. Sweden is the primary producer of this arsenic (Doyle, 2009, Fowler et al., 2022). The inorganic arsenic used and produced can bind to organic materials in the soil and be released for plant absorption, or leach into the groundwater. Metal-reducing bacteria are partly to blame here, since they have the ability to catalyze the breakdown of minerals containing arsenate, releasing arsenic into the surrounding water (Reyes et al., 2008). Aqueous arsenicals also have an effect on marine plants; algae, for example, have been found to contain extraordinarily high arsenic levels in its organic form already in 1922 (Jones, 1922).

2.4-3. Arsenic uptake

Arsenic is classified as a metalloid, sharing properties with both metals and non-metals. It is present in organic and inorganic forms as mentioned above, and in several oxidation states; -3, 0, +3, +5 (Cullen and Reimer, 1989). Trivalent inorganic arsenic, **arsenite** (As(III)), is generally regarded as more toxic than pentavalent arsenic, **arsenate** (As(V)), due to its higher cellular uptake and affinity for binding to proteins (Islam et al., 2015). Arsenic can accumulate and damage every organ in the human body and the highest concentrations are found in the liver and kidney (Fowler et al., 2022).

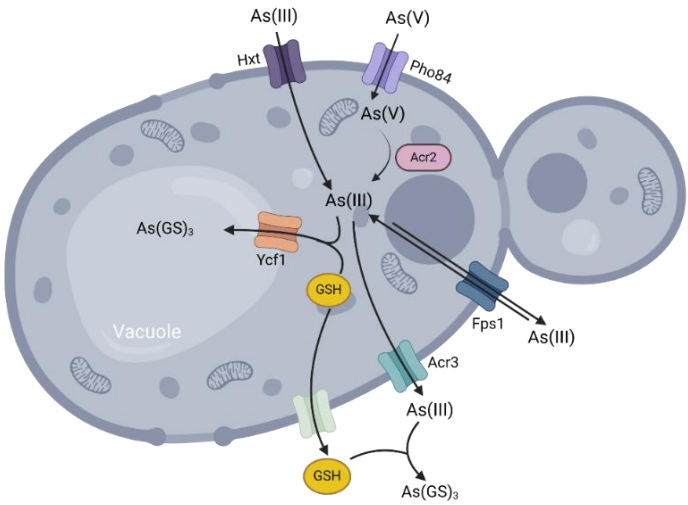

Figure 3 – Uptake and extrusion pathways of inorganic arsenic in yeast. As(III) can enter the cell via the aquaglyceroporin Fps1 or through hexose transporters (Hxt). As(V) is imported through phosphate transporters (Pho84) and gets converted to As(III) by Acr2 in the cytosol. As(III) can then either leave the cell via Fps1 (if reduced from As(V)), or exported via Acr3. Glutathione (GSH) can bind As(III), either to sequester in the vacuole (internal) or prevent entry into the cell (external). *Image created with Biorender.*

The uptake of arsenic depends on its oxidation state, as shown in Figure 3. As(III) is mainly imported via aquaglyceroporins (Fps1 in yeast; bidirectional channel) probably due to its inorganic state ($As(OH)_3$) structurally resembles glycerol which it normally transports (Wysocki et al., 2001). In addition, arsenite may also enter cells via hexose transporters (Tamás et al., 1999, Liu et al., 2004). Maciaszczyk-Dziubinska et al. demonstrated that the aquaglyceroporin Fps1 also plays a critical role in mediating the efflux of As(III) and maintaining tolerance to arsenate in budding yeast. Their research showed that yeast mutants lacking the Fps1 gene (*fps1Δ*) exhibited increased sensitivity to arsenate and reduced ability to extrude arsenite from the cell (Maciaszczyk-Dziubinska et al., 2012, Lee and Levin, 2018). The metalloid entry pathway is controlled: post-translational regulation of Fps1 is strongly repressed via the activation of the Hog1 pathway

when cells are exposed to arsenite (Wysocki et al., 2001, Lee and Levin, 2018, Thorsen et al., 2006). Arsenate has another way to enter the cell: As(V) is an analogue of inorganic phosphate and can thus utilize phosphate transporters (such as yeast Pho84 and Pho87) to get into the cell (Bun-ya et al., 1996). Once Inside the cell, As(V) can replace phosphate in biochemical reactions, for example by inhibiting ATP generation during oxidative phosphorylation (Islam et al., 2015, Shen et al., 2013, Porquet and Filella, 2007).

2.4-4. Detoxification

Due to the ubiquitous presence of arsenic in the environment, all organisms have developed mechanisms to evade its toxicity. The primary cause of resistance is a decrease in arsenic uptake by cells brought on by mutations that alter the structure of proteins involved in uptake and/or the activation of export mechanisms.

In mammals, inorganic As(III) is mainly metabolized via methylation in several steps; using methyltransferases creating **m**ono**m**ethyl**a**rsinic acid (MMAs) and **di**m**e**thyl**a**rsinic acid (DMAs) (Drobná et al., 2010). The methylated arsenic forms are then transported out of the cells by multiple ABC transporters, as well as by aquaglyceroporins and glucose permeases (Calatayud et al., 2012, Maciaszczyk-Dziubinska et al., 2012, Drobná et al., 2010). In bacteria, resistance is achieved by two transcriptional regulators ArsD and ArsR, an arsenate reductase ArsC that converts arsenate to the more easily extruded As(III), as well as two proteins ArsA and ArsB that together form an arsenical ATPase that exports As(III) from the cell (Ordoñez et al., 2009, Shen et al., 2013, Rosen, 1999).

The budding yeast *S. cerevisiae* has similar mechanisms of resistance; three adjacent (contiguous) genes called arsenical resistance (ARR or ACR) genes (Figure 3)(Bobrowicz et al., 1997). The arsenic is sensed by the AP-1-like transcriptional regulator ARR1 (also known as YAP8), that is activated by a modification of three cysteines by arsenic (Kumar et al., 2016, Wysocki et al., 2004). Yap8 then activates two genes only: an arsenate reductase ACR2, that converts As(V) to As(III) (Mukhopadhyay and Rosen, 1998, Mukhopadhyay et al., 2000); and a metalloid/H+ antiporter ACR3 located in the plasma membrane that exports the intracellular As(III) out of the cells (Wysocki et al., 1997). As(III) can also flow out of the cell via the bidirectional channel Fps1 when reduced from As(V) intracellularly (Lee and Levin, 2018, Maciaszczyk-Dziubinska et al., 2012). Methylation of the inorganic As(III) can occur in yeast by methyl-transferase Mtq2, creating methylated arsenite species shown to be more toxic than the inorganic form (Dong et al., 2015). Deletion of the *MTQ2* gene resulted in increased As(III) tolerance (Ren et al., 2011), and Lee and Levin showed that As(III) can be methylated to Mas(III) by the methyltransferase dimer Mtq2:Trm112 (Lee and Levin, 2018).

Arsenite can be sequestered by molecules such as glutathione (GSH) and phytochelatins (PCs). These are small thiol-containing peptides that exist in relatively high concentrations in the cytosol (low mM range) and are important for redox-mediated processes. GSH and PCs bind to As(III), forming complexes that are less toxic (Poole, 2015). These resulting chelated complexes become substrates for ATP-binding cassette (ABC) transporters and sequestered in the vacuole through Ycf1 and Vmr1 (Ghosh et al., 1999, Paumi et al., 2009, Maciaszczyk-Dziubinska et al., 2012). Evidence suggests that Ycf1 confers arsenic resistance. The deletion of both the *ACR3* and *YCF1* genes causes additive arsenite sensitivity, demonstrating that yeast

cells have two separate metalloid detoxification mechanisms with differing specificities (Ghosh et al., 1999, Tamás et al., 2005). Additionally, GSH links to protein thiol sites to prevent harmful metal binding and over-oxidation. GSH is one of the primary antioxidant molecules, and depletion of its intracellular pool is suggested as a mechanism of heavy metal toxicity (Wysocki and Tamás, 2010). Indeed, Thorsen et al. demonstrated that if yeast is chronically exposed to As(III), the cells export GSH to chelate the metalloid outside the cell. This chelation makes it inaccessible for uptake, lowers intracellular arsenic concentrations and thus leads to higher tolerance (Thorsen et al., 2007, Thorsen et al., 2012).

2.5. Metal and protein interactions

Typically, metals induce toxicity *in vivo* by disrupting protein and membrane functions, by interfering with nutrient uptake and redox reactions, and causing DNA damage (Wysocki and Tamás, 2010, Tamás and Wysocki, 2001). Arsenic, in particular, forms kinetically stable bonds to sulfur and carbon in organic compounds and at cellular level, its toxicity is linked to oxidative stress, epigenetic changes, genotoxicity, and altered protein function and activity. We have known since the 1960s that arsenite (+3) reacts with sulfhydryl groups of cysteine in proteins; and enzyme inactivation, altered protein conformation and aggregation by this mechanism is still considered to be the primary mode of arsenic toxicity (Ghosh and Sil, 2023, Wysocki et al., 2023, Webb, 1966). Studies have uncovered another harmful mechanism in which As(III) targets nascent proteins, disrupting their proper folding. Specifically, As(III) has been shown to inhibit the refolding of chemically denatured proteins *in vitro*, interfere with protein folding *in vivo*, and cause misfolding and aggregation of nascent proteins in living cells (Wysocki et al., 2023, Jacobson et al.,

2012, Tamás et al., 2014). Arsenate (+5) does not react with sulfhydryl groups though, it can, however, be reduced to arsenite in cells with the help of arsenate reductases in the process of excluding the metal (Stefanini et al., 2022).

2.5-1. Cysteine

Cysteines (Cys) are one of the least abundant amino acid residues, but when they are present, they are often located near active sites where they mediate catalytic and regulatory functions (Marino et al., 2010). Evolutionary studies suggest that cysteine residues have been enriched in genomes over time, with a positive correlation observed between cysteine abundance and the complexity of an organism (Miseta and Csutora, 2000). The unique chemistry of cysteine, primarily due to its thiol group (R-SH), allows proteins to form disulfide bonds (R-S-S-R), which are crucial for maintaining protein structure and stability (Wiedemann et al., 2020, Marino et al., 2010). These disulfide bonds can also serve as redox-active centers, playing significant roles in cellular redox homeostasis and signaling (Poole, 2015). Cysteines can change oxidation state depending on the surrounding environment's redox condition. The reversible transition between oxidation states of Cys has regulatory implications on protein interactions and activity (Garrido Ruiz et al., 2022, Bak et al., 2019). The importance of cysteine extends to the formation of metalloproteins, where cysteine residues often coordinate metal ions, contributing to the metal's proper placement and function within the protein. Furthermore, cysteine is involved in the synthesis of glutathione, a vital intracellular antioxidant that I mentioned in the previous section. The metabolism and regulation of cysteine itself are tightly controlled to prevent potential toxicity from its reactive thiol group (Stewart et al., 1998), though its production is stimulated by heavy metals such as As and Cd (Thorsen et al., 2007).

The ability of Cys to form disfulfide bonds and interactions within the protein depends on the structure of both the amino acid (a.a) sequence and the fold of the protein. Two neighboring Cys, or two Cys with one space in-between (CxC; where x is any a.a.) cannot interact, due to the rigid nature of a peptide bond. Both alpha helixes and beta-sheet structures allow for two cysteine to be closest when separated by two spaces (CxxC-motif). Studies have shown that, indeed, the CxxC-motif is present and well conserved in oxireductases and iron-sulfur proteins among others (Miseta and Csutora, 2000). It seems to be a trend especially for cysteines to appear in proximity of each other in protein structures, something that is called clustering (Poole, 2015). Proteins expressed by organisms living in harsh environments have been observed to have a higher degree of this cysteine clustering in regions related to metal binding or redox mechanisms – regions that need to be stable and function in these conditions (Beeby et al., 2005). Because of their redox-active state and propensity to form metal complexes, cysteines are also vulnerable to redox stress-induced overoxidation as well as toxic metal binding.

As(III) and mono-methylated As(III) can form both weak monodentate complexes and stable pluridentate complexes with sulfhydryl groups (like cysteine). When compared, binding affinity for As(III) to peptides with two closely spaced Cys residues had a tenfold higher affinity than singular cysteines (Kitchin and Wallace, 2005, Miseta and Csutora, 2000). Kitchin and Wallace conducted binding studies on synthetic peptides to determine how many residues can separate two cysteine for it to shift from pluridentate binding (high affinity) to monodentate (low affinity). For cysteines with two to five separating residues Cysteines with two to five separating residues have a high binding affinity, and interestingly, the affinity does not change considerably in peptides with up to fourteen residues between them. They reasoned that the conformation can easily bend in free peptides, bringing the

cysteine residues close enough together to form bonds with a single As(III)-ion (Kitchin and Wallace, 2005). When arsenite complexes with multiple cysteine residues, it can lead to alterations in the protein's tertiary structure or promote the formation of dimers, which can disrupt normal protein function and lead to toxic effects (Kitchin and Wallace, 2005). Nascent proteins, which are still in the process of active folding, are especially vulnerable to the complexing action of metals (Jacobson et al., 2012). Because the backbone of nascent proteins remains motile, the joining of sequentially distant residues by arsenite is possible, further increasing the potential for structural and functional disruptions (Tamás et al., 2014).

3. Protein homeostasis

Protein homeostasis, defined by the balance between protein synthesis and degradation, is essential for cell survival and growth. The quality control mechanisms that ensure proper protein folding are vital in maintaining this balance. Most proteins must achieve their native conformation (i.e., folded three-dimensional state) before they can function physiologically. This folding process occurs on the ribosome during translation, in the cytoplasm after ribosomal release, or within organelles such as the endoplasmic reticulum and mitochondria (Hartl and Hayer-Hartl, 2002). Failure to attain or maintain the correct native fold can lead to protein misfolding and aggregation, which may result from mutations, external stress conditions, errors during transcription and translation, or cellular aging. (Goloubinoff, 2016, Buchberger et al., 2010, Hartl et al., 2011, Labbadia and Morimoto, 2015). Protein misfolding and aggregation are detrimental to cells and organisms, and are associated with various human diseases, including metabolic, oncological, and neurodegenerative disorders (Labbadia and Morimoto, 2015).

To ensure protein homeostasis, cells rely on complex protein quality-control (PQC) systems. These systems include molecular chaperones which assist in the folding of proteins into their native conformation; and if proteins misfold, handle the disaggregation, refolding, sequestering, and degradation of non-native proteins (Hartl et al., 2011). Additionally, protein degradation systems, such as the ubiquitin–proteasome system (UPS) and the autophagy-lysosome pathway, are responsible for clearing misfolded and aggregated proteins from the cell (Goldberg, 2003, Balchin et al., 2016).

3.1. Protein synthesis and folding

Protein synthesis occurs in the cytoplasm or on the ER surface, where an mRNA sequence is translated into an amino acid sequence through the four stages of translation: initiation, elongation, termination and ribosome recycling. Mature, transcribed, mRNA is exported from the nucleus to the cytoplasm through nuclear pores. In eukaryotes, the 80S ribosome is composed of two subunits, the 40S small subunit and the 60S large subunit (Deuerling et al., 2019). Translation initiation occurs with the identification of the start codon on the mRNA by the 43S preinitiation complex and binding of the small ribosomal subunit (40S) to the mRNA and large subunit (60S). During elongation, aminoacyl-tRNAs sequentially bind to mRNA codons in the ribosome, bringing amino acids that form a growing polypeptide chain. The process continues until a stop codon (UAA, UAG, or UGA) is encountered on the mRNA, followed by the release of the polypeptide from the ribosome (Balchin et al., 2016). After translation termination, ribosomes are recycled, and all translational components are retrieved for utilization in subsequent rounds of translation. The newly synthesized polypeptide chain undergoes protein folding to attain its functional conformation (Deuerling et al., 2019). Here, any post-translational modifications, such as phosphorylation, glycosylation, and lipidation, may also occur to finalize the functional protein (Kramer et al., 2019). Proteins destined for specific subcellular compartments undergo targeting and transport mediated by signal sequences and specific transport machineries.

3.1-1. Molecular chaperones in folding

One of the major ways the cell protects and controls their proteins is by co-translational folding. The first folding stages are supported by a dedicated area on the ribosome itself. This area also acts as a hub for

organization of chaperones, modifying enzymes, and membrane targeting factors that will be interaction partners for the new peptides near the ribosomal exit tunnel. The vectorial emergence of polypeptides from ribosomes frequently prevents them from folding steadily until a whole domain is synthesized (Hartl et al., 2011). An increasing body of research suggests that these interactions take place in a specific order that creates a well-structured maturation process cascade. Precise timing of these co-translational interactions is necessary to maintain proteome integrity (Kramer et al., 2019). Eukaryotes have several ribosome-associated chaperone systems that support *de novo* protein folding; in yeast they include: Hsp70 Ssb, **RAC** (**r**ibosome-**a**ssociated **c**omplex) and **NAC** (**n**ascent polypeptide-**a**ssociated **c**omplex) (Deuerling et al., 2019, Zhang et al., 2017). Almost every newly translated polypeptide in yeast is associated with RAC-SSB and NAC. When protein folding stress occurs, cells lacking in NAC and RAC-SSB activity amass aggregated proteins and lose viability (Willmund et al., 2013).

Downstream chaperones in yeast interacting with either nascent or released polypeptides include prefoldin, the chaperonin TriC/CCT, the canonical Hsp70 Ssa with J domain proteins Sis1 and Ydj1 and nucleotide exchange factors, and the Hsp90 chaperone machinery ribosome-nascent chain complex (RNC complex) (Hipp et al., 2019). Despite not directly binding to ribosomes, these chaperones help *de novo* protein folding by interacting with nascent polypeptides and continue to work on them after the ribosome release (Kramer et al., 2019). These play an important part in protein clearance during stress as well, something we observed in **Paper I** and **Paper II** and will describe further in the section Protein aggregation.

3.1-2. The chaperones Hsp70, Hsp40 and Hsp110

Eukaryotes have developed a large chaperone system that associates with translating ribosomes and assists with folding (Albanèse et al., 2006). These proteins are important both at *de novo* folding, as well as denaturation of misfolded proteins. Many of these chaperones are referred to as heat shock proteins (HSPs) (Parsell and Lindquist, 1993). Hsp70 has a wide range of substrates and is one of the most prominent ribosome-associated chaperones (Kramer et al., 2019). The core mode of action of Hsp70 is that the C-terminal peptide binding domain interacts flexibly with hydrophobic regions of substrate polypeptides, and the N-terminal ATPase domain regulates this substrate binding and release cycle by switching between low affinity ATP and high affinity ADP states (Mayer and Bukau, 2005). Co-chaperones regulate Hsp70 function by modulating the ATPase cycle. The co-chaperone Hsp40 initiates the binding by recruiting a peptide chain to an Hsp70 bound to ATP in an open conformation. The interaction initiates ATP-hydrolysis altering Hsp70s conformational state to its closed form, efficiently ensnaring the emerging polypeptide chain preventing unwanted folding or interactions (see Figure 4) (McCarty et al., 1995). Release of the substrate is aided by the chaperone Hsp110, acting as a **n**uclear **e**xchange **f**actor (NEF). The ADP bound to the Hsp70 is exchanged with ATP and the substrate-binding domain is opened up, releasing the substrate and allowing Hsp70 to start a new substrate-binding cycle (Höfeld et al., 1995, McCarty et al., 1995). There are several Hsp70-Hsp40 chaperone systems, such as and the SSB-RAC and NAC systems.

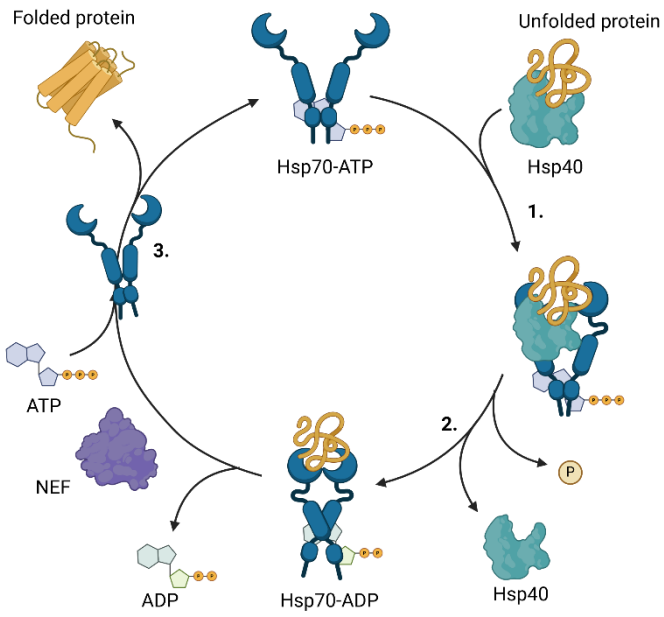

*Figure 4 - Hsp70 mediated protein folding. Hsp70-ATP in an open conformation binds to an unfolded peptide carried by Hsp40 (**1**), ATP-hydrolysis alters Hsp70s conformational state to its closed form (**2**). Hsp110 acting as a nuclear exchange factor (NEF), aid in the release of the protein and exchanges ADP to ATP, opening up Hsp70 conformation in the process (**3**). Image created with Biorender.*

3.1-3. SSA chaperones

The yeast SSA Hsp70 chaperones are a group of cytosolic proteins, comprising four members with similar functions: Ssa1, Ssa2, Ssa3, and Ssa4. Although they share functional similarities, each chaperone plays a distinct role in the processes of protein folding and refolding within the cell (Andersson et al., 2021, Mayer and Bukau, 2005). With the assistance of Hsp110 chaperone Sse1, these SSA Hsp70 chaperones aid in the folding of newly synthesized proteins, ensuring they achieve their proper conformations (Yam et al., 2005, Albanèse et al., 2006). Beyond protein folding, these chaperones are also involved in several other critical cellular functions, including protein translocation across membranes, the delivery of proteins to the proteasome for degradation

and the ubiquitin-dependent degradation of short-lived or transient proteins (Craig, 2018, Andersson et al., 2021).

3.1-4. SSB-RAC

Ribosome-associated complex (RAC) is made up of the heat-shock protein Hsp40 Zuo1 and the Hsp70 homolog Ssz1, which work in conjunction with the ribosome-attached Hsp70s Ssb1 and Ssb2 (Ssb1 and Ssb2 are functionally interchangeable in yeast, and only differs by 4 amino acids.; henceforth referred to as only SSB) (Pfund et al., 1998, Peisker et al., 2010). RAC acts as a co-chaperone, and via SSB stimulates ATP hydrolysis to promote substrate binding (Alamo et al., 2011). Recent cryo-EM studies revealed that RAC has an extended structure that binds to both the small 40S subunit and large 60S subunit of ribosomes near the polypeptide tunnel exit (Kišonaitė et al., 2023). This position brings the C-terminal substrate binding domain of the Hsp70 and the J-domain of the Hsp40 in proximity of the emerging polypeptide at the exit tunnel. Here, the J-domain of RAC is used to recruit the ATPase-active SSB chaperone to nascent substrates that require chaperone activity for co-translational folding as well as shielding nascent chains from misfolding (Kišonaitė et al., 2023, Willmund et al., 2013). However, RAC is not stably associated with ribosomes, and its cyclical binding seems to depend on the folding requirements of the translated protein (Gamerdinger and Deuerling, 2024).

3.1-5. NAC

The co-translational processing of cytoplasmic and nuclear (cytonuclear) proteins involves significantly different steps compared to secretory proteins. Secretory proteins need to remain unfolded and be transported to the endoplasmic reticulum (ER) membrane as they are synthesized. In contrast, cytonuclear proteins undergo co-

translational modifications at their N-terminus and must fold into their native three-dimensional structures within the cytosol. Given that both cytonuclear and secretory proteins are synthesized within the same cellular compartment, it is crucial to ensure that specific nascent chain processing factors bind precisely to the ribosome exit site. Recent studies have shown that recruiting factor NAC is tightly controlling this binding of co-translational factors, making sure each nascent protein ends up where it should (Gamerdinger and Deuerling, 2024, Jomaa et al., 2022).

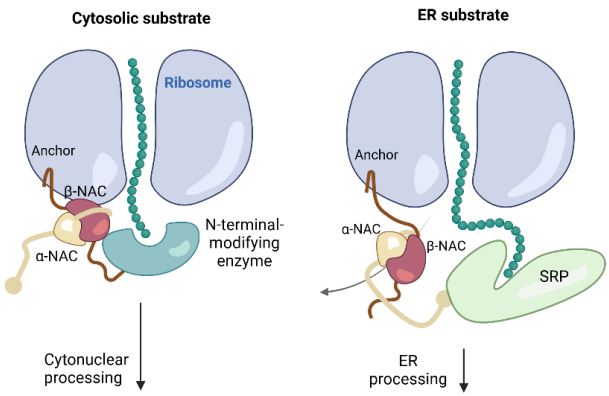

Figure 5 – Simplified model of the selective recruitment by the Nascent polypeptide-associated complex (NAC). NAC binds ribosomes through the anchor N-terminal β-NAC. Cytosolic substrates are selected by the NAC globular domain attaching to the ribosomal tunnel exit thus forming a binding platform for enzymes. ER substrates are reached by NAC globular domain detaching leading to the recruitment of SRP , taking the peptide to the ER for processing. Image modified from Gamerdinger et al. 2023, created in Biorender.

In budding yeast *S. cerevisiae*, the nascent polypeptide-associated complex, NAC, consists of stable heterodimers of Egd2 (α-NAC) together with Egd1(β1-NAC) or Btt1(β3-NAC) (Beatrix et al., 2000). The heterodimer contains a central globular dimerization domain from which four long flexible arms (N and C termini) protrude (see Figure 5). The β chain N-terminal acts as an anchor, interacting with the ribosome and flexibly tethers NAC to the periphery of the tunnel exit (Reimann et al., 1999, Wegrzyn et al., 2006). The central globular

domain makes a secondary contact with the ribosome by binding directly at the exit, enabling the free ”arms” to interact with the emerging nascent chain (Gamerdinger et al., 2023). Studies suggest that NAC binds to a ribosome already at translation initiation, inserting the anchor-β chain N-terminal into the empty tunnel with the globular domain right in-front of the tunnel exit (Wang et al., 1995). This is suggested to block nascent chain processing factors from binding the emerging chain or the inactive ribosome prematurely. As the peptide chain progresses, the β-domain is pushed out of the tunnel – binding to another site on the ribosome, while the globular domain of NAC binds next to the tunnel exit, ready to scan the nascent chain when it exits (Gamerdinger et al., 2019). This flexibility of the NAC domains enables it to be specific in its interactions; for example, if the globular NAC domain stays attached to the ribosomal tunnel exit, it promotes cytonuclear processing of proteins (Figure 5, left). This positioning blocks the access of co-translational ER-targeting factors such as SRP (signal recognition particle) while at the same time providing a binding platform for the N-terminal-modifying enzymes, ensuring that cytonuclear proteins are not mistargeted to the ER (Alamo et al., 2011, Gamerdinger et al., 2023). Enzymes are actively recruited by one of the flexible arms on NAC, and the stable binding of the globular domain to the tunnel exit is also crucial for the functional binding of the enzyme. ER-targeted proteins are processed by the globular NAC domain detaching from the exit tunnel; destabilizing N-terminal-modifying enzymes from binding, while freeing the SRP-binding site on the ribosome (Figure 5, right) (Jomaa et al., 2022, Gamerdinger et al., 2023).

In the event of protein homeostasis imbalance, NAC will fully detach from the ribosome and bind to the protein aggregates. This complex will then assist in protein aggregate clearance, preventing aggregate

accumulation. Moreover, this complete detachment also decreases the number of translating ribosomes – giving the cell more protection by preventing further protein synthesis and the ensuing accumulation of aggregated proteins (Alamo et al., 2011, Kirstein-Miles et al., 2013). Koplin et al found that in the absence of both NAC and Hsp70s SSB the levels of insoluble aggregates increase, and among those are ribosomal proteins and ribosomal biogenesis factors. This suggests that NAC and SSB has an unexplored role in ribosomal biogenesis as well (Koplin et al., 2010).

3.1-6. Ribosome-associated quality control

Part of the PQC system is the **r**ibosome-associated **q**uality **c**ontrol (RQC) pathway that is activated when ribosomes stall and collide during translation (shown in Figure 6, step 1). The stalling occurs due to the presence of a faulty mRNA that has been translated past its open reading frame (ORF) into the 3'-untranslated region (UTR). This leads to the translation of twelve consecutive basic residues, or translation of the poly(A) tail (Dimitrova et al., 2009). Reasons for stalling include absence of stop codons on RNA (non-stop mRNA; e.g. premature polyadenylation or endonucleocytic intermediates during RNA decay), premature 60S subunits that escaped the nucleus, or RNA/ribosomal damage due to external stressors (e.g. UV, or chemical stress) (Brandman et al., 2012, Shoemaker and Green, 2011). The translated basic peptides from the stalling electrostatically interact with the ribosomal exit tunnel, causing translation to slow down (Lu and Deutsch, 2008). Ribosomal stalling is toxic to the cell, partly due to aberrant peptides forming, and partly due to the need for recycling of the ribosomal subunit (Sitron and Brandman, 2020). Thus, the faulty synthesized protein peptides are marked for proteasomal degradation while they are still associated with the ribosomal 60S subunit (Joazeiro, 2019). The yeast RING domain E3 ubiquitin-protein ligase Ltn1 is the

key participant in RQC (Chu et al., 2009, Bengtson and Joazeiro, 2010). Chu et al first discovered the involvement of Ltn1 in targeting non-stop proteins from stalled ribosomes for ubiquitinylation and degradation by binding the large 60S subunit. Moreover, in Ltn1's absence, the products from stalling were found to accumulate on ribosomes (Chu et al., 2009).

In yeast, ribosomal stalling can be separated into a few steps, as seen in Figure 6. The stalled ribosomes are first identified by the E3 ubiquitin ligase Hel2 (also called Rqt1 for "RQC-trigger factor 1") that induces both no-go decay (mRNA are endonucleocytic cleaved and degraded) and the RQC system (Doma and Parker, 2006, Ikeuchi et al., 2019). Separating the 80S ribosome into subunits is a crucial first step, resulting in the 60S subunit still bearing the obstructing peptide and the 40S subunit free to be recycled. The mRNAs can thus be degraded by 5'-3' exosome complex and exoribonuclease Xrn1, preventing further translation of possible aberrant transcripts (Joazeiro, 2019). Hel2 together with E2 enzyme Ubc4 mediates K63-linked poly-ubiquitination of the ribosomal subunit 10S to activate the **R**QC-**t**rigger **c**omplex (RTC) that is responsible for the ribosomal splitting, in a still not fully understood mechanism (Matsuo et al., 2017, Ikeuchi et al., 2019).

RTC is composed of Slh1/Rqt2 (RNA helicase), Cue3/Rqt3 (ubiquitin-binding protein) and Rqt4 that together recognize the stalled ribosome (Matsuo et al., 2017). The splitting by RTC is then activated by the Hbs1/Dom34 complex; through hydrolysis of Hbs1 and conformational change of Dom34 they recruit the ATPase Rli1 whose activity stimulates ribosomal dissociation (Shoemaker and Green, 2011, Joazeiro, 2019). The now free 60S subunit is bound by the RQC complex consisting of Ltn1, Rqc1 and Rqc2. Ltn2 poly-ubiquitinates the nascent

polypeptide, and Rqc2 stabilizes Ltn2 and adds a CAT tail as a fail-safe to the ubiquitinated peptide as it drags out Lys-residues from the ribosome tunnel (Bengtson and Joazeiro, 2010). The Cdc48 ATPase and its cofactors, Ufd1 and Npl4, are recruited by Ltn1 and Rqc1 and attaches to the peptide chain. With energy from hydrolyzing ATP, Cdc48 and its cofactors together create mechanical force strong enough to remove the poly-ubiquitinated peptide from the ribosomal exit tunnel. The faulty peptide is then sent to the proteasome via the RQC components so that it can be broken down (Defenouillère et al., 2017).

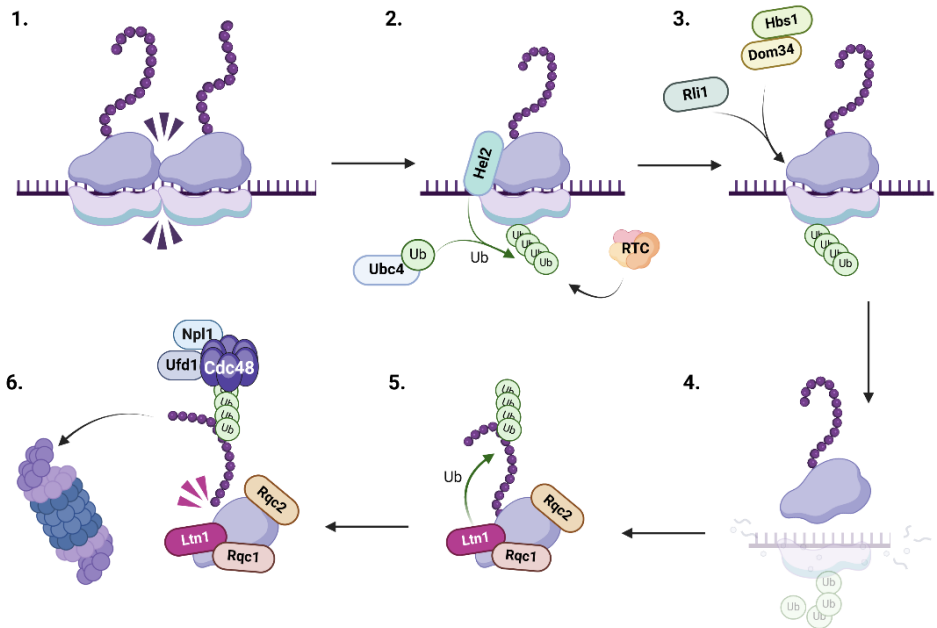

Figure 6 - Ribosomal quality control (RQC) for degradation of stalled peptides. ***1.*** *Ribosomes collide during translation.* ***2.*** *Hel2 recognizes the stalled ribosome and polyubiquitinates the small ribosomal unit together with Ubc4. RTC then triggers RQC systems.* ***3.*** *Dissociation of the ribosomal subunits are stimulated by Dom34, Hbs1 and Rli1.* ***4.*** *The small ribosomal subunit and the mRNA are both degraded.* ***5.*** *Ltn1 binds to the large ribosomal subunit stabilized by Rqc2, and polyubiquitinates the stalled peptide. Ltn1 and Rqc1 also recruits Cdc48 and its cofactors.* ***6.*** *Cdc48 together with Npl1 and Ufd1, creates enough force to detach the polypeptide, which is then degraded via the proteasome.* *Image created with Biorender.*

3.1-7. TriC/CCT chaperonin

The Hsp60 chaperonin family TriC/CCT, present in all eukaryotes, is primarily responsible for the de novo folding of actin and tubulin, among others, together with the co-chaperone prefoldin. The barrel-shaped chaperonin is comprised of two rings of eight subunits each creating a central cavity (Cct1-8 in yeast, CCTα-θ in mammalian cells). The equatorial domain contains an ATP-binding site, with a flexible linker to the apical substrate-binding domain where an ATP-hydrolysis driven reaction that alternates between conformational states until proper tertiary structure is achieved (Vallin and Grantham, 2019). An important cofactor to CCT in this process is prefoldin, a chaperone that stabilizes nascent forms of actin and tubulin and interacts transiently with the chaperonin to deliver them to the folding cavity (Vainberg et al., 1998). Not only newly synthesized proteins can utilize CCT for folding, but CCT can also assist proteins fold into larger complexes or hinder deleterious misfolding and aggregation (e.g. alpha-synuclein and huntingtin into amyloid fibers) (Nollen et al., 2004, Sot et al., 2017, Shahmoradian et al., 2013). CCT can also exist as stable individual monomers and recent studies has revealed that they are involved in processes beyond folding. For example, as a potential regulator of actin transcription, and several interactions involved in cell cycle progression in mammalian cells (Córdoba-Beldad and Grantham, 2024, Roobol et al., 1999).

3.1-8. Small heat-shock proteins

Small heat shock proteins (sHSPs) are a family of low molecular weight chaperones that bind denaturing proteins to either prevent aggregation or to refold them. If the rescue is unsuccessful, the sHSPs can deliver the protein to a degradation pathway (Malinovska et al., 2012). Yeast has two sHSPs: Hsp42 that form large barrel-like oligomers and are present in all conditions, and Hsp26 that is activated

only during stress conditions (Haslbeck et al., 2004b). The small chaperones are very responsive to changes in the environment such as changes in pH, temperature, phosphorylation, and in the case of oxidative and metal stress. The chaperones can then engage in transient or long-term interactions with substrates depending on the surroundings (Janowska et al., 2019). Haslbeck et al showed that during heat shock, the sHSPs prevented the aggregation of one-third of the cytosolic proteins (Haslbeck et al., 2004a).

3.1-9. Protein balance

Folded proteins are not very stable – evolutionary pressure on protein function causes inevitable selection of conformational states prone to misfolding and aggregation. Proteins are made of moveable components that cooperate to carry out a variety of biological functions. They must be flexible and able to adopt many conformations in order to accomplish this (Balchin et al., 2016). By exposing interactive surfaces in these alternate conformations; it creates possibilities for novel, advantageous interactions to aid protein function in the cell. The selection pressure on protein conformations encompasses stability, ability to fold, and if the protein functions correctly. As long as function is sufficient for cell survival, they are selected, thus threading the thin line of efficient versus aggregation prone when the interactive surfaces are too easily accessible (Gershenson, 2014). One example of this tension is susceptibility to mutation, either to the protein or to a component of the protein quality control system (PQC). Small changes in the sequence of proteins can tip the scale from functionally beneficial changes, toward protein misfolding and aggregation. Translation errors can also be detrimental, as misincorporation of amino acids can lead to the synthesis of aberrant proteins which might be more susceptible to misfold. Furthermore, environmental stress conditions, such as heat, chemical changes,

oxidative stress, or the absence of a binding partner can damage proteins. Metals can affect the correct folding of proteins – leading to loss of function and aggregation – either by binding the protein directly, or by oxidative stress or inducing translational errors. During the process of aging, the cell can go through all of the above, as well as accumulating misfolded and aggregated proteins due to a decline of the PQC system (Tyedmers et al., 2010).

Many compensatory cellular mechanisms have developed to reduce the number of high-risk protein structural states that can accumulate. Already at the amino acid level, sequences have evolved to minimize areas with high hydrophobicity and lowered net charge. On higher structure level, we see increased prevalence of β-sheet regions (Richardson and Richardson, 2002), as well as an inclination towards folding into a stable globular form (Gershenson, 2014, Monsellier and Chiti, 2007). Cells can also make adjustments in expression levels (Tartaglia et al., 2007) or protein turnover to decrease aggregation rates and relieve cell stress (Monsellier and Chiti, 2007). An example of this adjustment is in **Paper I**, where we show that yeast cells with reduced global translation have less protein aggregation and a higher resistance during arsenite stress.

3.2. Protein aggregation

Proteins can sometimes misfold or become damaged due to genetic mutations, environmental factors, or aging. These misfolded proteins can form aggregates that vary in shape and size, ranging from small oligomers to larger fibrils or insoluble amorphous clumps (

Figure *7*

Figure 7). However, cells can harness a controlled form of protein condensates for various functions though. For example, eukaryotic cells possess various dynamic membrane-less organelles, such as the nucleolus, Cajal bodies, stress granules, and P-bodies, collectively known as ribonucleoprotein (RNP) granules. These organelles can interact with RNA to carry out essential physiological functions (Wen et al., 2023).

The irreversible aggregation of misfolded proteins often represents a permanent loss of function and are detrimental for cells (Alberti and Hyman, 2021). The toxicity of these aggregates has been a frequent question that we encounter, and it depends on where the aggregates occur and how we define toxicity: e.g. single pathway disruption, stressed cells, or all the way to cell death (Mogk et al., 2018). Several factors influence the toxicity of aggregates including their stability, reactive surface properties, localization, and compartmentalization within the cell. These aggregates can disrupt normal cellular processes and interfere with the functions of organelles, such as physical damage to membranes (Lashuel et al., 2002), and induce stress-regulated translational shut-down (Smith and Mallucci, 2016) leading to cellular stress and dysfunction (Stefani and Dobson, 2003). Additionally, they may impair transport mechanisms and act as seeds for further misfolding of proteins, leading to toxic effects within the cell (Grima et al., 2017, Labbadia and Morimoto, 2015, Bäuerlein et al., 2017). In addition to proteins, these aggregates can also contain other molecular components, such as nucleic acids like RNA, which can exacerbate the accumulation of protein aggregates (Wallace et al., 2015).

Oligomers are small aggregates formed by a few units of misfolded proteins. They can range in size from dimers to larger assemblies. Although their exact role is debated, oligomers are generally considered toxic and can interfere with cellular functions, potentially contributing to the development of neurodegenerative diseases (Alam et al., 2019). **Fibrils** are elongated structures formed by aggregated proteins, often insoluble and arranged into larger complexes. Amyloid fibrils, characterized by their cross-beta sheet structure, are often associated with diseases such as Alzheimer's and Parkinson's disease and are formed by the aggregation of proteins like beta-amyloid, alpha-synuclein, or tau. **Amorphous aggregates**, in contrast to fibrils, lack a defined structure and appear as disordered clumps of misfolded proteins. They are diverse in form and shape and can be observed in various neurodegenerative diseases, contributing to cellular inclusions and dysfunction.

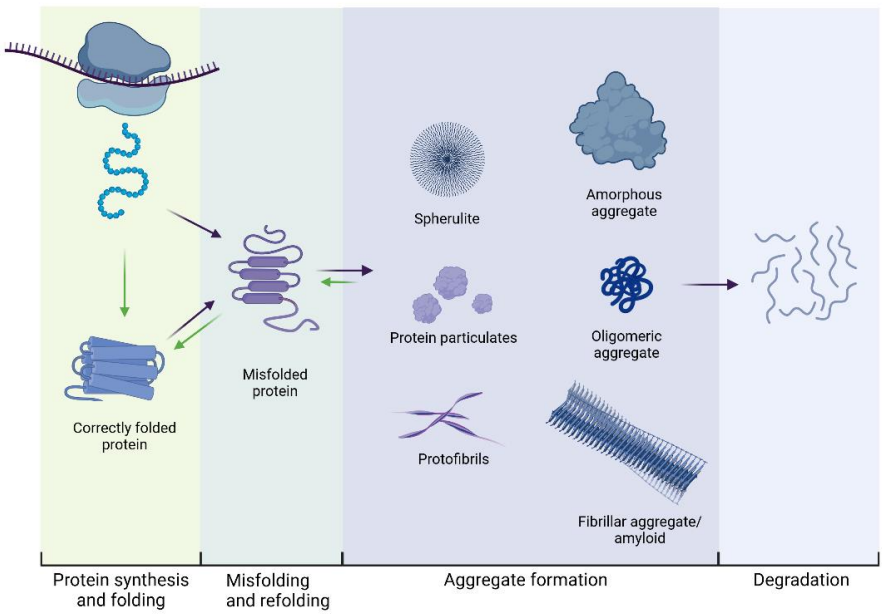

Figure 7 – Proteins can misfold and form aggregates. They can be small and oligomeric, large unstructured amorphous aggregates, or highly structured fibrillar aggregates. Cellular processes counteract aggregation by disaggregating and refolding of misfolded proteins. If aggregates form, degradation pathways are initiated. *Image created with Biorender.*

Protofibrils are intermediate aggregates that lie between oligomers and fully formed fibrils. They have a more defined structure than amorphous aggregates but are not as mature as fibrils. These structures are thought to be particularly toxic and may play a key role in the progression of amyloid-related diseases as well (Lashuel et al., 2002, Dearborn et al., 2016). **Spherulites** are spherical aggregates composed of radially arranged fibrils. These structures are less common but can be found in certain amyloid diseases. They represent another form of aggregated protein that can disrupt normal cellular functions. **Protein particulates** are small, insoluble aggregates that may form spontaneously in cells under stress. They can accumulate and coalesce into larger aggregates, contributing to cellular toxicity and organelle dysfunction. Intracellular inclusions or deposits are localized accumulations of aggregated proteins within a cell. Examples include Lewy bodies found in Parkinson's disease, composed mainly of alpha-synuclein, neurofibrillary tangles in Alzheimer's disease made up of hyperphosphorylated tau protein, and inclusion bodies observed in muscular dystrophy and Huntington's disease (Stefani and Dobson, 2003, Chiti and Dobson, 2017).

3.2-1. Protein Quality Control

Maintaining protein homeostasis is crucial for cellular function and survival. The **P**rotein **Q**uality **C**ontrol (PQC) system plays a key role in this process by ensuring that proteins are correctly folded, functional, and free from damage, as mentioned in the previous section.

In response to an excess of aberrant proteins, the regulation of the PQC network is primarily controlled through the activation of the master regulator **H**eat **S**hock **F**actor 1 (HSF1). Yeast Hsf1 triggers a highly conserved transcriptional program known as the heat shock response (HSR), resulting in a high upregulation of PQC factors (Solís et al.,

2016). Under normal conditions, the expression of the **h**eat **s**hock **r**esponse (HSR) components is kept at a basal level through a chaperone titration mechanism in which Hsp70 acts as a repressor of Hsf1. However, when chaperones like Hsp70 are diverted to bind misfolded or aggregated proteins, they are no longer available to suppress Hsf1, resulting in the activation of the HSR. Hsf1 activation increases the expression of several molecular chaperones, including Hsp70. As Hsp70 levels rise, they bind to Hsf1, thereby creating a negative feedback loop that ultimately leads to the repression of Hsf1 and the attenuation of the heat shock response once protein homeostasis is reestablished (Krakowiak et al., 2018). The HSR induces the production of various factors within the protein homeostasis network, such as Hsp104, to ensure the stability and proper folding of the proteome. Interestingly, many of the heat shock-responsive elements in the genome are also regulated by the general stress response transcription factors Msn2 and Msn4, which suggests a broader coordination between stress response pathways (Solís et al., 2016). **The PQC system** encompasses several mechanisms that work in both temporal and spatial manners to manage and mitigate protein misfolding and aggregation (see Figure 8).

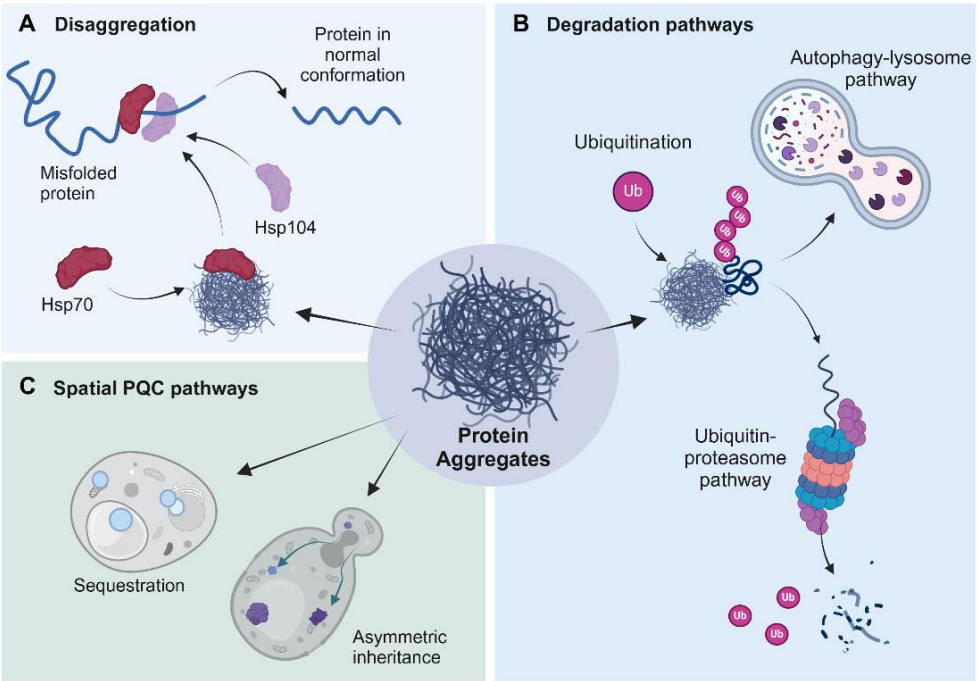

Figure 8 – Simplified scheme of protein quality control pathways in yeast. ***A****. Disaggregation via Hsp70-Hsp100s where an aggregated protein is disaggregated and refolded into a functional form.* ***B.*** *Ubiquitination of misfolded and aggregated proteins, leading to degradation either via the proteasome or autophagy-lysosome pathways.* ***C.*** *Spatial sequestration of aggregates into specific compartments in the cell. Protein aggregates can also be transported into the mother cell in asymmetric inheritance, to ensure that daughter cells have a fully functional proteome. The pathways often work in conjunction with each other to ensure protein homeostasis. Image created with Biorender.*

3.2-2. Disaggregation

One of the primary temporal mechanisms in PQC is protein disaggregation and refolding involving molecular chaperones such as Hsp104 (Figure 8A) (Sanchez and Lindquist, 1990). Yeast Hsp104 is a ring-forming oligomeric AAA+ chaperone crucial for disaggregating misfolded proteins and restoring them to their native states after heat and other stressors (Parsell et al., 1994). Hsp104 is a part of a larger Hsp100 family, a group with diverse cellular activities that uses ATP hydrolysis to remodel or dissociate protein complexes and unfold

bound substrates (Mogk et al., 2018). However, Hsp104 requires the activation of Hsp70 to start the disaggregation process (Zietkiewicz et al., 2004). Hsp70, as mentioned above, has versatile functions including folding of newly synthesized proteins and the prevention of their aggregation, and protein transport and degradation (Mayer and Bukau, 2005). Hsp70s interaction with the aggregate restricts the access of proteases and delivers the substrate chain to the core of Hsp104; directing the aggregate towards refolding rather than degradation (Glover and Lindquist, 1998). Hsp104 then pulls the substrate through the central core of its ring with energy from ATP hydrolysis, threading the substrate and detangling the misfolded polypeptides one-by-one (Figure 8A) (Lum et al., 2004). This temporal aspect of PQC allows cells to quickly respond to stress conditions that induce protein misfolding without it leading to degradation, thereby maintaining protein homeostasis.

3.2-3. Ubiquitin-Proteasome System (UPS)

The **U**biquitin-**P**roteasome **S**ystem (UPS) is one of the man pathways in protein degradation. This system targets misfolded or damaged proteins for degradation by tagging them with ubiquitin molecules (Figure 8B). The system works in 3 repeating steps: the E1 activator enzyme transfers ubiquitin to an E2 carrier enzyme, and together with an E3 ligase transfers ubiquitin to a substrate to be degraded (Nandi et al., 2006). The substrates are gradually charged with Ub, either at the N terminus (Met1) or at a lysine side chain of Ub, thus diverse linear or branching Ub chains are formed. Poly-Ub link acts as a strong signal, attracting endogenous Ub receptors of the proteasome or shuttle factors that leads ubiquitinated proteins to the proteasome (Oh et al., 2018, Nandi et al., 2006). There are E3 ligases with specificity for certain substrates other than the main Ub-tags in yeast, and their recognition

often depend on cooperation with Hsp70 chaperones - contributing to the selectivity and specificity of the UPS (Tyedmers et al., 2010).

The actual degradation is carried out by the 26S proteasome, a complex consisting of a 20S core capped by one or two 19S regulatory particles (Bard et al., 2018). Once the substrate is bound to the proteasome, the ubiquitin is removed by deubiquitinating enzymes to be recycled before the degradation commences. Then ATPase subunits on the base of the proteasome complex begin protein unfolding and translocation into the cylindrical core (Braun et al., 1999), where active subunits cleave the unfolded protein into short segments which diffuse into the cytosol for further processing (Finley et al., 2016). We found that the UPS pathway was the main contributor to As(III)-induced aggregate degradation, discussed further in **Paper** II.

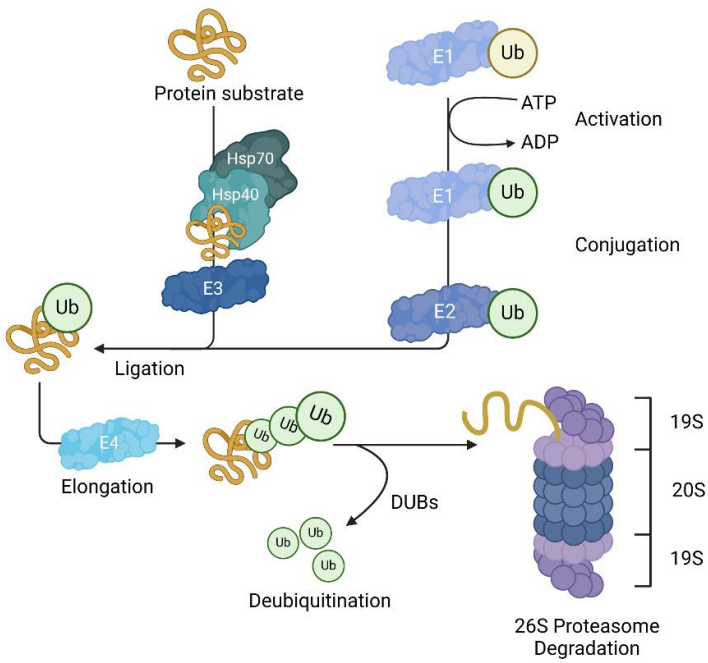

Figure 9-Ubiquitination (Ub) of protein. E1 activates via ATP hydrolysis and conjugates Ub to E2 and ubiquitinated via E3 ligase. The Ub chain is elongated by the repeat of previous steps and E4 elongation factor. Before degradation via the proteasome, the substrate is de-ubiquitinated by deubiquitinating enzymes (DUBs). *Image created with Biorender.*

3.2-4. Autophagy-lysosome pathway

Ub-chains can also join with autophagy receptors when the aggregate becomes too large for the proteasome to handle (see Figure 8). This activates autophagosomes, which engulfs protein aggregates and fuse with lysosomes to degrade them (Pohl and Dikic, 2019). Autophagy is another vital PQC pathway that deals with the degradation and recycling of cellular components, including proteins. Macroautophagy uses specialized, cytosolic, double-membrane structures that engulf large aggregates of proteins or damaged organelles in autophagosomes, which then fuse with lysosomes where the contents are degraded (Chun and Kim, 2018). This process not only helps in clearing misfolded proteins but also in recycling amino acids and other cellular materials, contributing to overall cellular homeostasis (Singh and Cuervo, 2011). The process is closely linked to the UPS; a decline in one of the degradation systems results in a compensatory up-regulation in the other (Korolchuk et al., 2009).

3.2-5. Spatial PQC mechanisms

Spatial protein quality control (sPQC) systems are involved in the segregation and compartmentalization of misfolded proteins within the cell, limiting their toxic effects (Figure 8C,). The protective inclusions not only relieve PQC factors from the burden of managing these proteins, but also prioritizes essential tasks during acute stress. The orderly sequestration of protein aggregates into distinct inclusions facilitates their clearance through repair or degradation (Kaganovich et al., 2008). This is achieved by enabling the efficient temporal and spatial accumulation of PQC components, which supports downstream processing. Retaining proteins for refolding after stress is often more energy-efficient than destroying them (Öling et al., 2014).

While the sPQC response varies depending on protein species and the type of stress involved, a common pattern of deposition sites has been revealed as a general sPQC response pathway. The common pathway begins with the formation of a lot of small foci throughout the cell, that coalesce into larger inclusions.

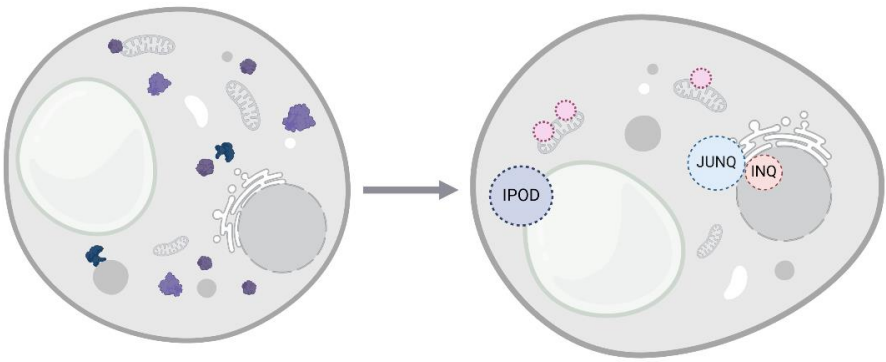

Figure 10 - Spatial protein quality control. Small foci form during initial stress and are sequestered to specific compartments at the vacuole(IPOD), nucleus(JUNQ/INQ) or mitochondria to either be refolded or degraded later on. Image created with Biorender.

Q-bodies: These are cytoplasmic compartments that temporarily store misfolded proteins, keeping them sequestered until they can be refolded or degraded. They are small peripheral foci that become visible during the initial response to protein folding stress (Escusa-Toret et al., 2013). The formation of CytoQs, which are a type of Q-body, depends on the endoplasmic reticulum (ER) and PQC factors such as the small heat shock protein Hsp42 and Hsp70s. This process enhances cellular fitness under stress conditions, although the absence of Hsp42 under normal growth conditions can increase lifespan, suggesting a context-dependent role for Hsp42 (Escusa-Toret et al., 2013). The active ATP-dependent fusion by Hsp42 into CytoQ formations are required for protein localization to sPQCs, which suggest CytoQ to be precursors to the dynamic JUNQ/INQ compartments (Sontag et al., 2017).

JUNQ/INQ (Juxta-Nuclear or Intranuclear Quality Control) and IPOD (Insoluble Protein Deposit): In yeast cells, two distinct compartments have been identified for protein quality control: the juxtanuclear quality control compartment (JUNQ) and the perivacuolar insoluble protein deposit (IPOD). Soluble, misfolded, ubiquitinated proteins are directed to JUNQ, while insoluble, terminally aggregated proteins accumulate at the IPOD near the vacuole. Disruption of the cytoskeleton has been shown to interfere with the targeting of proteins to both compartments, showing an importance of the network in gathering aggregates to the different compartments (Tyedmers et al., 2010).

JUNQ, also known as **INQ** under certain conditions, is situated near or inside the nucleus and serves as a main location for the concentration and processing of soluble or ubiquitinated proteins. These proteins can potentially be re-folded, otherwise they will be targeted for degradation (Miller et al., 2015). Several studies have expanded our understanding of the roles of these compartments, particularly under stress conditions. For example, it has been observed that INQ can form in response to genotoxic stress even in the absence of JUNQ formation (Gallina et al., 2015). Furthermore, some misfolded proteins localize exclusively to INQ and not to JUNQ, suggesting that INQ and JUNQ may serve as distinct quality control compartments, each with specific functions. The factors involved in both heat and genotoxic effects seems to be the same, including Hsp104, Sis1 and Hsp-like Btn2 (Gallina et al., 2015, Malinovska et al., 2012). Interestingly, INQ is not limited to processing nuclear proteins; it also contains misfolded proteins of cytosolic origin. These cytosolic proteins are imported into the nucleus through nuclear pores, a process that is facilitated by the co-chaperone Sis1 (Miller et al., 2015, Park et al., 2013). This trans-location likely involves additional protein homeostasis network

factors, which may vary depending on the specific characteristics of the misfolded proteins. In contrast, IPOD is positioned adjacent to the vacuole and is specialized in managing more insoluble protein aggregates. Unlike JUNQ, IPOD is less dynamic, showing minimal exchange with the surrounding environment, which indicates that it primarily harbors terminally misfolded proteins that are resistant to refolding or degradation (Kaganovich et al., 2008).

During yeast cell division, both JUNQ and IPOD are asymmetrically retained in the mother cell, ensuring that the daughter cell is largely free of damaged or aggregated proteins. Studies by Nyström's group show that proteins damaged by oxidation and aggregated proteins are almost exclusively retained by the mother during cell division (Aguilaniu et al., 2003, Liu et al., 2010). This asymmetric inheritance is critical for maintaining cellular health and longevity, as it allows the mother cell to manage protein damage while the daughter cell retains a functional proteome.

Mitochondria: Mitochondria play a role in PQC by housing specific proteases and chaperones that manage mitochondrial protein quality. They also assist in the degradation of damaged proteins through mitophagy, a selective form of autophagy targeting mitochondria (Pickles et al., 2018).

Sorting Factors: The processes involved in sPQC are not random; they are actively regulated and dependent on energy and various sorting factors. While no single sorting determinant has been identified, several factors—including molecular chaperones and the cytoskeleton—are known to influence the sequestration of misfolded proteins into specific PQC compartments. These sorting mechanisms are crucial for ensuring efficient protein quality control within the cell.

Several studies propose that protein aggregation, particularly for soluble proteins, may be driven by liquid-liquid phase separation (LLPS), a process that allows the formation of biomolecular condensates and membrane-less organelles in aqueous environments. LLPS has become an important concept in understanding protein homeostasis and stress responses, suggesting that aggregation is not merely a random process but rather a controlled mechanism for managing misfolded proteins (Riback et al., 2017, Brangwynne et al., 2009, Alberti and Hyman, 2021).

4. Nucleocytoplasmic transport

Eucaryotic chromosomes are housed within a nucleus, enclosed in a nuclear envelope (NE) composed of a double membrane of phospholipids. The processes of gene transcription and ribosomal biogenesis in the nucleus are segregated from the synthesis of proteins in the cytosol by a highly controlled transport mechanism; nucleocytoplasmic transport (NCT) allowing the cell to coordinate numerous processes key to its survival and growth (Oka and Yoneda, 2016). This results in a challenge for the cell to manage the large number of proteins and RNA to be transported in and out at the right time. Smaller molecules, such as metabolites and ions, can pass freely over the NE with the help of small transport proteins or by simple diffusion. Molecules larger than approximately 40 kDa, however, need assistance to cross the nucleocytoplasmic barrier through nuclear pore complexes (NPCs) that perforate the membrane (Panté and Kann, 2002).

4.1. Nuclear Pore Complex

Nuclear pore complexes are large barrel-shaped complexes consisting of around 30 nucleoporins of different characteristics (Cowburn and Rout, 2023, Kim et al., 2018b). The core consists of an octagonal cylinder of proteins (termed nucleoporins or nups) situated in the NE; consisting of outer and inner rings surrounding a central channel through which essentially all transport occurs (Figure 6). Outer rings help anchor the nucleoporins in place and provide structural stability to the NPC (Aitchison and Rout, 2012). Extending outward from the NPC core into the cytoplasm are cytoplasmic filaments, extended from a group of nups (so called FG nups) with large intrinsically disordered regions containing Phe-Gly (FG) repeats linked by predominately hydrophilic linkers (Radu et al., 1995, Stanley et al., 2017). These filaments play a role in capturing and guiding molecules that need to

enter the nucleus from the cytoplasm. Similarly on the other side, protruding into the nucleoplasm are filamentous structures known as the nuclear basket, for the recognition and processing of molecules leaving the nucleus (Cowburn and Rout, 2023, Jovanovic-Talisman et al., 2009).

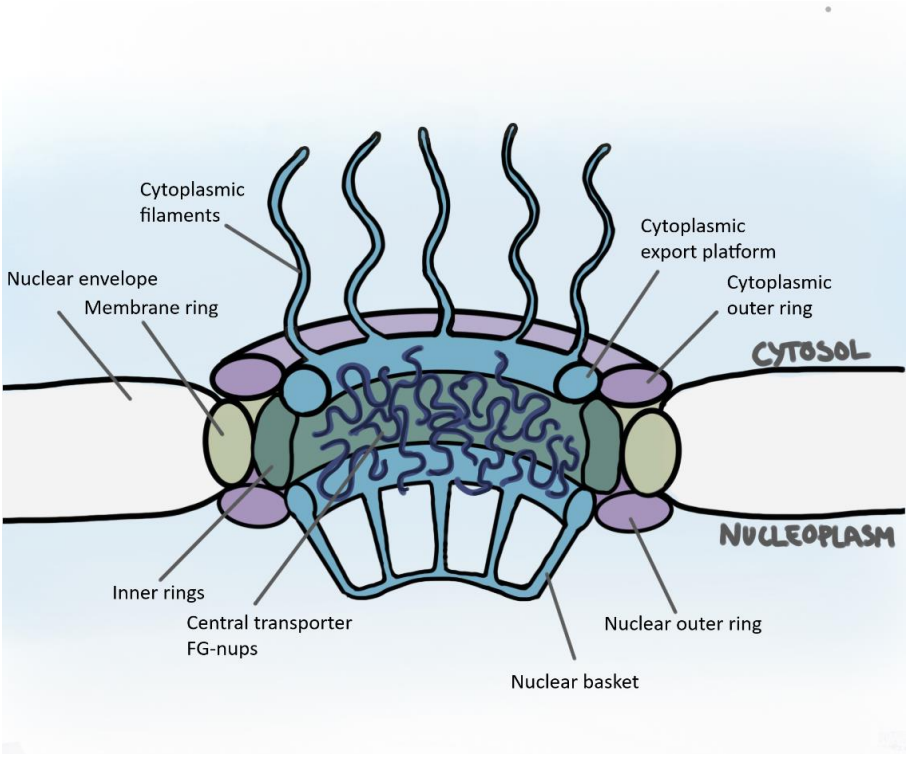

Figure 11 – A representation of the structure of yeast Nuclear Pore Complex with the major components labeled.

4.2. Nuclear transport

The majority of protein and macromolecule transport across the NPC is mediated by a conserved family of nuclear transport receptors (NTRs), β-karyopherins (Kaps), bringing molecules into the nucleus (importins), out of the nucleus (exportins) or in both directions (biportins/transportins) (Hicks and Raikhel, 1995, Wing et al., 2022). Importin α (yeast Srp1/Kap60; human KPNA1) was first discovered to

bind to NLS together with importin β (yeast Kap95; human KPNB1) (O'Reilly et al., 2011, Chook and Süel, 2011). This importin αβ complex is one of the main players of nuclear transport, though there is a whole family of Kaps helping. Indeed, *S. cerevisiae* has 14 Kaps in total with varying function and specificity towards nuclear localization signals, Ran-GTP and FG-repeat nucleoporins (Wing et al., 2022). Unlike the importin αβ complex, the other Kaps bind directly to their cargo (Aitchison and Rout, 2012). Cargo that needs transportation is recognized and bound to their NTR at folded domains and/or linear components known as nuclear localization signals or nuclear export signals (NLSs or NESs), depending on direction (Kalita et al., 2021, Chook and Süel, 2011). Although the sequences can be complex and differ quite a lot, the classic NLS is a stretch of basic residues (i.e. KKKRK) (Kalderon et al., 1984). The NTR-cargo complex then passes through the NPC by transiently binding FG repeats, crossing over to the other side and releasing its cargo (see Figure 12). However, not all proteins destined for NCT contains an NLS/NES; the range of cargo that passes through the NPC is huge and ranges from proteins, RNA, ribonucleoproteins to viruses (Oka and Yoneda, 2016).

The nuclear transport is driven by a formation and maintenance of a concentration gradient of GTPases at the NE. One of the key GTPases involved in nuclear transport is Ran (Ras-related nuclear protein). Ran exists in two nucleotide-bound forms, Ran-GDP (guanosine diphosphate) and Ran-GTP (guanosine triphosphate) (Bischoff et al., 1995). Ran-GTP is predominantly found in the nucleus and Ran-GDP is more abundant in the cytoplasm (Bischoff et al., 1995). This asymmetric distribution establishes a concentration gradient, influencing the directionality of cargo transport through NPCs (Stewart, 2007), one that can be reversed with increased cytoplasmic Ran-GTP (Nachury and Weis, 1999). During the import process, Ran-GTP binds to

importin β within the NPC. This binding provides energy for the disassembly of the import complex, leading to the release of the cargo into the nucleoplasm. Conversely, during export, the binding of Ran-GTP to exportins within the nucleus facilitates cargo binding, allowing the complex to exit the nucleus (Stewart, 2007, Cook et al., 2007). ATPases are involved in the formation and maintenance of the concentration gradients of nucleotide-bound Ran on both sides of the nuclear envelope (Izaurralde et al., 1997). Schwoebel et al. showed that ATP depletion leads to a decrease in RanGTP, which is essential for these transport processes, indicating an indirect ATP dependency even if the process itself is ATP-free (Schwoebel et al., 2002).

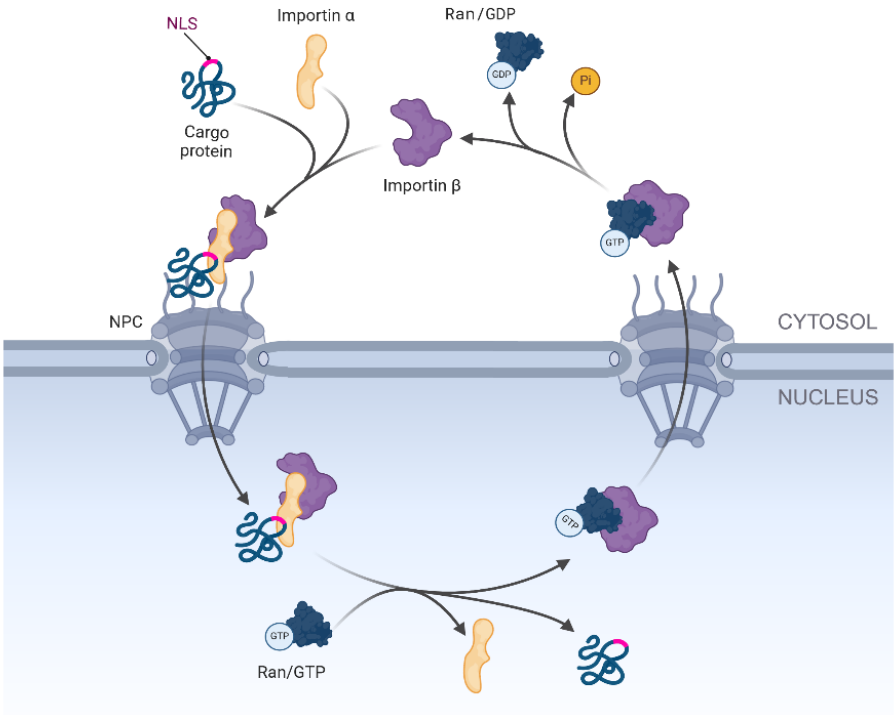

Figure 12 - Transport across the nuclear envelope via the importin αβ complex. Importin α recognizes an NLS sequence on a cargo protein in the cytosol, and together with importin β moves through the NPC by transiently interacting with FG-nups in the tunnel. On the nuclear side, the cargo is released via Ran-GTP and the importins are escorted out again to the cytosol. *Image created with Biorender.*

4.3. Functions beside NCT

Recent research has expanded our understanding of the NPC beyond its primary role in nucleocytoplasmic transport. While transport remains fundamental, the NPC also significantly influences various cellular processes occurring on both sides of the nuclear envelope (Strambio-De-Castillia et al., 2010). One notable component of the NPC is the nuclear basket. This basket is implicated in diverse functions such as transcriptional control, maintenance of SUMO homeostasis, regulation of cell cycle progression, organization of chromatin, and facilitation of RNA biogenesis (Strambio-De-Castillia et al., 2010, Kabachinski and Schwartz, 2015, Li et al., 2021).

The importin complex also serves a dual function: it acts as a chaperone by binding to nascent proteins co-translationally. Research by Seidel et al. (2023) demonstrated that some yeast importins bind to emerging peptide chains from the ribosomes during translation, shielding crucial domains such as nuclear localization signals (NLS) on cargo proteins. Proteins rich in basic amino acids, like ribosomal proteins and histones, are prone to aggregation in the presence of RNA. Co-translational binding by importins prevents such aggregation by shielding these basic domains (Seidel et al., 2023). This dual role enhances efficiency by eliminating the need for a handover from chaperone to importin, thereby reducing potential points of failure. Moreover, importins possess the ability to disaggregate and reverse aberrant phase transitions of cargoes bearing NLS, acting as molecular chaperones to ensure these proteins maintain their native structure and functionality (Jäkel et al., 2002, Hutten et al., 2020). *In vivo* studies have highlighted the therapeutic potential of importins in neurodegenerative diseases, where they have been shown to rescue neurodegenerative phenotypes by facilitating the correct trafficking of crucial proteins involved in neuronal function and survival (Odeh et al., 2022). This broader role of

importins underscores their significance not only in nuclear transport but also in cellular protein homeostasis and disease pathology, making them promising targets for therapeutic intervention.

Numerous eukaryotic cellular functions, such as signal transduction, cell cycle maintenance, and response to the environment depend heavily on Karyopherins and a working NCT. Thus, it should come as no surprise that defects in transport functions are linked to conditions such as cancer (Çağatay and Chook, 2018), viral pathogenesis (Miorin et al., 2020), and neurodegeneration (Kim and Taylor, 2017). Interestingly, in **Paper IV** I observe that arsenite binds directly to several proteins in both the karyopherin and nucleoporin families: causing them to mislocalize and aggregate. The interaction disrupts nuclear transport over time, pointing towards the reported role of metals in the emergence of cancer and neurodegenerative diseases mentioned earlier.

5. Neurodegenerative Diseases

The brain, a complex and highly specialized organ, relies on a fine balance of proteins and metals within the nervous system to maintain optimal function. Disruptions of this equilibrium can lead to a host of neurological disorders, among which neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease are particularly devastating. These disorders are characterized by the progressive degeneration of neurons, resulting in cognitive decline and motor dysfunction, which significantly impair the quality of life.

One critical aspect of maintaining neural homeostasis involves the regulation of metals in the brain. Essential metals, including iron, copper, and zinc, play important roles in various physiological processes such as enzymatic activity, neurotransmission, and the regulation of oxidative stress (Zoroddu et al., 2019, Jomova et al., 2022). The precise balance of these metals is crucial; both their deficiency and excess can have detrimental effects. In **P**arkinson's **d**isease (PD), dysregulation of metal homeostasis is increasingly recognized as a contributing factor (Greenough et al., 2013, Breydo and Uversky, 2011). Abnormal accumulation of metals, particularly iron and copper, can trigger oxidative damage, protein aggregation, and mitochondrial dysfunction, all of which are key features of Parkinson's pathology (Wojtunik-Kulesza et al., 2019, Crichton et al., 2012, Dexter, 2013, Valensin et al., 2016, Montes et al., 2014, Bharathi et al., 2007).

In addition to essential metals, exposure to toxic metals can interfere with the normal functioning of the nervous system, leading to neurotoxicity. For instance, exposure to lead is associated with an increased risk of PD and **A**lzheimer's **d**isease (AD) due to its capacity to induce oxidative stress and disrupt calcium signaling. Mercury can bind to thiol groups in proteins, causing oxidative damage and

impairing neuronal function (Chin-Chan et al., 2015, Farina et al., 2013). Cadmium exposure has been linked to the disruption of calcium signaling and mitochondrial dysfunction, contributing to neurodegeneration. Arsenic can induce oxidative stress and inflammation in neural tissues, exacerbating neurodegenerative processes (Thakur et al., 2021, Wang et al., 2013). Research has also shown that arsenic can alter the levels of neurotransmitters such as serotonin, dopamine, and norepinephrine in the brain, which are essential for mood regulation, motor function, and cognitive processes (Li et al., 2020). Cumulative lifetime exposure to these toxic metals is increasingly recognized as a key factor in the rising incidence of neurodegenerative diseases worldwide. For example, a study conducted in West Bengal, India, demonstrated a strong correlation between exposure to arsenic-contaminated groundwater and the prevalence of neurodegenerative problems (Mukherjee et al., 2003). The toxicological properties of metals enable them to accumulate in various tissues, such as bone, from which they can be gradually released into the bloodstream over time, potentially leading to chronic exposure and sustained neurological damage (Sinha and Prasad, 2020).

Previous research has highlighted the role of arsenic and cadmium in the aggregation propensity of alpha-synuclein (αSyn), a protein that plays a central role in the pathogenesis of PD. The aggregation of αSyn is known to potentiate its toxic effects on neurons, accelerating the progression of neurodegenerative diseases (Chin-Chan et al., 2015, Aung et al., 2015).

5.1 Parkinson's Disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer's disease (AD) and the most common movement disorder. The risk of PD increases with age, becoming significantly higher for individuals over 65 years old. PD is characterized by the degeneration of motor neurons, particularly dopaminergic neurons in the substantia nigra, and the presence of neuronal inclusions known as Lewy bodies (LBs). The main component of LB pathology is amyloid fibers of the protein α-synuclein (αSyn) (Spillantini et al., 1998). It recruits tubulin among a variety of other ubiquitinated proteins, including parkin (Chung et al., 2001), transglutaminase (Junn et al., 2003), and synphilin-1 into the aggregates. Besides PD, αSyn inclusions are found in several other neurodegenerative diseases, including the LB variant of AD, dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) (Crichton and Ward, 2013, Ito et al., 2023).

Duplication and triplication of the αSyn gene SNCA, along with various missense mutations (A53T, A30P, A53E, H50Q, G51D, or E46K), have been linked to early-onset PD, indicating that aberrant protein conformations and gene overexpression accelerate disease progression (Oczkowska et al., 2013, Polymeropoulos et al., 1997, Pasanen et al., 2014). While αSyn amyloid formation is clearly implicated in PD development, multiple modes of toxicity have been proposed, including the amyloids themselves, oligomeric intermediates, and αSyn interactions with lipid membranes (Sciacca et al., 2020, Cline et al., 2018). A significant portion of αSyn monomers in cells are bound to lipid membranes or chaperone proteins. Disruption of these interactions and oligomer–lipid interactions may also contribute to PD features such as mitochondrial dysfunction and oxidative stress, which accompany amyloid formation (Ito et al., 2023). Recent studies

have shed light on additional pathological roles that αSyn amyloid fibers may play. Horvath et al. demonstrated that αSyn amyloid fibers exhibit esterase and phosphatase activity *in vitro,* suggesting that these fibers could gain new enzymatic-like functions that alter the metabolite composition within cells. Such chemical reactivity represents a novel gain-of-function that may directly modulate disease progression by interfering with normal cellular metabolism (Horvath et al., 2023a).

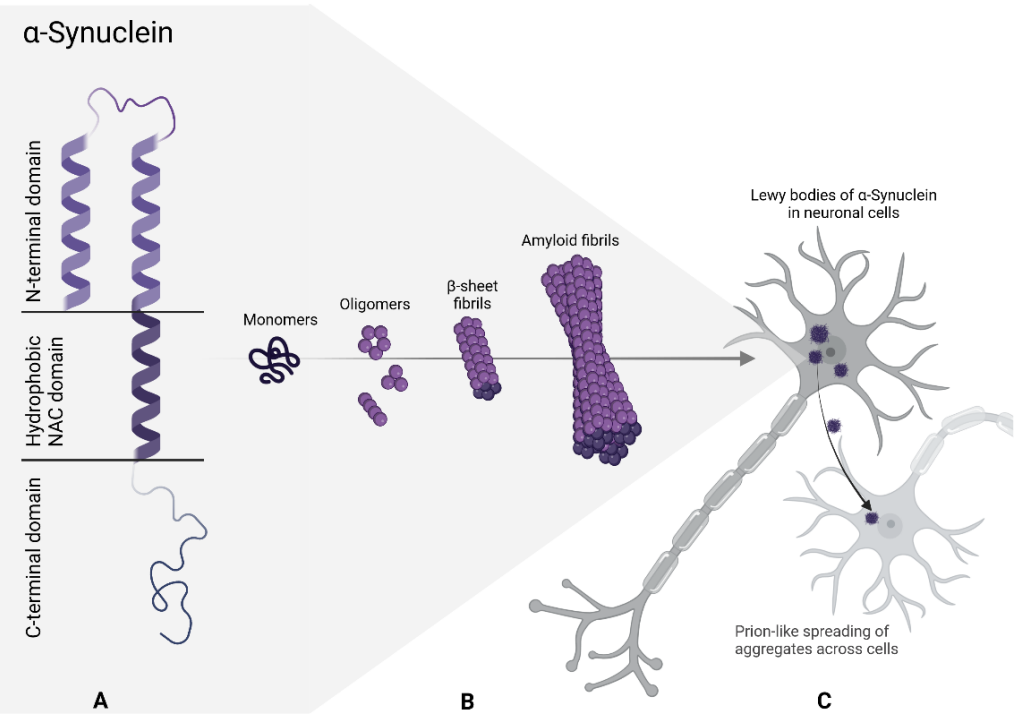

Figure 13 - Overview of αSynuclein (αSyn). ***A****: The different domains of αSyn: N-terminal region that interacts with membranes, NAC domain with the ability to form beta-sheets, and the flexible C-terminal domain where interactions with proteins or metals occur.* ***B****: The protein can aggregate and form oligomers. Oligomers can then build on each other (elongate) or act as seeds (nucleate) for more monomeric aggregation. The aggregates form long fibrils of cross-beta sheets that lump together into larger amyloid structures.* ***C:*** *Large lumps of amyloids can accumulate in neuronal cells, forming so-called Lewy Bodies that is a hallmark of Parkinson's disease. These aggregates can then spread to more cells in a prion-like manner, progressing the disease. Image created with Biorender.*

Under normal physiological conditions, αSyn is classified as a natively unfolded protein, exhibiting little to no organized secondary structure in solution. However, when αSyn binds to phospholipid vesicles, this interaction induces a dramatic conformational change in the protein, shifting from approximately 3% to over 70% alpha-helical content (Perrin et al., 2000). This structural flexibility is believed to be crucial for its normal physiological functions, while not fully elucidated, including roles in dopamine release, synaptic vesicle trafficking, membrane fusion, and vesicle clustering (Bisi et al., 2021, Perez and Hastings, 2004, Crichton and Ward, 2013, Luk et al., 2012).

The αSyn protein has three domains with different specialties: the N-terminal (1-60) is the region that interacts with membranes and can fold into α-helices; a middle domain (residues 61-95) called the non-amyloid-β component (NAC), a stretch of amino acids that can assume β-sheet structure, forming the core of amyloids; and finally the C-terminal region (96-140) where interactions with metals and small molecules can occur (Ulmer et al., 2005). Indeed, αSyn has been shown to bind metals *in vitro*, and imbalance of metal homeostasis is one of the hallmarks of PD (Uversky et al., 2001). Iron has been shown to accumulate in brain tissues, while copper decreased in tissues and increased in fluids, but both can be encountered in Lewy bodies in relatively high concentrations (Bush, 2000, Uversky et al., 2001). Several studies have linked the binding of metals to changes in amyloid formation kinetics, either to escalate aggregation or prevent it (Binolfi et al., 2010, Lorentzon et al., 2020, Alies et al., 2013).

The involvement of arsenic and cadmium ions in the formation and localization of αSyn amyloids is something I investigate in **Paper III**, both *in vitro* and within yeast cells. My findings indicate that amyloid structures formed in the presence of these heavy metal ions exhibit

distinct conformations. Additionally, the localization of αSyn within the cells is altered.

When αSyn monomers aggregate into amyloid fibrils with cross-β-sheet structure, they are thought to acquire toxic gain-of-functions similar to processes observed in other neurodegenerative diseases characterized by amyloid formation (Luk et al., 2012). Moreover, these αSyn amyloid fibrils are capable of spreading between cells, crossing the blood-brain barrier, and propagating pathology through mechanisms similar to prion diseases. These processes involve the direct transmission of αSyn fibrils from one cell to another, where they can induce the conversion of monomeric αSyn into additional amyloid fibrils (Prusiner et al., 2015). This is facilitated by elongation and secondary nucleation processes, which amplify the toxic species and further promote neurodegeneration (Gaspar et al., 2019, Horvath et al., 2023b).

Aim of the thesis

This thesis aims to investigate how the yeast *Saccharomyces cerevisiae* responds to heavy metal exposure, specifically arsenite and cadmium. This was done by evaluating the roles of protein quality control mechanisms in managing heavy metal-induced protein aggregates, as well as looking closer at the aggregate formation of specific proteins. Additionally, the study provides a comprehensive analysis of arsenic-binding proteins, with the goal to find novel targets and pathways.

Throughout this thesis, it has been shown that both cadmium and arsenic disrupt several key cellular processes including the induction of oxidative stress, interference with chaperone function and protein degradation pathways, disruption of protein homeostasis, and direct binding to proteins; thereby altering their conformation and cellular localization. Understanding these mechanisms may provide crucial insights into how environmental factors can accelerate metallopathies and metal-related disorders in cells.

Main findings

To maintain protein homeostasis, cells employ a range of protein quality control systems that assist in protein folding and maintenance. Given that arsenite (As(III)) disrupts protein homeostasis, , degradation pathways are needed to maintain a functional cellular environment. Thus, in **Paper I**, we examined the role of various protein quality control (PQC) pathways with regards to clearing away protein aggregates induced by arsenite, focusing on the ubiquitin-proteasome system (UPS), autophagy, the vacuolar degradation pathway, and disaggregating chaperones. Our findings indicate that the UPS is the primary pathway responsible for the clearance of As(III)-induced protein aggregates. While autophagy and the vacuolar degradation pathways also contribute to the clearance, they play a less significant role compared to the UPS. Chaperone activity was found to aid in the disaggregation and removal of As(III)-induced aggregates. However, As(III) appears to impair chaperone binding to these aggregates - probably by interfering with the substrate as the activity of the chaperones remain intact.

In **Paper II** we aimed to explore the significance of different protein quality control systems in their role to maintain protein homeostasis; specifically focusing on co-translational folding systems, molecular chaperones, the ribosomal quality control system, and ubiquitin ligases in the presence of As(III). Our study revealed that that As(III)-induced protein aggregates are not caused by increased ribosome stalling or defects in the ribosomal quality control (RQC) system. Interestingly, the loss of the ribosome-associated folding system SSB-RAC led to decreased protein aggregation and greater resistance to As(III), likely due to reduced global protein translation levels. While the Prefoldin complex plays a role in maintaining protein homeostasis during As(III) stress, the impairment of the TRiC/CCT complex does

not affect As(III)-induced protein aggregation. Furthermore, protein aggregates induced by As(III) were found to contain both K48- and K63-linked ubiquitin chains.

Chronic exposure to heavy metals leads to a wide range of diseases including cancer and neurodegeneration. Cd and As So, in **Paper III**, we investigated the effects of arsenic and cadmium on the formation of α-synuclein amyloids, a protein associated with Parkinson's disease. Our research show that both metals influence the aggregation process of α-synuclein amyloid formation, with cadmium accelerating nucleation and both metals altering amyloid fibril morphology. Structural analysis using atomic force microscopy revealed differences in fibril structure due to metal incorporation, mainly a change in fiber pitch. We found that both metals were incorporated into amyloid fibers, altering their structural properties, which may contribute to the increased toxicity. In yeast models, arsenic and cadmium disrupt the distribution and clearance of α-synuclein aggregates, increasing their toxicity and retention near the plasma membrane.

Arsenic can both cause and cure disease, due to the metalloid's ability to bind proteins and alter their folding and activity. Understanding arsenic-protein interactions is crucial for clarifying both its toxic and therapeutic effects. To further our understanding of how and what arsenic interacts with in cells, we turned to a large-scale arsenic-interaction study in **Paper IV**. In this study, we identified 174 arsenic-binding proteins in budding yeast by proteomic analysis. Among these, proteins involved in nucleocytoplasmic transport were significantly enriched. The arsenic-binding proteins had high cysteine-content and potential binding motifs, reaffirming the general notion that arsenic targets thiol groups in proteins. Arsenic binding caused nuclear import factors to mislocalize from the nuclear envelope and

aggregate in the cytosol, along with nuclear pore proteins that form the nuclear pore complex. This disruption led to impaired nuclear protein import and export, suggesting a model where arsenic binding causes mislocalization and aggregation of these proteins, thereby inhibiting nucleocytoplasmic transport.

Concluding remarks

The findings from **Papers I** and **II** expand our understanding of how cells maintain and regain protein homeostasis under stress, particularly under exposure to heavy metals like arsenite. The different protein degradation pathways such as the ubiquitin-proteasome system (UPS), autophagy, and chaperones work together to manage stress-induced protein aggregates and maintain cellular equilibrium. Our results in **Paper I** suggest that ubiquitination and degradation via the proteasome is the main pathway for dealing with As(III)-induced aggregates. Specifically, ubiquitin chains containing both K48 (**Paper I**) and K63 (**Paper II**) linkages play an important role in proteasomal degradation. This corroborates the major role of the ubiquitin-proteasome pathway during degradation of As(III)-induced protein aggregates, and also shows that the cell employs distinct strategies to manage aggregates formed under arsenic or cadmium stress compared to heat stress. The primary strategy under heat stress is the rapid heat shock response and synthesis of HSPs. This response is less selective compared to the heavy metal stress response, focusing broadly on stabilizing, refolding, and preventing further aggregation of a wide range of proteins.

It remains unclear exactly how metals modulate protein structure to promote different aggregation pathways, but our studies suggest an altered structure even at later aggregation stages (**Paper III**) perhaps

making them harder to deal with. Also, in **Paper I** we observe that arsenite alters protein aggregates in ways that limit chaperone binding to the substrate. Investigating how arsenic binding or post-translational modifications, such as cysteine oxidation, methylation or phosphorylation affect chaperone efficacy could help further our understanding of the mechanism behind this. These papers collectively emphasize the interplay between different protein homeostasis mechanisms—proteasome, autophagy, and chaperones. Understanding how these systems interact and sometimes compensate for one another during stress is crucial for developing targeted therapies in diseases where protein homeostasis is disrupted.

We also found that impairment of co-translational folding systems reduces protein aggregation and increases resistance to As(III). Notably, this impairment is accompanied by a decrease in global protein translation levels, underscoring the importance of translational control during As(III) stress. Translation can be regulated at multiple levels, including the availability of tRNA within the cell. Among the arsenic-binding proteins identified in **Paper IV**, we observe the targeting of tRNA ligases, tRNA methyltransferases, and translation initiation factors. Further investigation into these components would provide valuable insights into the mechanisms of translational control during stress conditions.

Paper III and **Paper IV** show how heavy metals disrupt protein homeostasis and nucleocytoplasmic transport, providing mechanistic insights into how environmental exposure can exacerbate or even trigger disease progression. The studies on arsenite and cadmium stress (**Paper III**) provide insights into how environmental toxins influence protein misfolding, particularly in proteins like α-synuclein, which is linked to Parkinson's disease. Since metals might influence α-

synuclein interactions with membranes, future studies could explore how non-essential metals impact α-synuclein's association with lipid membranes, potentially contributing to the membrane disruption seen in neurodegeneration.

Paper IV highlights a novel mechanism of arsenic toxicity by showing that arsenic binds to key nuclear transport proteins, leading to mislocalization and aggregation of importins, exportins, and nuclear pore proteins. One can draw parallels between the disruption of nuclear transport by arsenic and the nucleocytoplasmic defects observed in neurodegenerative diseases like Alzheimer's and Parkinson's. This discovery has implications not only for understanding arsenic's toxicity but also for its therapeutic use in cancer treatments, where arsenic's effects on nuclear transport could be exploited. Arsenic-containing compounds are already used in treatments for diseases like acute promyelocytic leukemia, and these findings may guide future strategies for designing arsenic-based therapies that selectively target cancer cells while minimizing toxicity to healthy cells.

The dose and exposure time is a tricky thing to study in a short-lived organism such as yeast, especially combined with the need to have higher dosage due to the yeast-specific arsenic defenses (ARR genes). The effects observed in humans after long time exposure is thus hard to replicate. Now we have a good foundation of proteins and specific pathways that bind and are affected by arsenic, most of which are conserved to some degree in higher organisms. A way forward would be studies in mammalian cells where we can better study long-time exposure at environmentally relevant concentrations, to see if the effects we saw in yeast are applicable.

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