On chaperone co-operation in temporal and spatial protein quality control

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Cover illustration: Sequential images of a dividing yeast cell illustrating the asymmetrical division of damaged proteins (cyan) counterstained with the nucleolus (magenta). Image capture and editing by Rebecca Andersson.

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Till minne av Eva

SCIENCE | noun

sci•ence | \ˈsī-ən(t)s\

Definition:

1. I don't know, but I am trying to find out, okay?

- Carlos the Scientist

Abstract

In order for a protein to be able to function correctly it needs to adopt its proper fold. Because of this, protein quality control (PQC) is vital at every level of cellular function. The central protein in PQC networks across all kingdoms of life are the Hsp70 molecular chaperones. In my thesis I present work into the adaptability of the chaperone network during different types of stress, and the functional variability between highly homologous yeast Hsp70 chaperones.

During heat stress, Hsp70s collaborate with other chaperones to sequester misfolded proteins into inclusion bodies and later resolve them. Hydrogen peroxide stress however requires additional activity from the peroxiredoxin Tsa1. Tsa1 is needed to recruit chaperones to misfolded proteins during oxidative stress. Interestingly, this is also true for stress caused by ageing, and increasing the level of Tsa1 can prolong the lifespan of yeast.

The Stress Seventy subfamily A (Ssa1-4) is an important Hsp70 family in yeast. Loss of Ssa1 and Ssa2 decreases cellular viability and lifespan even though Ssa3 and Ssa4 remain. Overproduction of Ssa4 can restore many but not all of the functions usually carried out by Ssa1/2, and restore a full lifespan. We show that preventing proteins from misfolding or sequestering them into inclusion bodies is enough to ensure a full lifespan, while disaggregation of inclusion bodies is not required for longevity assurance. We also describe a novel, Hsp70-dependent site for sequestration of misfolded proteins around the yeast nucleolus that forms after heat shock. The site is also the basis for the asymmetric segregation of damaged proteins in the nucleus during cell division, in a manner that is distinct from previously described asymmetry pathways in yeast.

Keywords: Molecular chaperones, Heat shock proteins, Hsp70 chaperones, Proteostasis, Ageing, Asymmetric damage segregation, Peroxiredoxins, Spatial protein quality control

Sammanfattning

Proteiner utgör stommen i alla cellers aktivitet och måste därför skyddas för att vi ska kunna leva och den process som möjliggör detta kallas för proteinkvalitetskontroll. Proteinkvalitetskontroll sköts av en särskild grupp proteiner, de molekylära chaperonerna. När vi åldras förlorar celler sin förmåga att upprätthålla många av de grundläggande aspekterna inom proteinkvalitetskontroll. Med åldrande kommer också med en ökad risk att drabbas av sjukdomar såsom Alzheimers och Parkinsons, där skadade och icke-funktionella proteiner på olika sätt stör och förstör celler i hjärnan. För att förstå och utveckla behandlingar för dessa typer av sjukdomar behöver vi veta mer om hur proteinkvalitetskontroll fungerar. I denna avhandling presenteras studier av de viktigaste proteinerna inom proteinkvalitetskontroll, Hsp70 chaperoner. Studierna har utförts i modellorganismen *Saccharomyces cerevisiae*, en vanlig art av jäst som delar många egenskaper med djur och människor.

Olika typer av cellstress, till exempel oxidativ stress och värmestress, drabbar cellen på olika sätt, och vi presenterar resultat som visar att även försvaret mot olika typer av stress kräver olika komponenter. Vid värmestress samarbetar Hsp70 med ett antal andra chaperoner för att skydda cellen. När celler istället stressas med väteperoxid, behövs dessutom peroxiredoxinet Tsa1 utöver de vanliga chaperonerna. Gamla celler behöver också Tsa1 tillsammans med Hsp70 för att hantera skadade proteiner, och jäst lever längre om nivåerna av Tsa1 ökas.

Vi visar att de Hsp70-funktioner som förebygger proteinskador och antigen bryter ner proteiner eller ansamlar dem ifall de skulle skadas, är essentiella funktioner för att jäst ska uppnå ett normallångt liv. Det här betyder att ansamlingar av skadade proteiner, ofta kallade aggregat, inte nödvändigtvis är skadliga om de hanteras korrekt och ansamlas på rätt ställe inom cellen. Processer som styr var

proteinaggregat placeras kallas för spatial, alltså rumslig, proteinkvalitetskontroll. Olika typer av proteiner aggregerar på olika platser inom cellen, och vi beskriver en tidigare outforskad plats inuti cellkärnan hos jäst där detta sker, kring den så kallade nukleolen. Efter en kort värmechock transporteras skadade proteiner till nukleolen med hjälp av Hsp70, där de sedan ansamlas tillfälligt. Vi föreslår att denna ansamling har en skyddande funktion då den bara ärvs till en av två celler efter celldelning, vilket möjliggör föryngring av jästens avkomma.

List of papers

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

I. Lifespan Control by Redox-Dependent Recruitment of Chaperones to Misfolded Proteins.

Hanzén S., Vielfort K., Yang J., Roger F., Andersson V., Zamarbide-Forés S., **Andersson R**., Malm L., Palais G., Biteau B., Liu, B., Toledano M. B., Molin M. and Nyström T.

Cell. 2016 Jun 30;166(1):140–51

II. Differential role of cytosolic Hsp70s in longevity assurance and protein quality control.

Andersson R., Eisele-Bürger A. M., Hanzén S., Vielfort K., Öling D., Eisele F., Johansson G., Gustafsson T., Kvint K. and Nyström T.

PLoS Genet. 2021 Jan 11;17(1)

III. Image-based analysis of the nucleolus associated protein deposit in yeast.

Andersson R., Syga L. and Nyström T.

Manuscript

Other publications

How and why do toxic conformers of aberrant proteins accumulate during ageing?

Josefson R., **Andersson R.** and Nyström T.

Essays Biochem. 2017;61(3):317–24.

Abbreviations

PQC	Protein Quality Control
SPQC	Spatial Protein Quality Control
Hsp	Heat shock protein
sHsp	small Heat shock protein
JDP	J-Domain Protein
NEF	Nucleotide Exchange Factor
Tsa1	Thiol-specific antioxidant 1
Prx	Peroxiredoxin
Srx	Sulfiredoxin
Trx	Thioredoxin
Hsf1	Heat shock factor 1
Ssa/Ssb/Ssz	Stress seventy subfamily A/B/Z
HSR	Heat Shock Response
IPOD	Insoluble PrOtein Deposit
JUNQ	JUxtaNuclear Quality control compartment
INQ	IntraNuclear Quality control compartment
HAND	Heat stress Associated Nucleolar protein Deposit
ROS	Reactive Oxygen Species
ERCs	Extrachromosomal Ribosomal DNA Circles
RLS	Replicative LifeSpan
CLS	Chronological LifeSpan
NTD	amiNo-Terminal Domain
CTD	Carboxy-Terminal Domain
NBD	Nucleotide Binding Domain
SBD	Substrate Binding Domain
UPS	Ubiquitin-Proteasome System
DUB	Deubiquitinase
CPY	CarboxyPeptidase yscY
ER	Endoplasmic reticulum

Contents

Introduction and aim of the thesis

The inside of any cell is a crowded milieu, yet the cell manages to carry out hundreds of thousands of specific, simultaneous activities to ultimately support the life of the organism. A potential problem in this tightly controlled machinery can be the machine components themselves; the proteins. Proteins need to adopt their native state, that is the secondary, tertiary and sometimes quaternary structure, that enables their function, and if this fails it can lead to aberrant reactions and loss of functional components needed for vital reactions. The outcome of this is oftentimes pathology and ultimately cell death. To prevent this cells produce molecular chaperones, a broad family of proteins that catalyses the proper folding and refolding of other proteins. Chaperone activities can range from the highly specific action of enzymes such as the disulphide isomerases that catalyses intramolecular disulphide bond formation in a redox dependent manner (Matsusaki et al., 2020) to the actions of the molecular chaperones that monitor the entire proteome and prevents misfolding and promotes refolding or destruction of damaged proteins (Rosenzweig et al., 2019).

Understanding the complex way in which chaperones interact and cooperate with each other and other cellular components to promote cellular fitness during times of stress is key to understanding how pathological conditions arise and how we can treat and prevent the associated diseases. My thesis is centred on the cytosolic Hsp70 family of proteins in the budding yeast *Saccharomyces cerevisiae*, the functional difference between different members in this family (Paper II) and their interaction partners during different types of stress (Paper I). I have also studied a novel, Hsp70-dependent site for misfolded protein deposition that forms during acute heat stress in the nucleolus (Paper III). In this thesis I will put my work into context by discussing different Hsp70-centered mechanisms and interactions at play during

stress-induced temporal and spatial protein quality control in the cytosol and nucleus of *S. cerevisiae.*

Stress from the perspective of a cell

A common way to study proteostasis is to introduce stress; that is something that will perturb a cell or an organism in a specific way, and then observe the outcomes and compare it to un-stressed systems. In the proteostasis field, the stressors can be physical, such as increased temperature; chemical, in the form of proteotoxic drugs; or genetic, like knockout of important genes for molecular chaperones or introduction of genes that code for toxic proteins. Ageing is also an important type of perturbation that introduces a system-wide stress that affects most cellular processes, not just proteostasis pathways.

Protein folding and misfolding

The amino acid sequence of a newly produced protein holds the key to what the native fold of the protein should look like, but getting there is not always achieved by the protein alone. The folding process follow the laws of thermodynamics, and reaching the native state involves burying hydrophobic regions of the proteins inside the structure, and exposing more hydrophilic parts of the proteins on the outside. Small proteins can often find this native state on their own, but larger proteins with more complex secondary and tertiary three-dimensional structures often require molecular chaperones to reach their native state (Hartl et al., 2011). The stability of folded proteins is challenged by extrinsic factors as well as intrinsic ones that can damage or otherwise hinder a protein to reach its native state. These can include things like mistranslation, mutations, pH-changes and heat. When a protein is damaged, it can partially unfold or misfold, which not only reduces its functionality but can also lead to toxicity and aggregation, see figure 1. While some proteins form insoluble protein aggregates that remain amorphous, some form highly structured amyloid aggregates that are stable and can act as seeds for other proteins to also adopt an amyloid conformation (Tyedmers et al., 2010a). Proteins that form amyloids often have intrinsically disordered regions and many

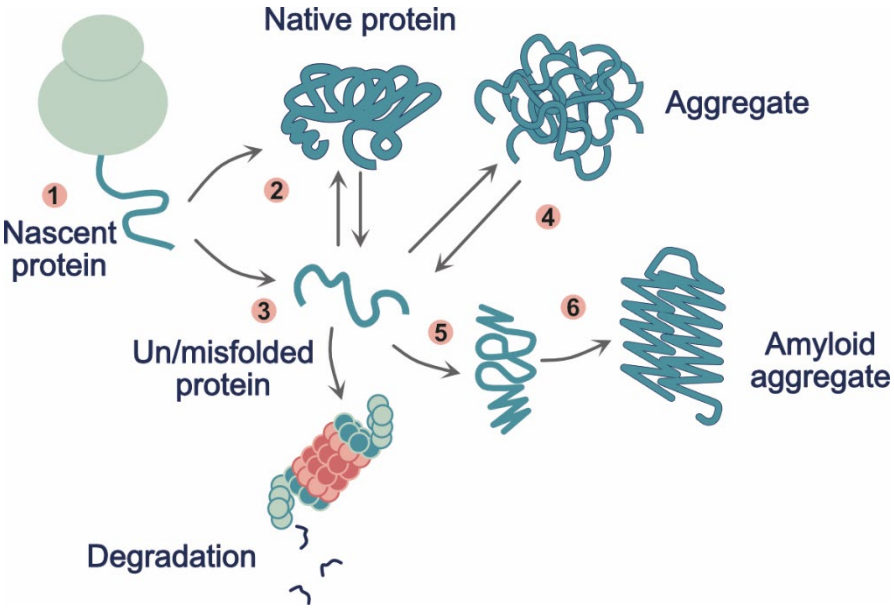

Figure 1: Newly synthesised proteins (1) are aided by chaperones during and after translation to reach their native state (2). If the folding is unsuccessful or if the native protein is damaged the protein can unfold or misfold (3). These proteins can form aggregates (4) or be degraded by for example the proteasome. Some proteins are prone to forming amyloid aggregates, which can either happen through protofibrillar monomers (5) that mature into full amyloid aggregates (6), or through aberrant reactions inside biomolecular condensates.

have the ability to form biomolecular condensates. Emerging studies point toward the mismanagement and disruption of biomolecular condensates as a pathway for amyloid formation and aberrant aggregation of disease proteins such as FUS and Huntingtin (Alberti and Hyman, 2021).

Heat stress

Heat stress is arguably the most used stressor in yeast studies of protein quality control (PQC) networks. This can be traced back to the discovery of the regulatory mechanisms allowing adaption to heat stress (Ritossa, 1962) and the nomenclature used in the field is heavily influenced by this and other early work. The Heat Shock Response (HSR) is the name for the coordinated transcription and protein

production that occurs after heat stress, which is controlled mainly by the transcription factor Heat Shock Factor 1 (Hsf1 in yeast). The proteins produced, called Heat Shock Proteins (Hsp), are in many cases molecular chaperones that protect the entire proteome from the harmful effects of the increase in temperature. The damaging effects of increased temperature can be seen throughout the cell, in the form of DNA damage, mitochondrial disruptions and increased production of reactive oxygen species (ROS), changes in plasma membrane permeability and thermal unfolding of proteins (see review by (Roti Roti, 2008)). In that sense heat stress is a very broad type of stressor, and the effects of it can be seen also in the intracellular architecture and organellar morphology (Keuenhof et al., 2021).

Chemical stressors

A multitude of proteotoxic drugs and chemicals exists that can either target specific cellular proteostasis activities, such as inhibiting the proteasome with MG132 or Hsp90 chaperone activities with Geldanamycin, or introduce damage to the entire proteome directly. An example of a drug causing proteome-wide defects is the proline analogue L-azetidine-2-carboxylic acid (AZC) that is mis-incorporated instead of proline as proteins are produced, which can potentially destabilise all newly produced proteins, and hydrogen peroxide that induces the formation of harmful oxidative damage on potentially all molecules inside the cell with oxidation-sensitive moieties.

Oxidative damage to proteins can cause either cleavage of the peptide backbone or damage amino acid side chains. The oxidative damage can be mediated directly by the ROS itself, but it can also be through secondary reactions with oxidised lipids and carbohydrates. Depending on the side chain that is affected, the damage and outcome can be specific, such as the formation of tyrosine crosslinks, but there are also general type of adducts that can be formed on several different amino acids (Levine and Stadtman, 2001). It is important to separate the irreversible oxidative damage done by ROS from the specific type

of oxidation events that are part of reduction-oxidation (redox) signalling and other vital redox activities, which are all reversible and essential to maintain fitness (Dahl et al., 2015).

Ageing

Ageing is perhaps one of the most all-encompassing stressors that an organism can experience, that combines both intrinsic changes and deficiencies with repeated extrinsic challenges from the environment.

Why do we age? – The age-long question

The ageing process is something that most of humans alive today have a personal relationship to, be it as a witness to a loved ones' increasing frailty to the experience of one's own ageing. Ageing, or senescence, can be loosely defined as the progressive physiological deterioration and heightened risk for debilitating disease and ultimately death that comes with an increase in age. Even though old individuals can be found in many species in the wild, senescence as a phenomenon that affects a large part, if not the majority, of the population is likely only found in humans (and non-human animals kept for companionship by humans), since out in nature, animals tend to die from non-ageing related causes before they reach an advanced age (Kirkwood and Austad, 2000). The central role of ageing in the human experience have led to many theories, both philosophical and scientific, as to why we age, from the early disproven wear and tear mechanistic model from the 19th century by Weismann, to the antagonistic pleiotropy model and disposable soma theories that were developed in the middle-to-late 20th century based in evolutionary theory (Kirkwood, 1977, Williams, 1957). The antagonistic pleiotropy model posits that ageing is a trade-off between effects of genes that increase fitness and reproductive capacity at a young age, while simultaneously decreasing fitness late in life (Williams, 1957). The disposable soma theory instead suggests that there is a limited amount of resources available to any organism, which prohibits them from investing too much into maintaining the body (or the soma) as that would lead to

lower levels of reproductive success (and vice versa) (Kirkwood, 1977, Kirkwood and Rose, 1991). In support of this are observations such as those in *Caenorhabditis elegans* that increasing life span through genetic tools often leads to reduction in fertility (Tissenbaum and Ruvkun, 1998). The disposable soma theory is rooted in the error catastrophe theory presented by Orgel, which hypothesise that there is an accumulation of translational errors with increasing age that affects vital factors in for example DNA maintenance, which eventually leads to a catastrophic failure and senescence. Diverting resources to repair to counteract these errors would lead to less resources available for reproduction (Orgel, 1963, Orgel, 1970, Kirkwood, 1977).

How do we age? – Mechanisms in the yeast ageing process

While humans tend to see and experience the symptoms of age from the organismal level, the changes associated with age start at the cellular and molecular level. In 2013 López-Otín *et al.* summarised the age-linked molecular and cellular changes into nine categories dubbed as the "Hallmarks of Aging" (López-Otín et al., 2013). The nine hallmarks outlined include: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and deregulated intercellular communication (López-Otín et al., 2013). All these changes occur throughout the entire organism and influence each other, meaning that ageing can't be traced to any one cause or organ.

Even though yeast is a single-celled organism several of the ageing hallmarks can be observed in yeast (Janssens and Veenhoff, 2016). While a colony of yeast can in some ways be thought of as immortal (anyone who has ever inherited their grandparents sourdough starter can attest to this), that is one can perpetually cultivate yeast as long as the culture is split regularly and fed new nutrients. However, individual yeast cells can only provide daughter cells a finite number

of times (Mortimer and Johnston, 1959). The reason for this apparent discrepancy in culture immortality and individual yeast cell mortality is in part the process of rejuvenation that occurs at every mitotic event in budding yeast (Mortimer and Johnston, 1959, Sinclair and Guarente, 1997, Aguilaniu et al., 2003, McFaline-Figueroa et al., 2011, Henderson et al., 2014), and the complete rejuvenation of all four spores from a meiosis event (Koch-Bojalad et al., 2020). While rejuvenation during meiosis leads to four young spores, mitotic rejuvenation saves the younger bud or daughter cell at the expense of the larger mother cell. Since every new daughter cell produced has a fully restored replicative potential, yeast cultures can be grown indefinitely even though individual yeast cells enter senescence along the way. This asymmetry during budding generates a rudimentary soma-germline pair, where the mother cell is the soma and the daughter the germ line, which is a prerequisite for senescence to occur (Kirkwood and Austad, 2000). Studies of ageing in yeast have led to the definition of individual yeast ageing factors that accumulate with age, are retained in the mother during budding and can extend or shorten lifespan if their levels are decreased or increased. To date these factors are extrachromosomal ribosomal DNA circles (ERCs) (Sinclair and Guarente, 1997), dysfunctional mitochondria (Lai et al., 2002, McFaline-Figueroa et al., 2011, Higuchi et al., 2013), vacuolar pH (Hughes and Gottschling, 2012, Henderson et al., 2014) and protein damage (Aguilaniu et al., 2003).

Lifespan assessment and methods for yeast ageing

To study ageing, one has to first find a way to generate a young and an old population to study and compare with each other. In yeast, two models for ageing are commonly used, replicative and chronological lifespan (RLS and CLS) (Longo et al., 2012). During *replicative ageing*, a population of yeast cells are maintained in a state where they continue to go through cell division until they fail due to cell-intrinsic reasons, while their progeny is removed repeatedly allowing for study of just the cells that have divided multiple times. This mimics certain, but not

all, aspects of ageing in mitotic tissues of metazoans. This can be achieved by growing the cells in a liquid media that is continuously refreshed and removing the newly budded daughters to avoid saturating the culture. Removal of daughters can be done by labelling the first generation of mother cells with biotin, followed by one or more rounds of magnetic bead isolations of the biotin-labelled mother cells after the cells have been allowed to grow (Smeal et al., 1996, Sinclair and Guarente, 1997). Removal of daughter cells can also be achieved by the use of the Mother Enrichment Programme (MEP) (Lindstrom and Gottschling, 2009), where cell cycle progression by newly budded cells is stopped and only mother cells can divide. This system is based on Cre-*lox* recombination with the expression of the Cre recombinase by a daughter-specific promoter and insertion of *lox* sites in the essential *CDC20* and *UBC9* genes. The Cre recombinase is further fused to an oestrogen receptor that will induce translocation of the recombinase from the cytosol into the nucleus when estradiol is added to the media, thus preventing the cell to proceed through the cell cycle (Lindstrom and Gottschling, 2009). The liquid culture approaches allows for the isolation of a large quantity of cells suitable for population analyses such as proteomics, transcriptomics and microscopy (see Paper I for an example). More precise scoring of the RLS potential of strains can be done with yeast cells seeded on agar plates and a micromanipulator that allows the experimentalist to remove daughter cells and count the number of budding events per cell (Kennedy et al., 1994, Kaeberlein et al., 1999) for examples see Paper I and II. This allows for very precise determination of the number of cell divisions by each cell, but cannot be done in bulk, even though high throughput approaches have been presented (McCormick et al., 2015). *Chronological ageing* is a type of post-mitotic ageing, where yeast cells are grown to stationary phase in a liquid culture, and then prevented to re-enter the cell cycle since the culture is kept dense and no new nutrients are added. The viability of the culture is assessed regularly to estimate what proportion of the culture

are able to exit the quiescence induced by the starvation/stationary phase and re-enter the cell cycle when transferred to an agar plate with fresh media (Longo et al., 1996, Fabrizio and Longo, 2003).

Proteins counteracting proteotoxic stress

Molecular Chaperones

Small heat shock proteins

Small heat shock proteins (sHsps) are so named because of their relative low molecular weight, typically between 10 to 20 kDa. sHsps are present in most, if not all, living organisms, from prokaryotes to vertebrates, and all share the characteristic C-terminal α-crystalline domain that is central to its function (de Jong et al., 1998), and through it almost all known sHsps form oligomeric complexes with variable numbers of subunits (Wotton et al., 1996, de Jong et al., 1998, Haley et al., 2000, Giese and Vierling, 2002). The sHsps counteract the aggregation of proteins during denaturing conditions on their own, in a manner not dependent on ATP (Horwitz, 1992, Jakob et al., 1993). The activity of sHsps can be modulated through post-translational modifications, but activation through heat shock is also an important regulating mechanism (Haslbeck et al., 1999, Franzmann et al., 2008). The main function of the sHsps is to bind unfolded proteins during stress which prevents them from terminally misfolding, and sequester them for later refolding (Ehrnsperger et al., 1997). Contrary to pure multimeric sHsp complexes, the binding and oligomerisation of sHsp:substrate complexes is mediated by the N-terminal domain, which is also the driver for functional differences between different sHsps (Stromer et al., 2004, Specht et al., 2011, Grousl et al., 2018). The main cytosolic sHsps in yeast are Hsp26 and Hsp42, with Hsp26 being mainly active during heat induced stress while Hsp42 has a ubiquitous activity with and without stress (Haslbeck et al., 2004).

J-domain proteins

The J-domain proteins (JDP), also known as 40 kDa heat shock proteins (Hsp40) are a diverse group of Hsp70 co-chaperones that aids

and provides specificity to Hsp70 folding and refolding activities. Generally, J-domain proteins can be divided into three types; I, II and III (or A, B and C), depending on the relative position of the J-domain within the protein, and the presence of a glycine/phenylalanine-rich domain in conjunction with the J-domain. The J-domain consist of four α-helices with a conserved histidine-proline-aspartic acid mini-domain separating helix II and III, which is crucial for its function (Kampinga and Craig, 2010). Upon binding to its Hsp70 partner, J-domain proteins will stimulate the ATP hydrolysis in the NBD domain of Hsp70, and in the case of substrate-binding J-domain proteins, will also bring the protein substrate to the Hsp70 chaperone (Szabo et al., 1994, Russell et al., 1999a, Kampinga and Craig, 2010). Through this action, the J-domain proteins drive the activity of Hsp70's but also brings an additional a layer of specificity to the system. In line with this, the Hsp70-association with different J-domain partners will change how misfolded proteins are dealt with in for example stress granules (Walters et al., 2015) and whether or not a protein gets refolded or degraded (den Brave et al., 2020). Yeast carries 22 genes that code for proteins with J-domains, which are found all throughout the cell (Walsh et al., 2004).

Hsp70 proteins

70 kDa heat shock proteins are the "work horses" of the molecular chaperone proteins and involved in everything from co-translational folding of nascent proteins to protein degradation. The breadth of Hsp70 activities are mediated in large part by the co-chaperones and other factors that interact with the Hsp70's. Hsp70 proteins consists of three main domains, the nucleotide binding domain (NBD) with its four subdomains (IA, IIA, IB and IIB) arranged around a central ATP/ADP-binding cleft, the substrate binding domain (SBD) consisting of the hydrophobic binding domain (SBDβ) and the lid domain (SBDα) that closes over the substrate when it is bound, and an intrinsically disordered domain in the C-terminus of the protein (Rosenzweig et al., 2019). When ATP is bound in the NBD domain, the

SBD is inaccessible to protein substrates, but binding to a substrate or JDP:substrate complex will introduce conformational changes in the NBD that allows for the hydrolysis of ATP to ADP in the nucleotide binding cleft and make the SBDβ accessible for the substrate, followed by clamping of the substrate by the SBDα lid domain (Fan et al., 2003). Release of the substrate is aided by a nucleotide exchange factor (NEF) that supports the exchange of ADP to ATP, which prompts the dissociation of the substrate from the SBD ((Rosenzweig et al., 2019), see figure 2). The substrate for Hsp70's are usually partially unfolded proteins with exposed hydrophobic amino acid stretches, and interactions with Hsp70's are thought to facilitate folding by preventing the stabilisation of folding intermediates that prevent proteins from reaching their native state, or by providing other folding machineries such as the Hsp90 chaperones with easily accessible substrates (Rosenzweig et al., 2019). Yeast has 14 Hsp70 proteins, of which 7 are found in the cytosol; the so-called Stress Seventy subfamilies A, B and Z (Ssa1-4, Ssb1-2 and Ssz1), with the Ssb and Ssz proteins being involved in co-translational folding and the Ssa proteins fulfilling other basal and stress related functions (Lotz et al., 2019).

In yeast, the Ssa family is as a whole essential, meaning that there needs to be at least one gene copy of either one of them that is expressed at a high enough level in order to ensure cell viability (Werner-Washburne et al., 1987, Hasin et al., 2014). The four Ssa proteins share a highly similar amino acid sequence but the respective genes do not share the same expression pattern, with *SSA1* and *SSA2* being constitutively expressed while *SSA3* and *SSA4* are induced during times of stress (Werner-Washburne et al., 1987). Even though they are homologous, there are functional differences between the inducible and the constitutive Ssa isoforms, which have been demonstrated by studying the behaviour of yeast prions in knock out

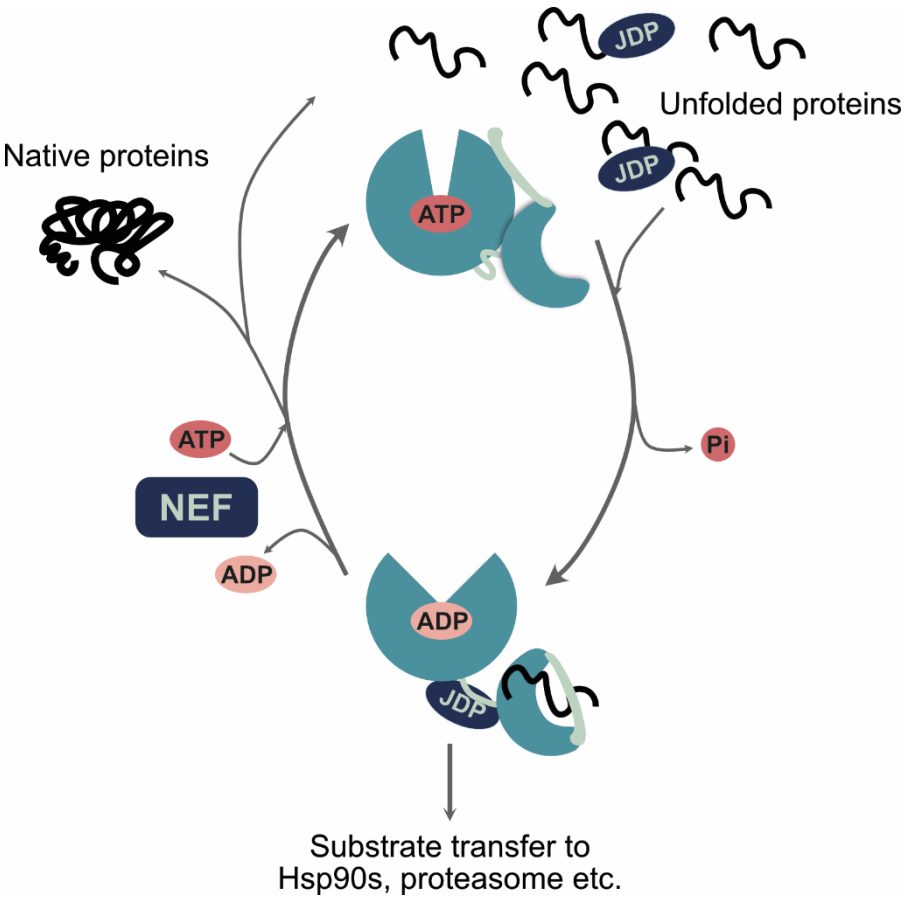

Figure 2: Hsp70 bound to ATP interacts with substrates and JDPs (top) which trigger the hydrolysis of ATP to ADP and a conformational change in the Hsp70 that closes the substrate binding domain around the substrate (bottom). The substrate can then be transferred to other PQC complexes or released from Hsp70 with the help of NEFs (left). The protein can then either fold or re-enter the Hsp70 cycle again (top).

strains (Sharma and Masison, 2008). The induced isoforms of Ssa confer a higher resistance to heat stress and cell wall damaging agents compared to the constitutively expressed isoforms, and they also have an increased capacity to propagate prions (Hasin et al., 2014). In a *ssa1*Δ *ssa2*Δ double mutant *SSA4* expression is induced (Boorstein and Craig, 1990), however this does not seem to compensate fully for the loss of Ssa1 and 2 as these strains are defective in several aspects of the

protein quality control machinery, have a growth defect and a shorter replicative lifespan compared to wild type strains (Craig and Jacobsen, 1984, Boorstein and Craig, 1990, Öling et al., 2014).

Hsp100 disaggregases

The Hsp100 chaperones are a family of chaperones that can be found in both prokaryotes and eukaryotes, but only in the mitochondria of metazoans (not in the cytosol or nucleus). The Hsp100 disaggregase family, ClpB in *E. coli* and Hsp104 in yeast, assembles into a hexameric ring with a central cavity through the middle. The ClpB/Hsp104 subfamily of Hsp100s is different from other Hsp100s due to their activity in protein disaggregation and refolding, while other Hsp100 proteins target their substrates for degradation by interactions with proteolytic partners (Parsell et al., 1994, Glover and Lindquist, 1998). The Hsp104 protein is regulated by Hsp70, which is necessary for Hsp104 to carry out its function. Hsp70 binds to the M-domain of Hsp104 and activates its ATPase domains (Rosenzweig et al., 2013, Lee et al., 2013), which triggers the threading of the protein substrate through the central cavity of the Hsp104 hexamer (Parsell et al., 1994, Glover and Lindquist, 1998, Lum et al., 2004). The protein substrates can then be either refolded on its own or require further interactions with chaperones to reach a native state.

Hsp90's, NEFs and chaperonins

The Hsp90 chaperones are a broad family of molecular chaperones with both specific and general substrates. Their function is different from the Hsp70/JDP complex in that they bind not unfolded substrates, but rather interact with already partially folded proteins. In doing so they stabilise and aid in the final maturation of the substrate. The function of the Hsp90s are implicit in a wide range of cellular activities due to their stabilisation of important client proteins through interaction with a variety of co-chaperones (McClellan et al., 2007, Li et al., 2012, Schopf et al., 2017).

Nucleotide exchange factors (NEFs) are proteins that catalyses the exchange of ADP to ATP in Hsp70 chaperones. There are three families of NEF proteins in yeast, Hsp110 (Sse1 and 2), Fes1 and Snl1, that interact with the Hsp70 chaperones. The Hsp110 family is essential and involved in a broad range of Hsp70s functions, while the Fes1 and Snl1 proteins are more specialised (Abrams et al., 2014).

The chaperonins are a family of chaperones that form multimeric structures with a central cavity wherein the chaperonin substrates are passed through to attain their native states, in a manner dependent on ATP. In yeast, an example of a chaperonin is the CCT/TriC complex that aids in the folding of both actin and tubulin monomers (Grantham et al., 2012).

Peroxiredoxins as non-canonical chaperones

The peroxiredoxin (Prxs) are most known as protein antioxidants found throughout prokaryotic and eukaryotic kingdoms of life (Wood et al., 2003). All Prxs have the potential to react with, or scavenge, hydrogen peroxide in a catalytic cycle that involves the oxidation of one of peroxiredoxin's cysteine residues, the peroxidatic cysteine. How this oxidation is resolved depends on how many reactive cysteines the protein has, which categorises them into one of three classes: 1-Cys, typical 2-Cys and atypical 2-Cys Prxs. In the case of typical 2-Cys Prxs the oxidised cysteine will form a disulphide bond with a resolving cysteine from another Prx monomer, making them functional homodimers in their catalytic cycle (see figure 3, top). The atypical 2-Cys Prxs on the other hand form the resolving disulphide bond with a cysteine residue on the same protein, making them monomeric (Wood et al., 2003). The 1-Cys Prxs only have one oxidisable cysteine, and their catalytic cycle is less understood but it is thought to involve the formation of complexes with other Prxs and glutathione, which supplies the resolving cysteine to the complex (Pedrajas et al., 2016). After disulphide formation between the peroxidatic and resolving cysteine of 2-Cys Prxs, they can be reduced

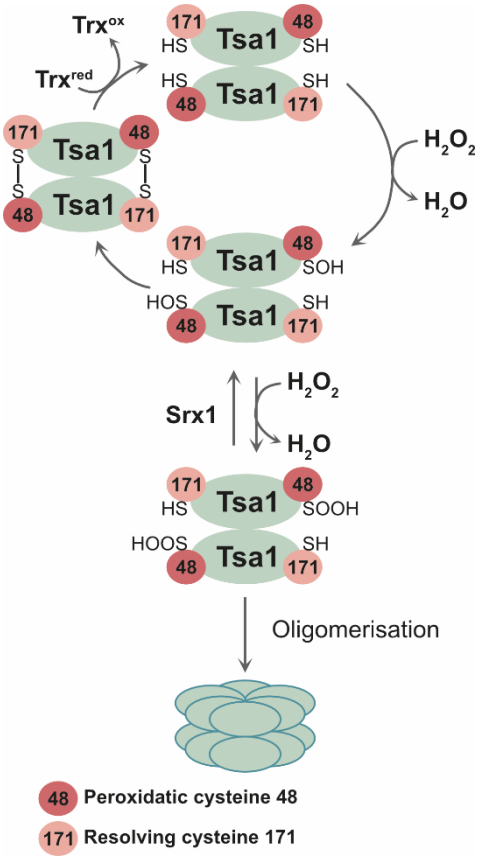

Figure 3: The peroxidatic cysteines (C48) on Tsa1 are oxidised by reaction with H_2O_2, which is resolved by formation of a disulphide bond with the resolving cysteine (C171). This bond can be reduced by the action of Trx. Further oxidation of the oxidised Tsa1 leads to hyperoxidation, which can trigger the formation of oligomers with chaperone activities. The hyperoxidation can be reduced by Srx1, which returns Tsa1 to the catalytic cycle.

by the actions of an oxioreductase, in yeast mainly the thioredoxin Trx1, which reduces the cysteines on the Prxs and resets the catalytic cycle (see figure 3, top).

The most abundant Prx in yeast is the Thiol-specific antioxidant 1 (Tsa1), a typical 2-Cys peroxiredoxin, with its peroxidatic cysteine at position 48 and the resolving cysteine at position 171. Tsa1 is found in the cytosol where it protects against oxidative damage from H_2O_2. It also has a special function at the ribosome where it safeguards against oxidative damage during translation (Trotter et al., 2008). Tsa1 has also been found to maintain genome stability and loss of Tsa1 leads to less efficient DNA repair (Huang and Kolodner, 2005).

During times of stress, such as heat and oxidative stress, the 2-Cys Prxs can exit their regular catalytic cycle and instead form a large multimeric complex (Schröder et al., 2000, Wood et al., 2003). Stress-induced oligomerisation is triggered by the reaction between an additional molecule of H_2O_2 with the already oxidised C48 (Cys-SOH)

which leads to the formation of a sulfinic acid (Cys-SOOH) (Schröder et al., 2000, Jang et al., 2004). As a multimer, Tsa1 have a chaperone-like function and can interact with misfolded proteins (Jang et al., 2004). When Tsa1 is hyperoxidised it is no longer a client for Trx1, and instead the sulphiredoxin Srx1 is necessary for the reduction of the hyperoxidised state of Tsa1 (Biteau et al., 2003), and this also triggers the resolution of the multimer of Tsa1 ((Jang et al., 2004), see figure 3, bottom), in essence making Tsa1 an oxidatively regulated molecular chaperone.

Protein degradation pathways

The Ubiquitin-Proteasome system

The Ubiquitin-Proteasome system (UPS) is a degradation system that degrades specific proteins in a targeted manner. The canonical targeting for UPS-mediated degradation involves the addition of monomers of ubiquitin in a polyubiquitin chain onto the target protein. Polyubiquitinylation is supported by three classes of enzymes: the E1 ubiquitin-activating enzyme, the E2 conjugating enzyme and the E3 ubiquitin ligase. The E1 enzyme catalyses the binding of one ubiquitin to the E2 conjugating enzyme, and the ubiquitin is then transferred from the E2 to the substrate protein by the E3 ligase enzyme. After the first ubiquitin monomer has been added to the protein substrate, subsequent additions of ubiquitin onto the first leads to the formation of a polyubiquitin chain that acts as a signalling complex ((Hershko and Ciechanover, 1998), see figure 4). Another mode of ubiquitinylation involves addition of multiple ubiquitin monomers to one protein, but at different amino acid residues, called multiubiquitinylation. This requires the support of an additional class of enzymes, the E4 multiubiquitin chain enzymes (Koegl et al., 1999, Hoppe, 2005).

How the ubiquitin chain is built, that is through which lysine residue the ubiquitin monomers are linked to one another, determines the outcome of the polyubiquitinylation. The most studied polyubiquitin

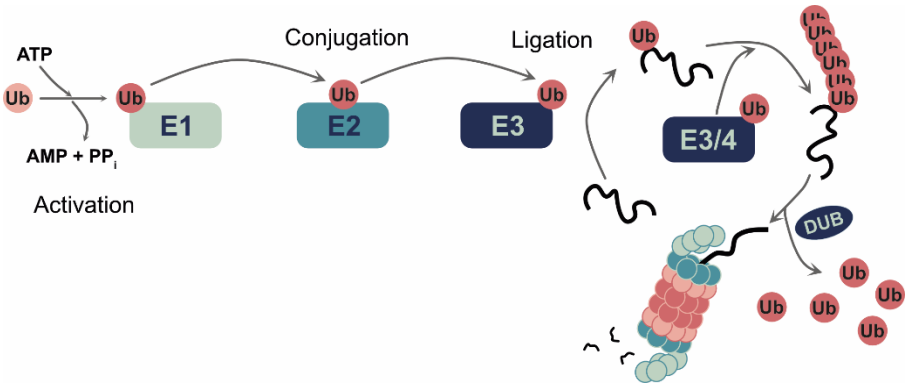

Figure 4: Ubiquitinylation is initiated by the activation of ubiquitin and binding of monomers to the E1 ubiquitin ligase. The ubiquitin is then transferred to the E2 conjugation enzymes and E3 ubiquitin ligases catalyses the addition of ubiquitin to a lysine residue on the target protein, upon which a polyubiquitin chain can be built by E3 and E4 enzymes. The polyubiquitin chain acts as a signal for targeting to for example the proteasome for degradation. Prior to degradation the polyubiquitin chain can be disassembled by DUBs.

signal is linked through lysine 48 (K48) on ubiquitin, which targets the substrate for degradation of the proteasome. Substrate specificity and polyubiquitin chain variability is mediated through the E3 ligases, and this enzyme family is the most variable and numerous member in the ubiquitinylation pathway, while most organisms tend to only have one E1 enzyme (Finley et al., 2012). This is exemplified in yeast, where one E1 ubiquitin activating enzyme, thirteen E2 ubiquitin conjugating enzymes and forty-three E3 ubiquitin ligases have been described (Lee et al., 2008). Another layer of regulation is also achieved by the action of the deubiquitinases (DUBs) that catalyses the removal of ubiquitin from substrate proteins (Suresh et al., 2020). While being an essential part of the proteasomal degradation of ubiquitinylated proteins, deubiquitinylation also acts prior to the proteasome and DUBs remove polyubiquitin chains or edit them for more efficient degradation, as exemplified by the DUB Ubp3 (Fang et al., 2016).

The proteasome is a large multimeric complex that degrades other proteins. The proteolytic activity of the proteasome takes place inside

the barrel-shaped core particle (20S), which contains the catalytically active β-subunits (Tanaka, 2009). In addition, a fully mature proteasome also has a lid subunit (19S) that has a regulatory function and increases the activity of the core particle and recognises the substrates targeted for degradation (Martinez-Fonts et al., 2020). The 19S also contain a deubiquitinase that deubiquitinylates the proteasomal substrates prior to proteolysis. The fully assembled proteasome that consist of both a lid and a core particle is usually termed a 26S proteasome (Tanaka, 2009).

Autophagy

Autophagy is an essential degradation pathway where cellular components are degraded in bulk through the formation of a membrane-enclosed autophagosome that fuses with the lysosome/vacuole where the degradation takes place. Autophagy is a flexible degradation pathway that can handle everything from organelles to inclusion bodies.

Temporal protein quality control

Refolding of unfolded and misfolded proteins

Yeast has a powerful refolding machinery that involves chaperones from several subfamilies. The central activity, that of transient binding and releasing of substrates is carried out by the Hsp70s, supported by JDPs and NEFs. Protein refolding *in vivo* is often studied in the context of proteins that have formed inclusion bodies or aggregates. The transient interactions of Hsp70s and partially unfolded proteins not part of aggregates is typically harder to track *in vivo* compared to the release of proteins from an inclusion/aggregate, which can be tracked based on changes in size and/or complete dissolution. In the chapter on spatial protein quality control there is a more extensive discussion of disaggregation and resolution of inclusion bodies.

In many cases, protein misfolding will trigger the activation of sHsps that bind to the protein substrates and form large oligomeric complexes (Ehrnsperger et al., 1997). These complexes maintain the folding capacity of the bound substrates so that they do not terminally misfold and are accessible for refolding after they dissociate from the complex. The resolution of sHsp:substrate multimeric complexes requires the cooperation from other molecular chaperones from the JDP, Hsp70 and Hsp100 families that work in tandem to dissociate the assemblies and refold the unfolded protein substrates sequestered by the sHsp complex (Lee and Vierling, 2000, Cashikar et al., 2005, Haslbeck et al., 2005). *In vitro* results of isolated proteins from both bacteria and yeast have shown that the refolding of substrates bound by sHsps require Hsp70s to displace the sHsps from the sHsp:substrate oligomer (Zwirowski et al., 2017). Full refolding capacity is however not achieved without the concerted activities of Hsp70 and Hsp104, at least *in vitro* (Glover and Lindquist, 1998). The activity of Hsp70-Hsp104 refolding/disaggregation needs to be tightly controlled however, since mutations that uncouple Hsp104 from Hsp70-mediated activation leads to cytotoxicity, possibly due to

destructive unfolding of properly folded protein complexes (Lipinska et al., 2013, Chamera et al., 2019). Compelling evidence for the Hsp70-Hsp104 mechanism *in vivo* can be found in the many studies that show a delay in disaggregation in *hsp104*Δ mutant cells ((Specht et al., 2011, Spokoini et al., 2012, Escusa-Toret et al., 2013, Miller et al., 2015a), Paper II and III), however the fate of the misfolded/disaggregated protein after it has been removed from the aggregate or inclusion is not always known.

While the traditional yeast refolding complex consist of Hsp70, a JDP and Hsp104, the alternative disaggregation complex of Hsp70, JDPs and Hsp110 (see below for further discussion) can also work together with sHsp:substrate complexes to promote dissolution (Duennwald et al., 2012), in yeast either with (Kaimal et al., 2017) or without the support of Hsp104 (den Brave et al., 2020).

The fate of a protein after it has been retrieved from an aggregate or inclusion body is determined by the chaperones involved in the process. This was recently illustrated in the context of the yeast nucleus where two chaperone systems work in parallel, one where the end result is refolding of the previously misfolded protein and one where the protein is degraded (den Brave et al., 2020). Once misfolded proteins have formed an inclusion body, supported by Btn2, they can be retrieved and either degraded or refolded. The degradation route is supported by Hsp70 together with Apj1 and Sse1, while the interaction between Hsp70, Sis1 and Hsp104 instead support the refolding of the misfolded protein (den Brave et al., 2020). How the flux between these pathways is regulated and how different substrates are triaged in the systems is still, excitingly, unexplored.

Chaperones in degradation pathways for cytosolic proteins

Proteosomal degradation can be facilitated through *cis*-acting elements; that is characteristics on the protein substrates themselves

target them for degradation. One such pathway is the N-end rule, wherein proteins are targeted for degradation based on the *N*-degron, the N-terminal amino acid and post-translational modifications made thereupon (Varshavsky, 2011). Degradation can also be promoted by specific sequence degrons, short amino acid sequences embedded in the protein that regulates its stability (Maurer et al., 2016, Geffen et al., 2016, Koren et al., 2018). However, cytoplasmic misfolded proteins often require chaperones in order to be properly targeted for ubiquitinylation and subsequent degradation. Many, if not all, UPS pathways for misfolded proteins converge on the cytosolic Hsp70s and their co-chaperones, which are required for delivery or access of the substrates to the proper UPS components. Chaperone involvement in UPS can be seen pre-ubiquitinylation, but it is also evident in post-ubiquitinylation recognition of substrates by the proteasome, and in the targeting of ubiquitin-independent substrates to the proteasome (Kandasamy and Andreasson, 2018). Stabilisation of misfolded proteins upon deletion of for example *SSA1* and *SSA2* has been observed for over three decades (see (Hayes and Dice, 1996) for a review that summarises the early findings), but exactly how different degradation pathways collaborate with molecular chaperone networks is still an open question.

Hsp70 chaperone networks and cytosolic E3 ligases

Reliance of the cytosolic Hsp70s for proper protein degradation in yeast was demonstrated by Park *et al*. with the use of mutated versions of carboxypeptidase yscY (Park et al., 2007). The carboxypeptidase yscY versions used either only lacks their endoplasmic reticulum (ER) signal sequence (ΔssCPY) or carries a destabilising mutation (ΔssCPY*/ΔssCPY*-GFP) as well (Park et al., 2007). The degradation of the substrates required the ubiquitin-conjugating enzymes Ubc4 and Ubc5, and was carried out by the proteasome and not the ER-associated degradation complex Cdc48-Ufd1-Npl4, which has been implicated in the degradation of other endogenous cytosolic proteins and protein constructs (Metzger et al., 2008, Medicherla and Goldberg,

2008). By use of a strain that lacked Ssa2-4 with a temperature sensitive (ts) allele of *SSA1* (*ssa1-45*) instead of the wild type allele, it was demonstrated that the Ssa proteins were necessary for degradation of the CPY-derivatives, and that much of the degradation could be supported by Ssa1 as the sole cytosolic Hsp70. The chaperone complex co-operating with Ssa1 to carry out the targeting of the CPY-substrates for degradation included the cytosolic JDP chaperone Ydj1, but neither Hsp90's, Hsp110's, sHsps or Hsp104 were found to be necessary at this point (Park et al., 2007).

As mentioned above, loss of both constitutive Ssa proteins Ssa1 and 2, leads to stabilisation of cytosolic misfolded proteins, even though the phenotype is not as extreme as in the *ssa1-45 ssa2*Δ *ssa3*Δ *ssa4*Δ strain (Park et al., 2007, Prasad et al., 2010, Öling et al., 2014, Lee et al., 2016), Paper II). One of the remaining Ssa proteins Ssa4 is induced in this mutant background (Werner-Washburne et al., 1987, Boorstein and Craig, 1990), but this induction is not enough to support efficient degradation of misfolded proteins. To further explore if this is just due to an overall lack of Ssa proteins or tied to a specific functional difference, we used a double mutant strain (*ssa1*Δ *ssa2*Δ) with a strong promoter driving the expression of *SSA4* (*ssa1*Δ *ssa2*Δ P$_{GPD}$*SSA4*). In the *ssa1*Δ *ssa2*Δ

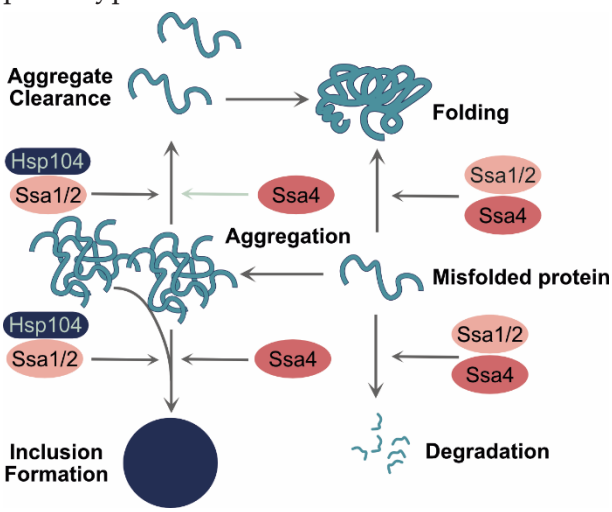

Figure 5: Ssa1/2 and Ssa4 can all support folding (top right) and promote degradation of misfolded proteins (bottom right) as well as promote the coalescence of CytoQs into larger inclusion bodies (bottom left). Ssa4 does however not co-operate with Hsp104 which leads to a reduced aggregate clearing capacity (top left). Adapted from Paper II.

P_{GPD}*SSA4* background, we could observe a destabilisation of two CPY-derivatives, ΔssCPY*-GFP and ΔssCPY*-Leu-myc ((Eisele and Wolf, 2008), see figure 5). By inhibiting protein production with cycloheximide treatment we followed the degradation of ΔssCPY*-GFP over time, and found that it was faster in the *ssa1*Δ *ssa2*Δ P_{GPD}*SSA4* strain compared to the *ssa1*Δ *ssa2*Δ strain, but not quite as efficient as in the wild type. The misfolding protein ΔssCPY*-Leu2-myc is essential in strains lacking *LEU2* if they are grown in leucine-free media, and loss of any protein that can destabilise ΔssCPY*-Leu2-myc will allow cells to grow even in the absence of leucine. While ΔssCPY*-Leu2-myc is stabilised in cells lacking Ssa1 and 2 and the cells grow well without added leucine, overexpression of *SSA4* in the *ssa1*Δ *ssa2*Δ background destabilised ΔssCPY*-Leu2-myc to an even greater extent compared to the wild type (Paper II). The different results between these two misfolded proteins could be related to the localisation of the misfolded proteins. ΔssCPY*-Leu2-myc is strictly reliant on degradation pathways in the cytosol mediated by Ubr1 (Eisele and Wolf, 2008, Amm and Wolf, 2016), while ΔssCPY*-GFP is degraded both by Ubr1 in the cytosol and San1 in the nucleus (Heck et al., 2010). The *ssa1*Δ *ssa2*Δ P_{GPD}*SSA4* strain also displayed a deficiency in the formation of nuclear inclusion bodies (Paper II) and Ssa4 doesn't show the same intranuclear accumulation pattern as Ssa2 ((Öling et al., 2014), Paper II). These data, together with the intermediate stabilisation of ΔssCPY*-GFP, suggests that Ssa4 may be a less effective chaperone partner in intranuclear protein quality control compared to Ssa1 or Ssa2. The paralog of Ssa4, Ssa3, has been implicated in mediating degradation of the cytotoxic α-synuclein protein through autophagy, something that could not be supported by either of the two constitutive Ssa proteins (Gupta et al., 2018), which also strengthens the role of the inducible Ssa proteins in cytosolic protein quality control. It is however unclear if ΔssCPY*-Leu2-myc can be redirected from UPS-mediated degradation in the absence of

Ssa1/2, and any changes in autophagic activity in *ssa1*Δ *ssa2*Δ P_{GPD}*SSA4* remains unexplored.

In an effort to identify the UPS and chaperone components involved in degradation of misfolded cytosolic proteins, ΔssCPY*-GFP was used in a targeted screen with a collection of knock-out mutant strains (Heck et al., 2010). The screen identified the nuclear San1 and the cytosolic Ubr1 E3 ligases as important in mediating the ubiquitinylation and degradation of ΔssCPY*-GFP in a pathway together with Hsp70s, Ydj1, but also the Hsp110 Sse1 (Heck et al., 2010), contrary to what was demonstrated before (Park et al., 2007). Ubr1 have, before the screen by Heck and colleagues, been implicated in the degradation of misfolded proteins in a manner outside of its established role in *N*-degron degradation (Eisele and Wolf, 2008, Nillegoda et al., 2010). The importance of Ubr1-mediated ubiquitinylation for the degradation of cytosolic proteins was further strengthened in another screen of ts-alleles of essential yeast proteins. Upon a shift to a high restrictive temperature, strains carrying these alleles *in lieu* of the wild type alleles will fail to grow. By expressing the ts-alleles in strains lacking different E3 ligases, almost a third of the ts-alleles was found to be stabilised in cells lacking *UBR1*, which also rescued the heat sensitivity of these strains (Khosrow-Khavar et al., 2012).

Hsp70 co-chaperones and NEFs in UPS pathways

While at first the Hsp110 Sse1 was thought to not be involved in Hsp70-mediated degradation by the UPS (Park et al., 2007), the involvement of Sse1 in this process has been demonstrated by others in later studies (Heck et al., 2010, Prasad et al., 2010, Guerriero et al., 2013, Kandasamy and Andreasson, 2018). Khosrow-Khavar *et al.* found that Sse1 was only partially required for Ubr1-mediated degradation of some misfolded substrates (Khosrow-Khavar et al., 2012). The suggested mechanism for Sse1/2 takes place downstream of ubiquitinylation in the transfer of Hsp70-substrates to the 26S

proteasome, where Sse1 is the bridge between the 19S regulatory particle and the Hsp70 that allows for efficient substrate targeting. In this way, Sse1 act in both ubiquitin-dependent and independent protein degradation (Kandasamy and Andreasson, 2018). On the other hand, the NEF Fes1 was shown to work together with Hsp70s to promote the ubiquitinylation of both artificial misfolding prone proteins as well as endogenous proteins misfolded through heat shock. Based on the accumulation of misfolded proteins bound to Ssa2 and the reduced association between substrates and Ubr1 in *fes1*Δ cell extracts, Fes1 was suggested to play a role in the transfer of substrate from Hsp70s to the E3 ubiquitin ligases (Gowda et al., 2013).

Ydj1 has been demonstrated to work together with the Hsp70s to target cytosolic misfolded proteins for degradation (Park et al., 2007, Heck et al., 2010), but Ydj1 is not the only JDP involved in the process. The essential type II JDP Sis1 was shown to be involved in the degradation of a misfolded short-lived version of GFP (slGFP) (Summers et al., 2013). While loss of Ydj1 lead to the accumulation of slGFP into insoluble aggregates that did not exhibit any fluorescence, reduction of Sis1 protein levels lead to slGFP forming soluble aggregates and fluorescence puncta in the cytosol. Increased production of Sis1, but not Ydj1, lead to an increase in the turnover of slGFP, which suggests that Ydj1 is involved in the stabilisation and folding of nascent immature slGFP and can maintain the protein soluble once it is folded, but targeting the native slGFP for protein degradation is rate limited by Sis1. Sis1's action is however not ubiquitous as it was not involved in the degradation of the von Hipple-Lindau protein. Just as with Ydj1-dependent substrates, Sis1-mediated protein degradation of slGFP was supported by both Ubr1 and the San1 E3 ligases (Summers et al., 2013).

Stress changes UPS pathways

Heat shock triggers widespread polyubiquitinylation of mainly cytosolic proteins, and interestingly it appears that E3 ligases

important for polyubiquitinylation and degradation of model substrates aren't necessarily involved in this effort (Fang et al., 2011). A targeted screen for ubiquitinylation defects after heat shock instead identified Hul5, a HECT-domain ubiquitin ligase, as important for heat stress mediated polyubiquitinylation. Upon heat shock, Hul5 translocates from its usual nuclear localisation into the cytosol, where it promotes ubiquitinylation of endogenous cytosolic substrates. The ubiquitinylation response during heat shock is important for post-heat stress recovery, as cells lacking Hul5 show a greater post-heat shock growth defect compared to wild type cells (Fang et al., 2011). Interestingly Hul5 is also able to promote ubiquitinylation of substrates in the absence of Ssa proteins, which suggests that it is the main enzyme involved in the increase in polyubiquitinylated proteins that is observed in cells lacking Ssa1 and Ssa2 (Öling et al., 2014), since many other E3 ligases require Ssa1 and Ssa2 in one way or another for substrate recognition (Heck et al., 2010, Prasad et al., 2010, Guerriero et al., 2013, Prasad et al., 2018).

The heat shock associated ubiquitinylation of cytosolic proteins is not completely abolished in cell lacking Hul5 (Fang et al., 2011), and another E3 ligase. Rsp5, is also active in this process (Fang et al., 2014). While there is some degree of substrate overlap, Hul5 and Rsp5 have distinct substrate pools and a complete abolishment of heat shock induced polyubiquitinylation is only achieved when both proteins are lacking or impaired (Fang et al., 2014). Rsp5 interacts with Ydj1, and this interaction is necessary for Ydj1-substrates to be ubiquitinylated by Rsp5 (Fang et al., 2014). While Rsp5 have a role in the targeting of membrane bound proteins to the lysosome/vacuole through K63-linked polyubiquitinylation, heat shock changes the ubiquitin linkage catalysed by Rsp5. This change is supported by the association with the DUBs Ubp2 and Ubp3, which remodel ubiquitin chains built by Rsp5 (Fang et al., 2016).

Different stressors induce different types of damage on proteins, which might require distinct pathways to safeguard cellular fitness. In support of this we have found that loss of Tsa1 leads to an increase in the build-up of ubiquitinylated proteins after hydrogen peroxide stress and conversely, overexpression of *TSA1* lead to lower levels of ubiquitinylated proteins. This could either mean that Tsa1 can protect against oxidative damage to the proteome, or hint at a role for Tsa1 in proteasomal degradation (Paper I). The former argument is supported by the fact that Tsa1 can protect against the oxidative damage mediated by increased mitochondrial ROS production, stemming from AZC-misfolded proteins and aggregates (Weids and Grant, 2014). The latter argument is supported by the fact that multimeric Tsa1 interacts physically with Ssa proteins, which might aid Hsp70 in targeting misfolded proteins for more efficient degradation. In line with this, Tsa1 is not just needed during times of overt oxidative stress, since the ΔssCPY*-Leu2-myc substrate is stabilised to the same extent in cells lacking *TSA1* as it is in the *SSA1/2* double deletion mutant, which indicates that Tsa1 has a role in the basal turnover of misfolding prone proteins (Paper I). In mammalian systems, degradation of oxidatively damaged proteins by the proteasome does not require substrate ubiquitinylation (Shringarpure et al., 2003), and whether or not peroxiredoxins somehow partake in this direct targeting to the proteasome would be an interesting avenue of exploration.

The cytosolic proteins most sensitive to damage and subsequent degradation are newly synthesised proteins, and proteins retain this sensitivity up to an hour post translation (Medicherla and Goldberg, 2008). This was suggested by Medicherla and Goldberg to represent the time it takes for proteins to reach their final subcellular localisation, to be assembled into the proper complexes and to be post-translationally modified correctly. During this window of time, proteins can be damaged from both heat and ROS and the damage leads to degradation, while the bulk of the "mature" cellular proteome is less sensitive to damage (Medicherla and Goldberg, 2008). The

degradation of these proteins are reliant upon the UPS but also the ER-associated Cdc48-Ufd1-Npl4 degradation complex (Medicherla and Goldberg, 2008).

Chaperones and organelle-associated degradation pathways for cytosolic proteins

The ER is important in protein degradation and has its own signalling and degradation pathways (the unfolded protein response; UPR, and ER-associated degradation; ERAD) that deal with proteotoxic stress that affects the organelle as well as the entire cell. The ER contains a number of E3 ligases spread out either in the membrane facing the cytosol (ERAD-C, Doa10 in yeast) or the membrane facing the ER-lumen (ERAD-L/M, Hrd1 in yeast). Cytosolic proteins containing degrons derived from ER-proteins require the Doa10 E3 ligase for efficient ubiquitinylation and degradation by the proteasome, and also requires Ssa1/2 and the JDP Ydj1 (Maurer et al., 2016). However, Doa10 also collaborates with Sis1 in the degradation of fusion proteins with degrons from known non-ER Doa10 substrates (Shiber et al., 2013). Shiber *et al* found that the co-operation between Sis1 and Ssa1/2 was carried out in a distinct manner, where Sis1 was required for substrate ubiquitinylation by Doa10, and Ssa1 and/or Ssa2 was necessary for the subsequent degradation of the substrate after polyubiquitinylation (Shiber et al., 2013). Other misfolded substrates originating in the cytosol seems to also rely on Sis1 for their ER-associated ubiquitinylation, which can be supported by either Hrd1 or Doa10 (Samant et al., 2018). The ubiquitinylation pattern created by Hrd1 and Doa10 is different from that of Ubr1 and San1, as Hrd1 and Doa10 catalyses polyubiquitin chains with K11 linkages, while Ubr1/San1 catalyses K48-linked chains, and both linkages is typically found on cytosolic misfolded proteins, indicating that there is a co-operation between the ER-associated and soluble E3 ligases (Samant et al., 2018).

The mitochondria has also been suggested as a partner in the degradation of misfolded cytosolic proteins (Ruan et al., 2017), and inclusion bodies have been known to localise in close proximity to mitochondria (Zhou et al., 2014, Babazadeh et al., 2019). Import into the mitochondria is suggested to be mediated by mitochondrial importers such as Tom70 and the degradation is dependent upon mitochondrial proteases. The targeting of cytosolic proteins to mitochondria for degradation is however independent of the cytosolic Ssa proteins, but Hsp104 activity is necessary for the process (Ruan et al., 2017).

Nuclear degradation pathways for cytosolic proteins

Transporting cytosolic proteins for ubiquitinylation and degradation inside the nucleus might seem counter-intuitive, but it appears to be an established route for many cytosolic misfolded proteins (Prasad et al., 2010, Prasad et al., 2018) and the yeast nucleus also has a higher concentration of proteasome subunits compared to the cytoplasm (Russell et al., 1999b). As was mentioned above, Ubr1 is not the only ubiquitin ligase important for cytosolic protein degradation, and there is an overlap with the nuclear E3 ligase San1 for many investigated misfolding cytosolic protein substrates (Heck et al., 2010, Khosrow-Khavar et al., 2012, Summers et al., 2013, Guerriero et al., 2013). San1 is a RING-domain ubiquitin ligase required for the ubiquitinylation and degradation of nuclear proteins, in tandem with the ubiquitin conjugating enzymes Ubc1 and Ubc3 (Gardner et al., 2005). It should also be noted that the clear distinction between Ubr1 as a cytosolic and San1 as a nuclear E3 ubiquitin ligase has been challenged, as Ubr1 has been observed in the nucleus, and known cytosolic Ubr1-substrates remain dependent on Ubr1 even after they are transported in to the nucleus via the addition of a nuclear localisation sequence to the protein (Prasad et al., 2018).

The first step for the degradation of cytosolic proteins in the nucleus is transport of the protein across the nuclear membrane. In the case of

purely cytosolic substrates, this is not enabled by the misfolding protein itself, but instead it is mediated by the Hsp70s Ssa1 and Ssa2, independently of each other (Prasad et al., 2010). One kind of substrate specificity in nuclear protein degradation arises here at the translocation step since there is a size discrimination for the Hsp70-mediated import of misfolded proteins into the nucleus, where larger substrates are excluded from import (Amm and Wolf, 2016). Hsp70 involvement in nuclear degradation doesn't stop at the translocation step, as Ssa1 has been shown to promote the direct interaction between San1 and at least one of its substrates (Guerriero et al., 2013), however the generality of this is contested (see below). Ydj1 and Sis1 have both been suggested to cooperate with Hsp70s in the import of cytosolic misfolded proteins to the nucleus and their subsequent degradation (Prasad et al., 2018). Ydj1, together with Sse1, is described as the primary JDP and NEF needed for transport of substrates in the nucleus, while Sis1 is needed for both the transport but also the subsequent polyubiquitinylation and degradation of substrates in the nucleus (Prasad et al., 2018). Corroborating this is the observation that sequestration of Sis1 in cytosolic insoluble aggregates with polyglutamine expanded proteins (polyQ) lead to a loss of nuclear UPS-mediated degradation (Park et al., 2013).

While many studies have shown a requirement of Ssa proteins for the targeting of misfolded proteins to the San1 E3 ligase (Heck et al., 2010, Prasad et al., 2010, Guerriero et al., 2013, Prasad et al., 2018), how this targeting is mediated is not clear. A suggested model by the Gardner group is based on data that shows that San1 can interact with its substrates on its own without the Ssa proteins (Rosenbaum et al., 2011), and the key role of the Hsp70s is ensuring substrate solubility so that the interaction can take place (Jones et al., 2020). This is based on the apparent variability in the need for Ssas and chaperoning proteins in the nucleus (in this case, Cdc48 (Gallagher et al., 2014)) correlating with the solubility of the substrate, and characteristics of the substrate itself directly determining its availability to San1

(Fredrickson et al., 2013, Gallagher et al., 2014, Jones et al., 2020). This is in line with the reasoning presented by Prasad and colleagues when they observed that their misfolding protein substrates formed large cytosolic inclusions in the *ssa1*Δ *ssa2*Δ mutant which prevented nuclear import, concurrent with a decrease in San1-mediated ubiquitinylation and degradation (Prasad et al., 2010). Whether or not this model of Hsp70-San1 interplay is true for other E3 ligases and their substrates remains to be seen. A similar relationship has been observed between prefoldin and Ubr1 by the use of thermosensitive mutants of essential proteins such as guk1-7-GFP, a GFP-tagged thermolabile variant of Guk1 (Comyn et al., 2016). Guk1-7-GFP is dependent on Ubr1 for its ubiquitinylation (Khosrow-Khavar et al., 2012). Loss of subunits of prefoldin, a chaperone complex that co-operates with chaperonins, leads to the inclusion of guk1-7-GFP into insoluble aggregates which decreases its ubiquitinylation, suggesting that the protein substrates for Ubr1 must be maintained in a soluble state by prefoldin in order for the ubiquitinylation to occur (Comyn et al., 2016).

Temporal PQC during ageing

PQC systems are challenged during ageing, and proteostasis decline is one of the "hallmarks of ageing" (López-Otín et al., 2013). This is also reflected in the type of diseases that afflict the ageing population. As humans age, there are large-scale changes in the chaperone network (the so-called chaperome) (Brehme et al., 2014). By combining published brain transcriptome datasets from humans and the worm *Caenorhabditis elegans* with RNAi screens, a subnetwork of protective chaperones was identified. This subnetwork is specifically repressed in the human brain during ageing and in neurodegenerative diseases (Brehme et al., 2014), which demonstrates the interconnectivity between proteostasis, ageing and disease. An example of how ageing and neurodegeneration could directly be linked is through the fact that the Hsp70 cognate protein HSC70 and the Hsp90 protein HSP90β

in humans are needed to maintain the disease-linked α-synuclein protein from toxic oligomerisation (Burmann et al., 2020), and HSC70 and HSP90 proteins are specifically repressed during ageing (Brehme et al., 2014), which could leave cells more vulnerable to α-synuclein toxicity. However, the transcriptome changes associated with age are not uniform, as different tissues have different age-related transcriptome profiles which is exemplified in mitotic and post-mitotic tissues from mice (de Toda et al., 2016). The question also remains whether or not the transcriptome and protein levels directly translate to a decreased proteostasis capacity (Josefson et al., 2017).

In *C. elegans*, a widely used model organism in the ageing field, there is a decline in proteostasis networks that manifests itself as a failure to respond to heat stress and to maintain misfolding-prone proteins in a functional state, that can be observed already in early adulthood (Ben-Zvi et al., 2009). Well known age-deterministic pathways, such as the insulin/insulin-like growth factor 1 signalling pathway regulated by DAF-2, requires HSF-1, as the lifespan extension seen in *daf-2*$^{-}$ and *age-1*$^{-}$ is abolished upon *hsf-1* downregulation or knock out (Hsu et al., 2003, Morley and Morimoto, 2004), and *hsf1*-inhibition itself shortens lifespan (Garigan et al., 2002). Similarly, an increase in lifespan can be achieved by introducing multiple copies of *hsf-1* in the worm genome (Hsu et al., 2003) or by upregulating genes downstream of *hsf1-1* or *daf-16* that encode Hsps (Walker and Lithgow, 2003, Hsu et al., 2003). Lifespan extension by boosting proteostasis pathways, by for example overproduction of the metacaspase Mca1 or the peroxiredoxin Tsa1, is documented in yeast ((Hill et al., 2014) and Paper I), and loss of key genes such as *SSA1/2*, decreases lifespan (Craig and Jacobsen, 1984, Werner-Washburne et al., 1987, Öling et al., 2014), Paper II). Chaperones also become limiting with advanced replicative age in yeast, which destabilises key cyclins needed for cell cycle progression, directly tying proteostasis failure to cell senescence and death (Moreno et al., 2019).

One problematic aspect of ageing is the fact that many times the effector proteins in PQC systems are themselves targets for age-related damage. This can be seen in human fibroblasts where HSC70 is a target for oxidative damage (Unterluggauer et al., 2009). It is also seen in the proteasome, as it can be inhibited by the accumulation of aggregated proteins which leads to a detrimental feedback loop (Holmberg et al., 2004, Andersson et al., 2013, Ayyadevara et al., 2015). Restoring proteasome capacity by promoting aggregate clearance (Andersson et al., 2013), or boosting the production of proteasomal subunits can extend the lifespan (Nguyen et al., 2019), although this is a marginal extension in yeast (Andersson et al., 2013).

Spatial protein quality control

Protein aggregation can both be a boon and a bane; aberrant protein conformers are the culprit in many human diseases and the introduction of one toxic aggregation-prone protein can affect the stability of a wide range of other essential proteins (Olzscha et al., 2011), cause defects in how other aberrant proteins are managed (Chakrabarti et al., 2011), disrupt vital nuclear functions (Gasset-Rosa et al., 2017, Grima et al., 2017) and disrupt PQC pathways by sequestering chaperones, thereby inhibiting their normal function (Park et al., 2013). If the aggregation is not dealt with properly it can lead to impairment of important cellular processes such as DNA-repair (Mediani et al., 2019), and it can disrupt cytoprotective processes such as stress granule formation (Mateju et al., 2017). On the other hand, proper management of misfolded/unfolded proteins promote cell survival, and transient aggregation/accumulation of certain proteins can be part of an adaptive response that promotes cell survival after stress (Audas et al., 2016, Azkanaz et al., 2019). Being able to properly regulate protein aggregation into protective inclusion bodies and preventing toxic aggregation that interfere with cytosolic processes is therefore key to maintain homeostasis and to ensure cellular survival. The importance of this is made explicitly clear when looking at the number of human diseases where protein aggregates are part of the pathological process, like Alzheimer's, Parkinson's, Huntington's and Amyotrophic Lateral Sclerosis.

Traditionally, there has been a distinction made between amyloid(-like) and non-amyloid, amorphous misfolded protein substrates in the study of SPQC, since their behaviour, resolution/degradation pathways and chaperone interactions are, at least to some extent, distinct (Kaganovich et al., 2008, Specht et al., 2011, Escusa-Toret et al., 2013). Recently, the proteostasis field have been introduced to a new idea, namely liquid-liquid phase separation (LLPS), which drives the formation of "biomolecular condensates" and membraneless

organelles (Banani et al., 2017). This has been suggested as a possible explanation as to how protein aggregation occurs, especially when it comes to proteins with intrinsically disordered domains and proteins that interact with nucleic acids (Alberti and Hyman, 2021). In this chapter, the active formation of chaperone-substrate complexes as a response to protein misfolding will be called inclusion bodies, and whenever there is data on the state on the protein in question, if it is in an amyloid state, included in a phase separated compartment, terminally misfolded in an aggregate, or part of a chaperone:substrate complex that can be reversed it will be stated explicitly.

The role of spatial protein quality control in cellular fitness

When the phenomenon of protein aggregation was first discovered in the context of Alzheimer's disease, it was believed that the protein aggregates observed within Alzheimer patient samples were the causative agent of disease. However, the process is not as simple as disease proteins clumping together and disrupting the cell, instead it seems like protein accumulation into inclusion bodies is an active process that promotes cellular fitness during times of stress, and that pathological aggregation represents a deviation from the spatial protein quality control (SPQC) pathways. Studies of an aggregation prone mutant version of the cystic fibrosis transmembrane conductance regulator protein (CFTR) ΔF508 lead to the description of the first known SPQC site, the so-called "aggresome" (Johnston et al., 1998). ΔF508 accumulated in cells after either the proteasome was inhibited or the production of the protein was drastically increased (Johnston et al., 1998). The ΔF508-aggregate was insoluble, and localised to the MTOC (microtubule-organising centre), where it was enveloped by the intermediate filament protein vimentin. Further studies revealed that not only vimentin co-localise to the aggresome, but also Hsp70, JDP co-chaperones and Hsp60 chaperonins can be found at the site, as well as the 20S proteasome, seemingly

independent of the presence or absence of an exogenous misfolding protein (Garcia-Mata et al., 1999, Wigley et al., 1999). The proteasome was also demonstrated to be important for the clearance of the misfolded proteins that localise to the aggresome, since proteasome inhibition lead to build-up of aggresome material and a decrease in degradation of the misfolding proteins associated with the aggresome (Johnston et al., 1998, Garcia-Mata et al., 1999).

The formation of the aggresome is not a random process, but instead an active one that relies on a functional microtubule network (MT), which was revealed early on when the inclusion body was first described (Johnston et al., 1998, Garcia-Mata et al., 1999). Misfolded proteins form aggregates in the periphery of the cell, and are transported along microtubuli in a process dependent on the dynein/dynactin motor complex (Garcia-Mata et al., 1999, Johnston et al., 2002). Furthermore, Garcia-Mata and colleagues showed that even though the intracellular architecture appear to be disrupted by the presence of the aggresome, crucial processes such as ER-to-Golgi vesicle transport are not interrupted, indicating that there is a tolerance for the type of disturbance caused by the aggresome (Garcia-Mata et al., 1999). The association between the microtubule network and aggregated proteins is mediated through the cytosolic Histone Deacytelase 6 (HDAC6) that binds both polyubiquitinylated proteins and the dynein motor complex which is necessary for the deposit of misfolded proteins at the aggresome (Kawaguchi et al., 2003). This transport route is however exclusive to ubiquitinylated misfolded proteins, and misfolded, non-ubiquitinylated proteins can still be found at the aggresome in HDAC6 knockout cells while the polyubiquitinylated substrates remain in peripheral foci (Kawaguchi et al., 2003).

The formation of cytoprotective inclusion bodies have been demonstrated using a wide variety of protein substrates involved in human pathologies. PolyQ mutant forms of exon one of the huntingtin

(mHttQ) protein is prone to misfold, is cytotoxic to neurons, and forms intranuclear inclusion bodies. Rather than being causative of cell death, the mHttQ inclusion bodies are associated with an alleviated risk of cell death and, in support of this notion, suppression of inclusion body formation leads to an increase in apoptosis in cultured striated neurons (Saudou et al., 1998). It is rather the non-aggregated, diffuse, or oligomeric, form of mHttQ that is associated with cell death, and the formation of inclusion bodies leads to a decrease in the diffuse form of mHttQ as well as a decrease in risk for cell death (Arrasate et al., 2004, Miller et al., 2010). Similarly, by screening for antibodies that associate to different structural forms of mutant huntingtin, it was shown that the antibody that recognises the form of mHttQ associated to the highest risk of neuronal death recognises an epitope found in a compact mHttQ monomer, and not the form of mHttQ present in inclusion bodies (Miller et al., 2011, Peters-Libeu et al., 2012). Modifying the polyQ expansion can also modulate the toxicity of mHttQ. Adding the proline-stretch from native Htt to a mHttQ with 103 polyQ residues (here called mHttQP) renders the construct less toxic for yeast and it forms a single inclusion body instead of many small ones (Duennwald et al., 2006, Dehay and Bertolotti, 2006). This aggregation pattern is disturbed in yeast mutants with a defective ribosomal protein quality control complex (RQC), and instead of forming a single, actin-associated inclusion, multiple small foci of mHttQP appear in the cell. This is associated with an increase in cytotoxicity, which is mediated by RQC components interacting with Hsf1 (Yang et al., 2016). The toxicity of soluble versus insoluble mHttQ is potentially mediated by the large interactome of soluble mHttQ compared to mHttQ in insoluble cellular fractions (Kim et al., 2016). In yeast, this becomes evident as soluble versions of mHttQ exhibits disruptive interactions with the type 1 myosins Myo3 and 5, which interrupts endocytosis (Berglund et al., 2017). α-synuclein, a disease protein implicated in Parkinson 's disease, also follows the same pattern regarding toxicity as mHttQ, as

promoting formation of large over small inclusion bodies by inhibition of the deacytelase SIRT2 decreases cell death in human neuroglioma cells. The same drug-mediated inhibition also protected a subset of α-synuclein-sensitive neurons in both flies and cultured midbrain sections from mice from cell death, suggesting that promoting α-synuclein inclusion body formation is cytoprotective (Outeiro et al., 2007).

In SPQC, the question isn't always if a protein should aggregate or not, but instead the central question is where the misfolding protein should be deposited. Rerouting misfolded proteins that form insoluble aggregates in the JUxtaNuclear Quality control (JUNQ) compartment to the Insoluble PrOtein Deposit (IPOD) instead can lessen the toxicity of these proteins (Weisberg et al., 2012). An aggregation-prone version of superoxide dismutase-1 (SOD1G93A) co-localises with other misfolded proteins in the JUNQ, and decreases the mobility of these substrates and prevents their proteasome-mediated degradation. This is accompanied with a decrease in cell survival and destabilisation of misfolding-prone proteins. Sequestering SOD1G93A to the IPOD by fusing it with mHttQ alleviates the proteostasis defects and increases cell survival (Weisberg et al., 2012). Conversely, other proteins can induce toxicity when they are present in the cytosol instead of the nuclear compartment. Using artificial aggregation substrates as well as mHttQ proteins it was demonstrated that the presence of these proteins in the cytosol, but not the nucleus, negatively impacted the translocation of proteins into the nucleus and export of mRNA from the nucleus (Woerner et al., 2016).

In yeast, the spatial sequestration into SPQC compartments becomes especially important when the Hsp70-machinery is limited (Ho et al., 2019). In cells lacking the disaggregase Hsp104 and the Hsp70 NEF Fes1, Hsp70 activities are reduced. The double mutant *hsp104*Δ *fes1*Δ has a synthetic sick genetic interaction with *BTN2*, and *HSP42* to a lesser extent. Hsp42 and Btn2 are important in cytosolic and nuclear

inclusion body formation and acts as sequestrases/aggregases in the respective compartments (for further discussion see below). Sequestration of misfolded proteins by Btn2 and Hsp42 becomes vital in preventing spatial proteostasis collapse in the *hsp104*Δ *fes1*Δ mutant, as deleting *HSP42*, *BTN2* or both in the double mutant leads to signs of uncontrolled protein aggregation. The authors of the study argue that the prevention of proteostasis collapse could work through the protective sequestration of misfolded proteins by Hsp42 and Btn2, which restricts interaction with Hsp70s and the misfolded proteins, ensuring that there is enough Hsp70 to go around for other basal Hsp70 activities such as folding of nascent proteins (Ho et al., 2019). In cells that lack Hsp42 but have an otherwise intact chaperone network, loss of Hsp42 actually increases lifespan (Saarikangas and Barral, 2015), which suggests that even though sequestration of misfolded proteins is necessary and cytoprotective during certain conditions, it is also an energy-consuming process that can have negative consequences.

Synthetic Genetic Arrays in yeast spatial PQC

S. cerevisiae is a well-established eukaryotic model organism that has been extensively used due to its ease of cultivation, genetic manipulation and high degree of homology to metazoan organisms, including mammals and humans. Studying PQC in yeast is made simpler by the availability of strain collections such as the yeast GFP clone collection (Huh et al., 2003) and the yeast deletion collection (Giaever et al., 2002). Such technological advances makes *S. cerevisiae* unique among eukaryotes and opens up for large-scale, genome wide screen experimental approaches such as the di- and trigenic Synthetic Genetic Array (SGA) analysis (Tong et al., 2001, Kuzmin et al., 2021) and its derivatives, which have been used to uncover new pathways in proteostasis ((see for ex. Paper I and (Liu et al., 2010, Song et al., 2014, Hill et al., 2016, Zheng et al., 2017)) and to create ground-breaking tools such as the CellMap (Costanzo et al., 2016). The SGA analysis is based on a haploid query strain that carries a deletion of a

gene of interest, and a haploid genome-wide single deletion array library. The query strain is mated to each strain in the library and the resultant diploids are sporulated and the double deletion haploid with the correct mating type is selected for. After that, the double deletion library can be analysed for growth during a wide range of conditions. In the traditional SGA analysis the primary measurement is colony size of the generated "double mutants" that reveal any genetic interactions, specifically so-termed synthetic lethal or sick genetic interactions where the combination of two viable single deletions yield a double mutant that is either inviable or sicker than expected from the sickness of either of the two single deletions on their own (Tong et al., 2001, Tong et al., 2004), see Paper I for an example). Further, the SGA methodology has been used to generate libraries where a fluorescently tagged version of one or several proteins, instead of a knockout, are systematically introduced into the single deletion library, which can be used in high content microscopy screening approaches to study the behaviour of proteins and/or organelles ((Vizeacoumar et al., 2010) for examples, see (Hill et al., 2016, Babazadeh et al., 2019).

Spatial PQC in the yeast cytosol

CytoQs – the first line of SPQC defence

When cells first encounter stress, particularly heat stress, the first visible phenotype is the appearance of many small, peripheral, dynamic protein foci which have been given many different names, including CytoQs, peripheral aggregates, stress foci and Q-bodies (Specht et al., 2011, Spokoini et al., 2012, Malinovska et al., 2012, Escusa-Toret et al., 2013, Miller et al., 2015a). These foci, here referred to as CytoQs, are typically formed by non-amyloidogenic misfolded proteins, and they coalesce over time to fewer, larger inclusion bodies (Paper II, (Specht et al., 2011, Escusa-Toret et al., 2013)). Many non-amyloidogenic misfolded proteins relies on the sHsp Hsp42 to form CytoQs. In the absence of Hsp42 cells mainly form nucleus-associated

inclusions, as demonstrated using both a thermolabile variant of Ubc9 (Ubc9^{ts}), VHL and Hsp104-GFP (Specht et al., 2011, Malinovska et al., 2012, Miller et al., 2015a). The sorting activity of Hsp42 to peripheral aggregates is regulated through Hsp42's N-terminal domain (NTD) and can't be supported by the other sHsp, Hsp26, in yeast cytosol. However, fusing the Hsp42 NTD to Hsp26 restores the peripheral aggregation phenotype in the absence of Hsp42 (Specht et al., 2011). The NTD domain of Hsp42 is unique in that it contains two intrinsically disordered domains (IDDs), one of which mediates the direct interaction between misfolded proteins and Hsp42, and is necessary to form CytoQs, and one regulatory IDD which interferes with the Hsp42:substrate interaction by a yet to be determined mechanism (Grousl et al., 2018). The requirement of Hsp42 for CytoQ formation is however not absolute and varies between substrate and stress treatment, as we have shown that loss of Hsp42 does not disrupt the initial CytoQ formation or clearance of the misfolding protein guk1-7-GFP after severe heat stress (Paper III), and even though CytoQ formation of Ubc9^{ts} is reported to be severely impacted in a *hsp42*Δ mutant, the formation is not completely abolished (Escusa-Toret et al., 2013). In conflict with Miller and colleagues (Miller et al., 2015a), Hsp104-GFP has also been reported to still form peripheral foci in the absence of Hsp42 (Babazadeh et al., 2019). Loss of Hsp42 does however lead to slower disaggregation of Hsp104-GFP foci during recovery after heat shock (Babazadeh et al., 2019). There is precedent for sHsp involvement in protein disaggregation, as sHsp have been shown to destabilise aggregates, mainly amyloid, and promote their resolution through the interaction with chaperone disaggregation machineries (Cashikar et al., 2005, Duennwald et al., 2012).

The dynamics of CytoQs are determined as much on their formation as on their coalescence and disaggregation, and this might provide a key to understanding the conflicting reports on the importance of Hsp42. The coalescence and disaggregation appears linked and the disaggregase Hsp104 is the an essential factor involved in this process

((Specht et al., 2011, Spokoini et al., 2012, Escusa-Toret et al., 2013, Miller et al., 2015a), Paper II and III). Disaggregation of inclusion bodies requires Hsp104 in concert with JDPs and Hsp70 ((Parsell et al., 1994, Glover and Lindquist, 1998, Lee et al., 2013, Mogk et al., 2015), Papers I-III). It has been suggested that the disaggregation activity of Hsp104 drives the merge of two CytoQs into one through the solubilisation of misfolded proteins from one CytoQ followed by their subsequent addition into the other by the action of Hsp42, which leads to the observed coalescence (Escusa-Toret et al., 2013). This model proposes a balancing act between Hsp42 and 104, where loss of one can tip the scale towards either aggregation or resolution, but might leave a window for either phenomenon to occur. This idea is strengthened by the fact that loss of both Hsp42 and 104 has no discernible effect on CytoQ formation, but the coalescence is disrupted, without the substrate degradation being affected (Escusa-Toret et al., 2013). This model of a tug-of-war between Hsp42 and 104 could also explain why the guk1-7-GFP behaves differently compared to Ubc9^{ts}, if this protein is less accessible to Hsp104 mediated disaggregation (Paper III).

Apart from just Hsp42 and Hsp140, there is an extensive chaperone network required for the efficient handling of CytoQs that cooperate to coalesce and ultimately resolve the inclusion bodies (Glover and Lindquist, 1998, Winkler et al., 2012). At the centre in this cooperative are the cytosolic Hsp70s, in yeast mainly the constitutively produced Ssa1 and Ssa2. Loss of both of these two proteins leads to the formation of one or a few large persistent inclusion bodies, often observed before stress (Shiber et al., 2013), instead of the multiple CytoQs found in wild type cells after stress. Ssa1 and 2 are also necessary for the coalescence of inclusion bodies and disaggregation/degradation of the misfolded proteins found therein ((Shiber et al., 2013, Escusa-Toret et al., 2013, Öling et al., 2014), Paper II), and Hsp104 is not recruited to protein aggregates without Ssa1 and/or 2 ((Glover and Lindquist, 1998, Winkler et al., 2012), Paper II). The formation of multiple CytoQs

and their coalescence into fewer inclusions can be rescued by increasing the level of heat stress induced Ssa4 in an *ssa1*Δ *ssa2*Δ background (Paper II). However, Hsp104 is still absent from these inclusions, which correlates to them persisting longer in the cell (see figure 5). The difference in Hsp104 interaction between Ssa4 and Ssa1 is tied to the NTD of Ssa1, as we have shown that exchanging the Ssa4 version of this domain with the one from Ssa1 promotes the Hsp104 recruitment to inclusion bodies by Ssa4 (Paper II). The amino acid residues on the NTD of Ssa1 that is important for the interaction with Hsp104 (Doyle et al., 2015) are conserved in Ssa4, which means that the source of the differential interaction is either other sites in the NTD domain, or could be coming from differing interactions with NEFs or other co-chaperones between the two different proteins (Paper II). The latter is supported by data from human cells, since even though there is high homology between human Hsp70 proteins, they display differences in their interaction partners, which in turn influence their interactions with substrate proteins (Serlidaki et al., 2020). Ssa4 is also reported to interact weakly with Ydj1 and the Hsp90 substrate v-Src, in comparison to Ssa2, which leads to a less efficient Hsp70-Hsp90 co-operation and folding of the substrate. The difference between Ssa2 and Ssa4 was traced in this case to the CTD of the Ssa proteins, as the Hsp90-dependent folding pathway could be supported by a Ssa4-Ssa2 hybrid protein with the CTD from Ssa2 and likewise disrupted by using a Ssa2-Ssa4 hybrid protein with the CTD from Ssa4 (Gaur et al., 2020). Further, the interaction differences between Ydj1 and Ssa2/4 stemmed from the NTD, with the NTD from Ssa4 mediating a weaker interaction with Ydj1 compared to the one from Ssa2 (Gaur et al., 2020).

Disaggregation of protein aggregates and inclusion bodies is not only mediated through JDP-Hsp70-104 activity, but can also be supported by the Hsp110 family of chaperones. The Hsp110 chaperones are nucleotide exchange factors to Hsp70s, but they also have a separate, true chaperone function that involves binding to misfolded proteins

which promote their disaggregation from aggregates (Shorter, 2011, Mattoo et al., 2013). Since there are no Hsp100-chaperones in metazoans, the JDP-Hsp70-110 disaggregation machinery have been presented as a possible metazoan counterpart to the bacterial, fungal and plant Hsp100-containing disaggregation complexes (Shorter, 2011, Rampelt et al., 2012, Mattoo et al., 2013, Nillegoda et al., 2015). The disaggregation activity of Hsp110 is conserved in yeast (Shorter, 2011, Kaimal et al., 2017, den Brave et al., 2020), and Hsp110 activity is necessary for Hsp104-dependent disaggregation, in a manner that is not dependent on Hsp110 ATPase activity, but rather its direct interaction with Hsp70 (Kaimal et al., 2017). In the process of CytoQ disaggregation, this becomes evident as loss of one of the two yeast Hsp110 chaperones, Sse1, leads to a decreased rate in CytoQ clearance as well as degradation of the misfolded protein substrate (Escusa-Toret et al., 2013).

In contrast to heat induced stress, H_2O_2-stress requires the peroxiredoxin Tsa1 in order for the JDP-Hsp70-Hsp104 machinery to recognise and localise to misfolded proteins (see figure 6). During oxidative stress, Tsa1 is found in an oligomeric complex catalysed by the sulfinylation of its catalytic cysteine, cysteine 48 (Jang et al., 2004), and in this state Tsa1 recognises aggregates and misfolded proteins ((Jang et al., 2004, Weids and Grant, 2014), Paper I). Sulfinylated, oligomeric Tsa1 also physically interacts with Ssa1, and this interaction is necessary during H_2O_2-stress for Hsp70 and Hsp104 to

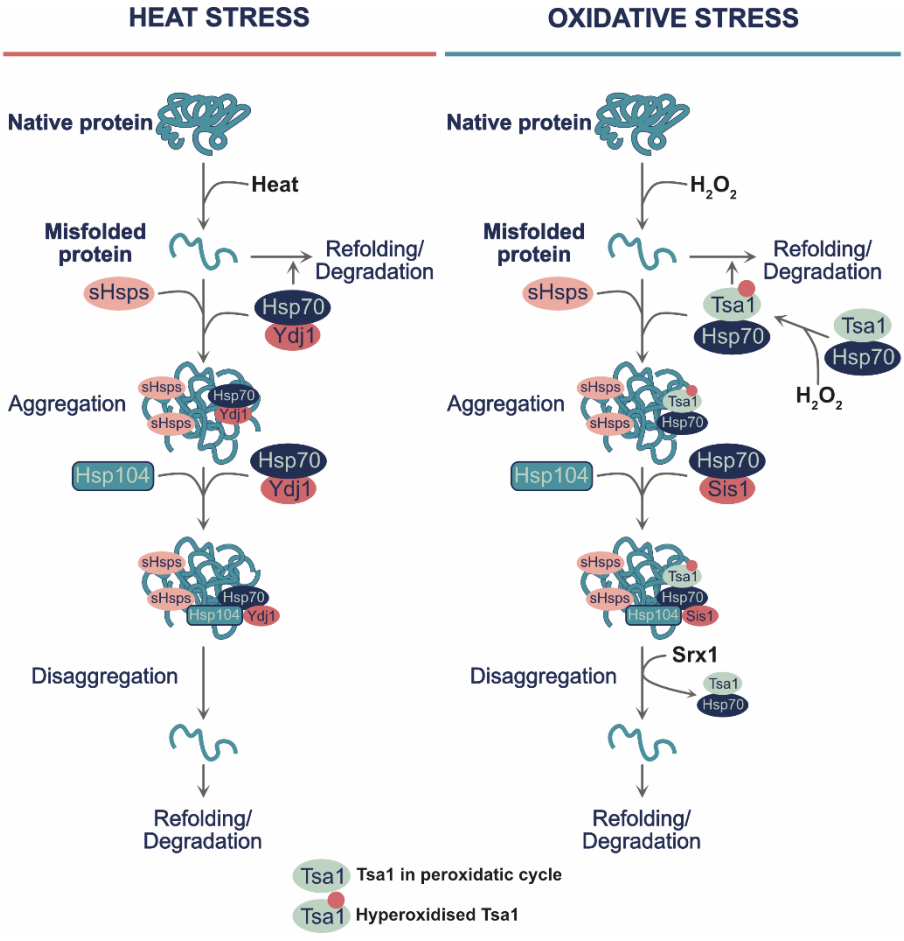

Figure 6: During heat stress (left), misfolded proteins are recognised by sHsps, Hsp70s and the JDP Ydj1 which can either promote the refolding or degradation of the protein, or sequester them in inclusion bodies. Hsp104 is recruited to the aggregate by Hsp70 and the aggregate can be resolved and the proteins either degraded or refolded. During oxidative stress (right), Tsa1 is hyperoxidised and acts as a molecular chaperone. Tsa1 is necessary for Hsp70s and Hsp104s to localise to misfolded proteins, and the primary JDP partner is not Ydj1 but rather Sis1. Resolution of protein aggregates requires Srx1 activity to reduce the hyperoxidised Tsa1. Modified from Paper I.

localise to misfolded proteins. Misfolded protein substrates and other chaperones, namely the sHsps Hsp26 and 42, still localise to inclusion bodies during H_2O_2-stress in the absence of sulfinylated Tsa1. The

disaggregation and resolution of the inclusion bodies formed by H_2O_2-stress are also tied to the redox cycle of Tsa1, as inclusion bodies persist in the absence of the Tsa1-dedicated sulfiredoxin Srx1, which reduces sulfinylated Tsa1 (Paper I). Heat and H_2O_2-stress also have different JDP co-chaperone requirements. During heat stress, Ydj1 is necessary for efficient disaggregation of inclusion bodies (Escusa-Toret et al., 2013), but during H_2O_2-stress Ydj1 is dispensable, with Sis1 fulfilling the JDP role (Paper I). There is evidence that type I JDP co-chaperones are sensitive to oxidative inactivation, and this might explain why Ydj1 does not participate in resolution of protein aggregates after H_2O_2-stress (Choi et al., 2006). Tsa1's role in PQC is further strengthened by the fact that *TSA1* has a synthetic sick genetic interaction with many chaperone genes, in particular JDP genes, suggesting that *TSA1* and Sis1 in a parallel pathway to these JDP chaperones.

The Insoluble PrOtein Deposit

One of the two inclusion bodies first described in budding yeast was the perivacuolar IPOD. Using a set of both amyloid and non-amyloid misfolding protein substrates, the IPOD was defined as the site where non-ubiquitinylated amyloid substrates where accumulated, in contrast to the JUNQ where ubiquitinylated protein substrates where deposited (Kaganovich et al., 2008). The partitioning between the two sites is changeable, as an increase in temperature can drive the JUNQ-exclusive substrate Von Hipple-Lindau (VHL) protein into both the IPOD and the JUNQ compartment. Inhibition of ubiquitinylation of JUNQ substrates lead to their accumulation at the IPOD, while enhancing the ubiquitinylation of the yeast prion Rnq1 by tagging it with a ubiquitinylation signal promote its deposit into JUNQ as well as in the IPOD. The difference in ubiquitinylation status of the substrates is also reflected in the co-localisation of the proteasome at the JUNQ (Kaganovich et al., 2008). Ubiquitinylation as a triage for misfolded proteins into either peripheral or juxtanuclear deposition sites is however not an absolute rule, as both ubiquitinylated and non-

ubiquitinylated non-amyloidogenic misfolded proteins can be found in both peripheral and juxtanuclear sites (Summers et al., 2013, Shiber et al., 2013). Many studies show that non-amyloidogenic misfolded proteins will pass through CytoQ inclusions to ultimately end up at perivacuolar inclusion sites, which are then identified as the IPOD. After oxidative stress, non-amyloidogenic proteins have been observed to co-localise with amyloidogenic proteins at the IPOD, which suggests that this is a common site for multiple protein substrates (Tyedmers et al., 2010b). There is however a distinction between non- and amyloidogenic protein substrates in targeting to the IPOD (Specht et al., 2011, Hill et al., 2016, Kumar et al., 2017) and the question remains whether or not they co-aggregate at the same site or if their sequestration is spatially distinct (Kryndushkin et al., 2012).

Amyloidogenic substrates that are targeted to the IPOD are modified by chaperones, with Hsp104 being especially important. Hsp104 localises to both the IPOD and JUNQ, and ectopically increased levels of Hsp104, as well as the Hsp70 Ssa1 and the JDP Sis1, disrupts the formation of single mHttQ inclusion bodies, which leads to multiple foci per cell and a larger fraction of soluble mHttQ found in protein extracts (Krobitsch and Lindquist, 2000). The ultimate localisation of amyloidogenic proteins is also modulated by the ribosomal quality control complex (RQC), as loss of the RQC-associated E3 ligase Ltn1 or Rqc1 (which recruits Ltn1 to stalled translation products), leads to the formation of intranuclear inclusion bodies of mHttQ. The accumulation observed in *ltn1*Δ cells is mediated through Rqc2 and transport of CAT-tailed peptides through the nucleopore. These nuclear inclusion bodies of mHttQ leads to an increase in cytotoxicity compared to sequestering mHttQ in the cytosolic IPOD (Zheng et al., 2017).

Spatial PQC in the yeast nucleus

Since its introduction, the actual localisation of the JUNQ (Kaganovich et al., 2008, Spokoini et al., 2012) has been suggested to be intranuclear

instead of juxtanuclear (Intranuclear Quality compartment; INQ) (Miller et al., 2015a). Whether or not the JUNQ is actually INQ or if INQ and JUNQ represents two distinct compartments is still an open, exciting question.

As mentioned before many, but not all, protein substrates that accumulate in JUNQ/INQ are ubiquitinylated, and there is an enrichment of proteasomes at the site (Kaganovich et al., 2008). By using $Ubc9^{ts}$ and Ssa2-GFP, it was demonstrated that clearance of JUNQ induced by heat shock was delayed in the absence of the deubiquitinase Ubp3, and overproduction of Ubp3 can counteract JUNQ formation in cells even in the absence of Ssa1 and 2 in a manner dependent on the proteasome (Öling et al., 2014).

SPQC in the yeast nucleus is characterised by Btn2 and Cur1, two protein paralogues that have been implicated in the inheritance and curing of prions (Wickner et al., 2014). Cur1 and Btn2, together with the Sis1 chaperone, are also important for the partitioning of non-amyloidogenic, misfolded proteins to peripheral and nuclear/juxtanuclear sites. In a model presented by the Alberti lab, Btn2 directs misfolded proteins to either a peripheral site, if in complex with Hsp42, or to the JUNQ site if in complex with Sis1. Sis1 is in turn transported into the JUNQ/nucleus together with Cur1, when not in complex with Btn2 and a misfolding protein substrate (Malinovska et al., 2012). The role of Btn2, Sis1 and Hsp42 has been further specified by others, with a notable difference that the JUNQ compartment is instead referred to as INQ (Miller et al., 2015a). Miller and colleagues suggest that the localisation of misfolded proteins to either the cytosol or the nucleus are not dependent upon Hsp42 or Btn2, but rather that their aggregation into inclusion bodies are. This notion was verified using both biochemical detection of insoluble proteins and the presence of inclusion bodies visualised by microscopy (Miller et al., 2015a). In this model, the intranuclear aggregation is dependent on Btn2, while cytosolic aggregation is

mediated by Hsp42, with both proteins being dubbed "compartment-specific aggregases" (Miller et al., 2015a, Miller et al., 2015b). However, the dependency of Btn2 and Cur1, just as with Hsp42, in substrate sorting and inclusion body formation is not universal for all substrates, as guk1-7-GFP does not rely on either of the proteins to form intranuclear foci (Paper II). However, *CUR1* and *BTN2* have an epistatic interaction in inclusion body clearance as loss of *CUR1* leads to a decrease in clearance rate and loss of *BTN2* leads to an increase in clearance rate, but the double mutant behaves like wild type cells (Paper III).

Further studies into Btn2 have revealed that this protein perform many of the activities that sHsps does in cytosolic PQC, as it has a holdase function *in vitro* and can aid Hsp70-mediated disaggregation of misfolded proteins (Ho et al., 2019). Btn2 is also structurally related to sHsps as it harbours an α-crystalline-like domain. Btn2 promotes the formation of INQ and interacts directly with Sis1 through its NTD, which is a necessary interaction in order for the JDP-Hsp70-100 disaggregase complex to be recruited to INQ. The NTD is however dispensable for the interaction with protein substrates and the formation of the INQ, but INQs formed with Btn2 lacking the NTD persists for longer in the cell. The C-terminal domain (CTD) of Btn2, and its central, α-crystalline-like domain, mediates the interaction with the misfolding protein (Ho et al., 2019).

The nucleolus as a spatial PQC site

The nucleolus has been implicated in PQC, and early observations show how Hsp70-proteins translocate from the nucleoplasm into the nucleolus during heat shock in metazoan cells (Welch and Feramisco, 1984). More recent findings have shown that this translocation of Hsp70s is part of a response to stress important for protein quality control in the nucleolus (Azkanaz et al., 2019, Mediani et al., 2019, Frottin et al., 2019), and it allows for unfolded proteins to translocate into the nucleolus during stress (Nollen et al., 2001). Upon heat stress,

misfolded proteins enter the nucleolus and interact with the nucleolar protein nucleophosmin, which acts as a scaffold for phase separation of the proteins. Transient heat shock triggers a phase transition to a more solid-like state in the nucleolus, which is tied to the presence of the misfolding proteins that adopts the same low mobility state (Frottin et al., 2019). This is however a reversible state, and Hsp70s can resolve these structures and refold the misfolded proteins upon recovery from stress (Nollen et al., 2001, Azkanaz et al., 2019, Mediani et al., 2019, Frottin et al., 2019). The reversible aggregation of misfolded proteins in the nucleolus has been suggested to occur within a subnucleolar structure called nucleolar amyloid bodies (NoABs), after an observation that the previously defined nucleolar aggresomes (NoAs) (Latonen et al., 2011) and nuclear amyloid bodies (A-bodies) (Audas et al., 2016) might actually be the same structure (Mediani et al., 2019). Different stressors can trigger the formation of NoABs and they contain both proteins and RNA (particularly mRNA and other polyadenosinylated RNA species) (Latonen et al., 2011, Mediani et al., 2019). The formation of NoABs have been proposed to be dependent upon the accumulation of aberrant polypeptides that arise from premature stops in translation (defective ribosomal products, DRiPs) inside the nucleolus, since inhibition of translation both stops the build-up of DRiPs and inhibits the formation of NoABs and the aggregation of other misfolded proteins inside the nucleolus after proteasomal or heat stress (Mediani et al., 2019).

Together with misfolding protein substrates, a wide variety of endogenous proteins also translocate to the nucleolus during heat stress in mammalian cells (Audas et al., 2016, Frottin et al., 2019), and while there are many differences in the yeast nucleolus compared to mammalian nucleoli, this phenomenon has also been observed in yeast (Gallardo et al., 2020). In *Schizosaccharomyces pombe*, a selection of nuclear proteins translocate to the nucleolus during heat stress, where they form a ring around the nucleolus. The main proteins involved in these nucleolar rings (NuRs) are nucleopore complex

proteins, particularly from the intranuclear basket subcomplexes, proteins involved in mRNA transport, cell cycle regulating proteins and chaperones (Gallardo et al., 2020). The release from the NuRs coincided with post-heat shock recovery and re-entry into the cell cycle, and required an active Hsf1-mediated heat shock response and the disaggregase Hsp104 (Gallardo et al., 2020). The budding yeast nucleolus has been shown to be enriched for proteins that aggregate into insoluble aggregates after a short, severe heat shock (Wallace et al., 2015) and similarly to the *S. pombe* NuRs, translocation and ring-formation around the nucleolus by chaperones have been observed with Ssa2, Apj1 and Sis1 ((Öling et al., 2014, Feder et al., 2020), Paper III), as well as misfolding proteins (Paper II and III). In the case of Sis1, this nucleolar segregation is thought to be a part of the heat shock response regulation, as the translocation of Sis1 from the nucleoplasm frees Hsf1 from Hsp70-mediated repression, which initiates the transcription of Hsf1-regulated genes (Feder et al., 2020). We have shown that misfolded proteins accumulate in this ring-shaped deposit when yeast cells are subjected to a short, severe heat stress, and have thus dubbed this formation as the Heat-stress Associated Nucleolar Deposit (HAND) (Paper II and III, see figure 7). A similar phenotype in *S. pombe* has also been observed (but not explored) with another thermolabile mutant of Guk1, guk1-9-GFP, and a mis-localised, misfolding mutant of Rho1 (Cabrera et al., 2020). The sequestration of the misfolding protein guk1-7-GFP (Khosrow-Khavar et al., 2012, Comyn et al., 2016) to the HAND requires the two constitutive Hsp70s Ssa1 and Ssa2, and cannot be supported by the heat-inducible Ssa4 even if the production of the protein is increased with the use of a strong promoter (Paper II). Other chaperones required for HAND formation are unknown, but the two nuclear-acting JDPs Sis1 and Apj1 are not necessary on their own, which either means that they have overlapping functions in this process or that neither is necessary (Paper III). Loss of Apj1 does however destabilise the HAND during recovery, but there are instead more cells with intranuclear inclusion

bodies in *apj1*Δ cells both before stress and after 40 minutes of recovery (Paper III).

As mentioned above, Btn2 and Cur1 are not needed to form intranuclear inclusions of guk1-7-GFP, but the loss of Cur1 leads to a stabilisation of the HAND (and other, mainly nuclear, inclusions) in post-heat shock recovery. Loss of both Cur1 and Btn2 has no effect on HAND stability. During recovery, the HAND is cleared from the cells, but will in some cases pass through a phase where the deposit forms a perinucleolar inclusion body (Paper III), that might be related to the INQ (Miller et al., 2015a). This would make the HAND into a type of nuclear CytoQ that can be either resolved or deposited into the INQ, much in the same way as CytoQs can be deposited into IPOD or IPOD-like inclusions in the cytosol. The clearance of guk1-7-GFP from the HAND and/or INQ is slower compared to CytoQs and other inclusion bodies in the cytosol, many of which appears to be close to mitochondria (Paper III). This has been observed before, as CytoQs close to mitochondria resolve faster compared to those that are distal to the organelle (Babazadeh et al., 2019). Just as with cytosolic inclusion bodies, Hsp104 is necessary to clear the HAND (Paper II and III), but there has been no observation of Hsp104 being physically located at this inclusion site after stress ((Feder et al., 2020), Paper II). The clearance of proteins from the HAND is influenced by to the substrate as well, as the pro3-1-GFP misfolding protein (Khosrow-Khavar et al., 2012) is cleared from almost all CytoQs and HANDs within 40 minutes (Paper III), while roughly 50 % of cells with guk1-7-GFP still have HAND inclusions at that time point (Paper II and III). The chaperones located at the HAND are also cleared from the HAND at different rates, with Ssa2 being removed at a faster rate compared to Sis1 (Paper III). The clearance of Ssa2 from the HAND is retarded in the absence of Ubp3, indicating that substrate deubiquitinylation and possibly proteasomal degradation is necessary for resolution of the HAND (Öling et al., 2014). Interestingly, disrupting LLPS processes by the use of the chemical 1,6-hexanediol (Kroschwald et al.,

2017), revealed that both the pro3-1 and guk1-7 proteins adopt a solid-like state when they are associated with inclusion bodies after heat stress, indicating that their solubility is not directly tied to the clearance rate. The chaperones at the HAND are however in a more liquid phase and sensitive to LLPS disruptions (Paper III), which has been observed in other cases of protein aggregation (Wallace et al., 2015, Klaips et al., 2020) and demonstrates a similarity between how non-toxic, non-amyloidogenic misfolding proteins and polyQ proteins interact with the chaperone machinery ((Klaips et al., 2020), Paper III).

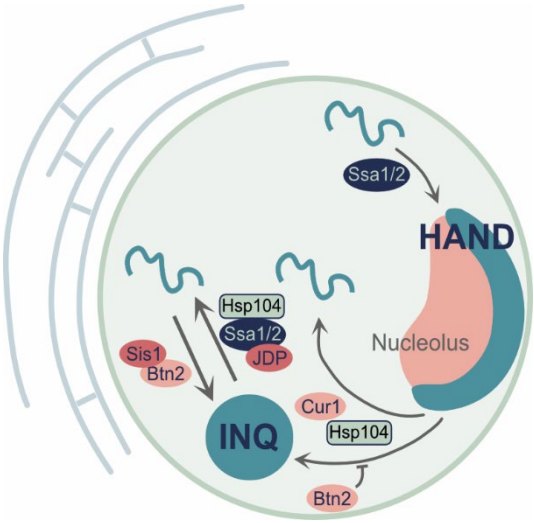

Figure 7: Inside the yeast nucleus the HAND can form around the nucleolus after a short heat stress. The HAND formation is dependent on Ssa1 and Ssa2, and its resolution is dependent on Hsp104. Btn2 and Cur1 have opposite effects on the HAND, where loss of Cur1 stabilises the HAND and loss of Btn2 promotes the clearance of all intranuclear aggregates. INQs formed either through the HAND or independently of it, can be cleared by the activity of Hsp70s, JDPs (Apj1 and Sis1) and Hsp104, alternatively Sse1.

Intracellular trafficking in yeast SPQC

Initial findings in the SPQC field showed that microtubuli depolymerisation lead to a failure in foci coalescence and IPOD and JUNQ formation in yeast, which mimics the findings from mammalian cells (Garcia-Mata et al., 1999, Johnston et al., 2002, Kaganovich et al., 2008). Defects in inclusion body formation was also demonstrated by prevention of actin polymerisation by the use of Latrunclin A (Specht

et al., 2011). The involvement of the tubulin and actin cytoskeletons are however points of contention as both the actin and the microtubuli networks have been reported as dispensable in foci formation in later studies. In the case of microtubuli, the effect of drug-induced MT depolymerisation on foci formation demonstrated by Kaganovich and colleagues (Kaganovich et al., 2008) can be traced to non-specific activities of the drug in use, as using a drug-insensitive mutant yeast strain yields the same results vis-à-vis the wild type (Specht et al., 2011). The effect of actin disruption is also contested from the initial findings (Escusa-Toret et al., 2013). One source of the confusion could be tied to the treatment used to study the process, as the study which have seen a reliance on the actin cytoskeleton have used both proteasome inhibition and heat stress (Specht et al., 2011), while the study that show no such connection only used heat stress (Escusa-Toret et al., 2013). Whether or not this demonstrates that there are different SPQC pathways in cells for when the proteasome is inhibited compared to when it is active is unanswered at this point. It is notable though that the formation of the aggresome in mammalian cells is always reliant on the MT network, regardless of proteasome status (Johnston et al., 1998, Garcia-Mata et al., 1999, Kawaguchi et al., 2003).

While the effect of direct disruption of the cytoskeleton remains unclear, there is compelling evidence that actin-based vesicle trafficking is a crucial component in protein aggregate sorting and coalescence of CytoQs into inclusion bodies (Hill et al., 2016, Babazadeh et al., 2019) as well as transport of amyloidogenic prion proteins into the IPOD (Kumar et al., 2016, Kumar et al., 2017). Hsp104 directly interacts with several proteins directly involved in vesicle trafficking, and boosting the protein levels of Vac17, a vacuole-specific myosin receptor, increases the percentage of cells with successful inclusion body formation after 90 minutes of heat stress and loss of Vac17 leads to the opposite phenotype (Hill et al., 2016). The Myo2-binding domain of Vac17 is necessary in order for Vac17 to support inclusion body formation. Communication with endocytic vesicle

trafficking is also important, which is mediated in part through partnership with Vps1, a direct Hsp104-interacting protein involved in vesicle fusion to the vacuole. The role of Vac17-mediated IPOD formation seems to be restricted to non-amyloidogenic proteins (Hill et al., 2016, Kumar et al., 2017), which strengthens the notion of different pathways depending on the nature of the misfolded protein being processed (Specht et al., 2011). However, the pathways do seem to converge on the actin cable network, as depletion of Myo2 or Tropomyosin 1 (Tpm1) by tagging either protein with an auxin-destabilised degradation tag, followed by auxin treatment, leads to the appearance of multiple aggregates of amyloidogenic protein substrates and a failure to deposit these substrate into the IPOD (Kumar et al., 2016). The dynamin Vps1, but not Vac8 or Vac17, is also necessary for the prion domain of the [*PSI*$^{+}$] prion to localise to the IPOD, which strengthens the idea that there are distinct pathways with shared commonalities for both amyloid and non-amyloid substrates in inclusion body formation (Kumar et al., 2017).

High-content microscopy screening for mutants that fail to coalesce Hsp104-GFP CytoQs into larger inclusions revealed that several vesicle transport complexes are critical for proper CytoQ handling, in particular ER-to-Golgi transport and tethering of the late endosome vesicles from the Golgi to the vacuole, in close proximity to mitochondria and vacuole-mitochondria contact sites. This transport pathway in turn is involving Myo2 through its actin-interacting activities (Babazadeh et al., 2019). By boosting ER-to-Golgi vesicle trafficking through increasing production of the essential t-SNARE syntaxin Sed5, both inclusion formation and clearance of Hsp104-GFP and guk1-7-GFP aggregates was increased beyond levels of the wild type. In contrast to the wild type, the clearance of inclusion bodies in Sed5-overproducing strains relied on vacuole-associated peptidases and not the 26S proteasome, and Sed5 promotes inclusion body formation close to mitochondria instead of JUNQ/INQ (Babazadeh et al., 2019). This suggests that there are two pathways for inclusion body

formation and clearance, one that is mediated through the proteasome and promotes inclusion body formation at JUNQ/INQ, and one pathway that is proteasome-independent that relies on vacuole-mediated degradation and inclusion body formation close to the mitochondria (Babazadeh et al., 2019). This might explain why there is a reliance on the actin network for aggregate handling when the proteasome is inhibited (Specht et al., 2011), but when the proteasome is active there is little to no effect on inclusion body formation by drug-induced actin depolymerisation (Escusa-Toret et al., 2013). How these potential pathways interact and communicate with each other to maintain proteostasis remains an open question.

An alternate, cytoskeleton independent route for inclusion body formation have been proposed to be meditated through the cortical ER. Disruptions in the cortical ER lead to the appearance of a $Ub9^{ts}$-GFP inclusion body already before stress and a decrease in degradation of the misfolded protein (Escusa-Toret et al., 2013). The association with the cortical ER is mediated through Ydj1 which is required for inclusion body formation at the ER (Escusa-Toret et al., 2013).

Asymmetric segregation of damaged proteins

Not sharing is caring

One important aspect of the connection between inclusion bodies and cellular trafficking pathways is that it underlies the asymmetrical segregation of damaged proteins, a process that causes the rejuvenation of one cell at the expense of the other during mitosis. Asymmetric segregation of protein damage during cell division was first observed in yeast (Aguilaniu et al., 2003), where it was found that during mitosis, mother cells retain oxidatively damaged proteins so that the progeny would be damage-free. In the short-lived *sir2*Δ mutant this process is disrupted, which suggests that damage retention by dividing cells contributes to the fitness of newly formed cells (Aguilaniu et al., 2003). Sir2-dependent asymmetry not only

promotes the unequal segregation of protein damage into the mother, but also the selective inheritance of factors needed for oxidative stress management into the daughter. This leads to an increase in stress–tolerance in the newly budded progeny, and even though the daughter inherits similar levels of ROS as the mother, this is quickly dealt with after cytokinesis in the daughter (Erjavec and Nyström, 2007).

Asymmetric inheritance of damaged proteins has since its discovery in yeast been observed in several metazoans, including mammalian species. In cultured human and hamster cell lines, the mHttQ protein is asymmetrically segregated during mitosis when incorporated into the aggresome. Cells that fail to include mHttQ into the aggresome were unable to progress through mitosis (Rujano et al., 2006). There is some evidence that this process is important *in vivo* in humans as well. Patients with spinocerebellar ataxia type 3 that produce a polyglutamine expanded version of ataxin-3 will accumulate misfolded polyglutamine-rich aggregates in the epithelial cells of the intestinal crypts, but the corresponding stem cells at the base of the crypts remain free of these inclusions. This suggests that the stem cells are able to pass on their inclusion bodies asymmetrically to the cells that commit to differentiation (Rujano et al., 2006). In *D. melanogaster*, the same process has been observed in the intestinal crypts, with the stem cells segregating damaged proteins (identified by their 2,-4-hydroxynonenal adducts) into the cells that later commit to differentiation into an epithelial cell, while the stem cell remains damage free (Bufalino et al., 2013). In other stem cell niches the process is the reverse, with both neural and germline stem cells retaining the damaged proteins in favour of the differentiating cells. This lead to the hypothesis that the cell with the shortest chronological lifespan will inherit protein damage, while the long lived cell will be formed damage free (Bufalino et al., 2013). Evidence that the inheritance of damaged proteins can alter cellular function can be seen in cancer cell lines that produce mHttQ, where the cells that inherit inclusions of

mHttQ have decreased proliferative capacity, but are more resistant to stress (Bufalino and van der Kooy, 2014). Human embryonic stem cells also segregate proteins destined for degradation asymmetrically during self-renewal divisions (Fuentealba et al., 2008), and during early differentiation in murine embryoid bodies asymmetric segregation of protein aggregates through direct interaction with vimentin is essential for cell survival (Pattabiraman et al., 2020).

Mechanisms behind asymmetric segregation

The asymmetric segregation of damaged proteins and aggregates into the mother has been proposed to be achieved by stochastic diffusion of aggregates, with the bud neck acting as a restriction point (Zhou et al., 2011). This model has been refuted by several studies that instead demonstrate that inclusion bodies and aggregates are not in fact diffusing freely, due to their extensive contact with organelles and the cytoskeleton (Liu et al., 2010, Liu et al., 2011, Spokoini et al., 2012, Zhou et al., 2014, Song et al., 2014, Hill et al., 2016) and because the implications in bud neck size that would allow for the failure of damage retention in certain mutants simply are not met (Song et al., 2014). Similar principles govern the asymmetric inheritance of aggregates in mammalian systems, where interactions between the aggregates and the intermediate filament vimentin ensure the asymmetric segregation of damage (Ogrodnik et al., 2014, Pattabiraman et al., 2020). The disaggregase Hsp104 is heavily implied in the asymmetric segregation of damaged proteins and inclusion bodies, with Hsp104 mediating connection between misfolded proteins and segregation machineries. As described above, generation of asymmetry involves the sirtuin Sir2, and interestingly the loss of asymmetry in *sir2*Δ mutant cells can be restored by overproduction of Hsp104, which also restores the short replicative lifespan of *sir2*Δ to wild type levels (Erjavec et al., 2007). Damage inheritance is also tied to the actin cytoskeleton, as depolymerisation leads to loss of damage asymmetry and damage retention in the daughter. This has a great impact on the fitness of daughters born to replicatively aged mothers,

where there is a build-up of carbonylated, damaged proteins, but no discernible effect on progeny from young mothers with low levels of damage (Aguilaniu et al., 2003, Erjavec et al., 2007). The connection between Sir2 and Hsp104-dependent aggregate asymmetry has been shown to be mediated by the actin cytoskeleton and the polarity machinery during budding. The polarisome is located at the bud tip and where it assembles actin generating a flow of actin cables in retrograde into the mother from the bud (Liu et al., 2010). Several of the genes involved in polarisome formation and polarity, such as *BNI1*, *BUD6* and *RHO3*, as well as conditional mutants of *ACT1* and *MYO2* all have synthetic sick genetic interactions with *SIR2* (Liu et al., 2010). Cells lacking Sir2 also have decreased actin folding capacity, which leads to a build-up of nascent, unfolded actin monomers. This can be traced to increased inhibitory acetylation of the CCT chaperonin complex that folds actin (Liu et al., 2010). By using continuous heat shock that allows for *de novo* formation of aggregates in the bud, it was shown that loss of polarisome proteins and destabilisation of Myo2 and Tpm1 lead to a failure to segregate Hsp104-GFP aggregates into the mother, while disruption of endocytic vesicle trafficking from the bud into the mother did not affect the damage asymmetry. Instead, Hsp104 was found to interact directly with cortical actin *in situ*, which lead to the suggestion that the retrograde flow of aggregates from the bud into the mother is mediated through direct interaction between Hsp104-bound aggregates and actin cables flowing into the mother from the polarisome (Liu et al., 2010). The direct contact between Hsp104, aggregates (in this case CytoQs formed by mHttQ) and actin cables have also been demonstrated by three dimensional structured illumination microscopy (Song et al., 2014), and Hsp104 and the actin-binding protein Abp140 (Liu et al., 2011). Hsp104 also has been suggested to play a direct role in stabilisation of polarisome proteins and through this mechanism support the retrograde flow of actin-interacting aggregates into the mother (Tessarz et al., 2009). The role

of Sir2 in establishment of aggregate asymmetry is further strengthened as many essential genes that show a synthetic sick interaction with *SIR2* also have defects in aggregate asymmetry visualised by Hsp104. The main essential genetic circuits included are involved in ER-to-Golgi transport pathways and actin-associated transport and stability (Song et al., 2014).

Asymmetry pathways involved in yeast converge on many of the same cellular processes (see figure 8). One example of this is the inheritance of mitochondria, which also relies on the actin cable network (Fehrenbacher et al., 2004). During budding, there is an asymmetry of mitochondrial function between mother and daughter cells, where mitochondria in the daughter are more reduced and produce less ROS compared to mitochondria in the mother (McFaline-Figueroa et al., 2011). When yeast divide, the mitochondria have to migrate into the daughter against the retrograde flow of actin from the bud tip. This is the basis for the selective inheritance of fitter mitochondria into the daughter since they exhibit a faster migration rate compared to dysfunctional mitochondria (Higuchi et al., 2013). Loss of Sir2 affects the mitochondrial inheritance, which is suggested to be mediated through the decrease in retrograde flow of actin from the polarisome and other actin-cable dysfunctions that *sir2*Δ mutant cells exhibit (Higuchi et al., 2013).

The attachment of aggregates and inclusion bodies to organelles is also an important part in establishing asymmetry and ensuring that damage is retained in the mother. Misfolded proteins either at the JUNQ or the IPOD are associated with the nuclear outer membrane and the vacuole respectively. Disrupting these inclusions by deleting *HSP104* leads to movement of smaller aggregates into the bud, but forcing the misfolding proteins to accumulate at the IPOD the damage asymmetry could be restored even in the absence of Hsp104 (Spokoini et al., 2012). The IPOD and JUNQ have also been reported to be located in close proximity to mitochondria and the ER (Kaganovich et al.,

2008, Zhou et al., 2014), and Myo2-regulated mitochondrial inheritance has been shown to impact inclusion body inheritance into the daughter (Böckler et al., 2017). The importance of the vacuole has been expanded upon in further studies. By performing a high content microscopy screen for single mutants that have defects in Hsp104-GFP aggregate inheritance, Hill and colleagues found that loss of key genes involved in vacuole inheritance lead to reduced retention of aggregates in the mother during mitosis (Hill et al., 2016). The inheritance complex involved consist of Vac17, Vac8, Myo2 and Act1, where Vac17 is the limiting factor due to its low abundance and its key position as an adaptor protein mediating the contact between the vacuole (through Vac8) and the actin cable (through Myo2). Just as with inclusion formation, Vac17 needs to bind to Myo2 in order to promote inclusion body retention in the mother, and the process also requires functional vesicle trafficking and fusion to the vacuole (Hill et al., 2016).

Diffusion barriers in the cortical ER have also been proposed to be involved in damage retention in the mother, which is mediated by the association of farnesylated Ydj1 and protein aggregates and supported by a diffusion barrier at the bud neck. Disrupting either the diffusion barrier or inhibiting the farnesylation (and thus cortical ER tethering of Ydj1) lead to daughter cells prematurely forming age-associated inclusion bodies visualised by Hsp104 (Saarikangas et al., 2017). An ER membrane-associated diffusion barrier have also been described in murine neural stem cells that controls the segregation of, amongst other things, damaged proteins during mitosis (Moore et al., 2015). The mitochondria, in close proximity to the ER, has also been proposed to underlie the inclusion body asymmetry in mother-daughter pairs. In this model, the aggregates interact with the mitochondria through the mitochondrial outer membrane protein Fis1, involved in mitochondrial fission, which is necessary to prevent inheritance of the aggregates into the bud. The association between the mitochondria and the inclusion bodies also decreases with age, which

lead to an increase in inheritance of aggregates into the bud (Zhou et al., 2014).

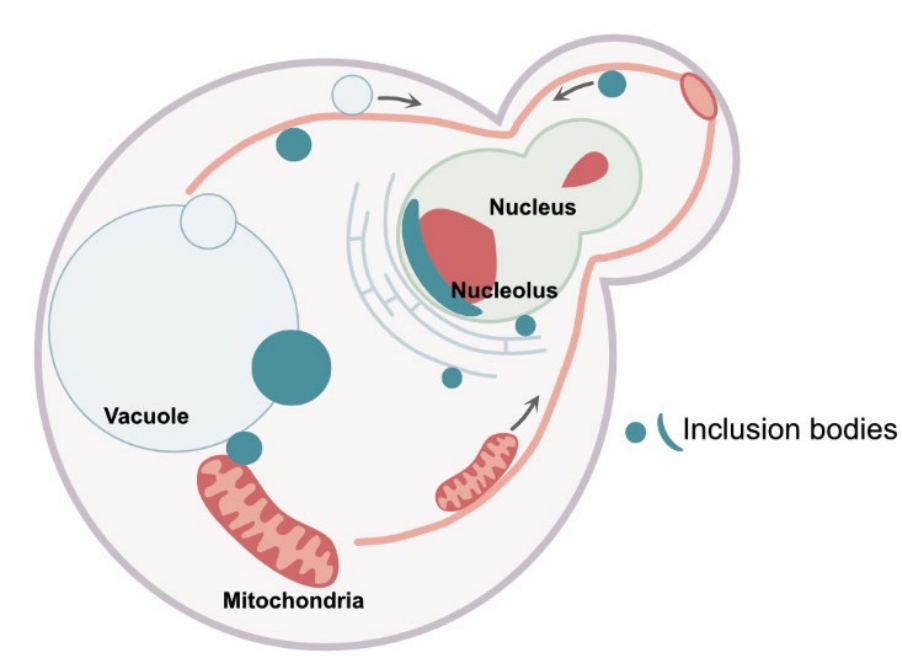

Figure 8: Asymmetric inheritance of damaged proteins and inclusion bodies is mediated by attachment to organelles, such as the vacuole, mitochondria, the ER and the nuclear membrane. Aggregates that form inside the bud move along the actin cables that flow from the bud tip into the mother, while organelles such as mitochondria and vacuoles move against the retrograde flow. Inside the nucleus, damaged proteins are retained in the mother in the HAND associated to the nucleolus.

The suggested mechanisms for how protein damage asymmetry is achieved during mitosis have thus far been centred on cytoplasmic aggregates and pathways. Budding yeast has a closed mitosis with an intact nuclear envelope, which means that any aggregates present in the nucleus during division cannot be regulated in the same way as cytoplasmic aggregates. There are however defined pathways for asymmetric segregation for non-protein damage. Nuclear aging factors, such as ERCs (Sinclair and Guarente, 1997, Park et al., 1999), are also inherited asymmetrically into the mother during mitosis

(Shcheprova et al., 2008). The inheritance is potentially established by anchoring the ERC to the nuclear pore through the nuclear basket proteins Mlp1 and 2, as demonstrated with artificial ERC-like plasmid constructs (Shcheprova et al., 2008). During anaphase the nuclear pore complexes are retained in the mother through a diffusion barrier established by the septin ring at the bud neck, and disrupting this diffusion barrier by deleting key septin genes leads to ERC inheritance by the daughter. The lack of a diffusion barrier disrupts the rejuvenation usually seen by the daughter, as progeny produced later in life by a mother had a shorter lifespan than progeny produced during the first few divisions (Shcheprova et al., 2008). This model relies on data that show that daughters do not inherit nuclear pores from the mother but that they are rather inserted *de novo* into the nuclear membrane of the daughter (Shcheprova et al., 2008). This is however contested, as other have shown that nucleopores can be inherited into the daughter from the mother, which means that mechanisms for ERC inheritance still has many unanswered questions (Khmelinskii et al., 2010).

We have shown that the nucleolar-associated HANDs formed during severe heat stress is retained in the mother during cell division, in a process different from those previously described for yeast (Paper III). While loss of *HSP104* leads to a disruption in guk1-7-GFP CytoQ handling (Paper II and III) and an increase in protein aggregate inheritance into the daughter (Tessarz et al., 2009, Liu et al., 2010, Hill et al., 2016), the HAND is still preferentially inherited by the mother when the nucleolus segregates into the bud. Loss of *SIR2*, important for both ERC (Shcheprova et al., 2008) and protein damage asymmetry (Aguilaniu et al., 2003, Liu et al., 2010, Song et al., 2014, Erjavec and Nyström, 2007, Erjavec et al., 2007), also does not perturb the asymmetric inheritance of the HAND. Disrupting the nucleopore diffusion barrier by deleting *BUD6* or *SHS1* (Shcheprova et al., 2008) had no effect on nucleolus-associated aggregate inheritance, but the vast majority of cells lacking either Shs1 or Bud6 did not divide while

the HAND structure was present which suggest that there might be a link between progression through the cell cycle and nuclear aggregate management. It could also be related to an increase in the delay in cell cycle resumption after heat shock in these strains that allow the cell to clear the HAND from the cell prior to entering telophase (Paper III). Exactly how protein damage asymmetry linked to the nucleolus is established remains an open question.

Spatial PQC in yeast at the end of life

As mentioned before there is an apparent failure of the proteostasis network with advanced age, which is one of the "hallmarks of ageing" as defined by López-Otín and colleagues (López-Otín et al., 2013, Janssens and Veenhoff, 2016). SPQC systems are affected by the observed proteostasis failure with increasing age, which becomes apparent in the loss of protein damage asymmetry during budding of old mother cells (Aguilaniu et al., 2003, Erjavec et al., 2007, Zhou et al., 2014), and in the fact that boosting SPQC systems, by for example overproduction of the vacuole inheritance factor Vac17, can prolong lifespan beyond that of wild type cells (Hill et al., 2016). In addition, as cells age, the tethering of inclusion bodies to organelles such as mitochondria decreases, which can explain a potential route that protein damage can leak into the daughter cell and disrupt the rejuvenation process (Zhou et al., 2014). Organellar functions are also compromised as cells age, which can negatively impact proteostasis. An example of this is the loss of vacuolar acidity that occurs during ageing in yeast mother cells, that negatively impacts mitochondrial membrane potential and mitochondrial function (Hughes and Gottschling, 2012). The loss of vacuolar pH is tied to the asymmetric inheritance of a cell membrane proton pump that pumps protons out from the cell, which limits the acidification of the vacuole in the mother (Henderson et al., 2014). The decrease in vacuolar acidity in turn affects the storage of nutrients and amino acids in the vacuole,

which contributes to observed mitochondrial defects (Hughes and Gottschling, 2012).

Loss of Ssa1 and Ssa2, the two constitutive cytosolic Hsp70s in yeast, leads to a markedly reduced replicative lifespan ((Craig and Jacobsen, 1984, Werner-Washburne et al., 1987, Öling et al., 2014), Paper II), which can be restored by over production of the Ssa1/2 heat-inducible orthologue Ssa4 (Paper II). We show that Ssa4 restores many of the necessary PQC functions that are impaired in the *ssa1*Δ *ssa2*Δ mutant, such as degradation and/or refolding of misfolding proteins and prevention of build-up of insoluble proteins. Increased production of Ssa4 can also support the coalescence of CytoQs into fewer inclusion bodies in the absence of Ssa1/2, but there is a failure in the clearance of inclusion bodies, and a higher prevalence of cells with one guk1-7-GFP inclusion body already before stress, compared to wild type. Since Ssa4 overproduction fully restores lifespan in the *ssa1*Δ *ssa2*Δ mutant strain, but not all proteostasis functions of Ssa1 and Ssa2, we suggest that not all Ssa1/2 activities are necessary for longevity. Mainly, we suggest that the disaggregation and clearance of inclusion bodies is dispensable for a full lifespan (Paper II).

During ageing, oxidatively damaged proteins build up inside the cell (Aguilaniu et al., 2003, Erjavec and Nyström, 2007, Erjavec et al., 2007) and an increase in ROS production can lead to a decrease in replicative lifespan (Heeren et al., 2004), which highlights the role that oxidative processes play in the ageing process. In line with this, overproduction of the peroxiredoxin Tsa1 leads to an increase in the replicative lifespan of yeast (Paper I). Tsa1 has several functions related to DNA stability and damage repair (Huang and Kolodner, 2005), but we show that overproduction of Tsa1 has no positive impact of any of those processes and there is no difference in H_2O_2-scavanging in the Tsa1 overproduction strain, which suggests that the increase in replicative lifespan in this strain is mediated through a different pathway (Paper I). As described before, Tsa1 is required for Hsp70 and Hsp104

chaperones to localise to inclusion bodies formed by sHsps and damaged proteins during H_2O_2-induced stress. Inclusion bodies that form during ageing are similarly dependent on Tsa1 for the proper chaperone recruitment, and loss of Tsa1 leads to an accumulation of ubiquitinylated proteins in the insoluble cellular fraction as well as an increase in cells with foci of the misfolding protein ΔssCPY*-GFP. In turn, overproduction of Tsa1 decreases the number of cells with foci of both Hsp104-GFP and ΔssCPY*-GFP in aged cells, indicating that Tsa1 positively affects PQC pathways late in life. The lifespan extension seen in the Tsa1 overproducing strain is furthermore dependent on a functional UPS-system, Ssa1 and Ssa2, and the PQC-linked ageing pathway dependent on Sir2, which indicates that Tsa1's role in prolonging lifespan is through promoting a functional proteostasis network (Paper I). However, lifespan extension through Tsa1 overproduction does not only act through proteostasis, but also by direct inactivation of nutrient signalling through the Ras-PKA pathway, which highlights an interesting connection between metabolism and proteostasis in the ageing process (Roger et al., 2020).

Early in replicative ageing, cells form an Hsp42/Hsp70/Hsp104-positive focus that is separate from the JUNQ/INQ, IPOD and P-bodies; the so-called Age-associated PrOtein Deposit (APOD) (Saarikangas and Barral, 2015). The APOD forms early after the first few cell divisions in the mother cell, and persists through the entire life of the cell. Cells that have formed the APOD will retain it after being subjected to heat stress, but there is no apparent delay in overall CytoQ clearance. Neither is the UPS system impaired in young to middle-aged cells that have an APOD inclusion; instead they degrade cytosolic proteasomal substrates at a faster rate compared to cells without an APOD (Saarikangas and Barral, 2015). In accordance with how CytoQ formation and clearance is balanced between the aggregating actions of Hsp42 and the disaggregating action of Hsp104 (Escusa-Toret et al., 2013), so too is the formation of the APOD regulated, where a loss of Hsp42 abolishes the formation of the APOD,

and a loss of Hsp104 (or either of Ssa1 or Ssa2) leads to a premature formation of the site. In line with this, overproduction of Hsp42 also leads to a premature formation of APOD while overproduction of Hsp104 abolishes its formation. The APOD is stringently inherited by the mother cell during mitosis, but abolishing the APOD by deleting *HSP42* leads to an increase in lifespan compared to the wild type (Saarikangas and Barral, 2015). Inducing the APOD by deleting *HSP104* shortens lifespan (Erjavec et al., 2007, Saarikangas and Barral, 2015), but does not lead to asymmetry defects of the APOD during mitosis (Saarikangas and Barral, 2015), in contrast to what has been reported for other types of inclusion bodies (Erjavec et al., 2007, Liu et al., 2010, Spokoini et al., 2012). In light of this, the authors suggest that the APOD is both an ageing marker and the basis for asymmetric damage segregation during ageing, but that it might also induce ageing in the mother cell (Saarikangas and Barral, 2015).

Thesis summary

Main findings

Paper I

- Increased production of Tsa1 can prolong the replicative life span of yeast in a mechanism that is proteostasis-dependent but separate from Tsa1's activities in maintaining DNA stability and H_2O_2-scavenging
- Tsa1 is necessary in order for Hsp70s and Hsp104 to localise to H_2O_2-induced aggregates, but not aggregates formed by heat stress
- Hyperoxidation and a shift to the oligomeric chaperone-form of Tsa1 is necessary for Tsa1 to support its function in proteostasis, and the resolution of inclusion bodies is linked to the reduction of sulfinylated Tsa1 by Srx1
- Protein aggregation during ageing also requires Tsa1 for the proper chaperone recruitment, and Tsa1 overproduction can decrease aggregate formation in aged cells

Paper II

- Ssa4 can support many, but not all, vital Hsp70 functions in a strain lacking the constitutive Ssa proteins Ssa1 and 2. The supported functions include protein degradation, prevention of aggregation of misfolded proteins and the formation of inclusion bodies during recovery after stress
- Ssa4 cannot recruit Hsp104 efficiently to aggregates after heat stress, does not restore the nucleolus-associated aggregation site, and does not support efficient clearance of aggregates and inclusion bodies after stress
- Overproduction of Ssa4 can restore the lifespan of short-lived *ssa1*Δ *ssa2*Δ cells, suggesting that clearance of protein

aggregates is not necessary to support a full life span if the aggregates can be handled correctly

Paper III

- Chaperones and misfolding proteins accumulate in a semi-to-full circle around the nucleolus after a short severe heat shock, which we nicknamed the HAND (Heat stress Associated Nucleolar protein Deposit)
- We have developed an automated image analysis tool for ImageJ that can identify the HAND in microscopy images
- The formation of the HAND is regulated in a different manner compared to previously described intranuclear SPQC sites such as INQ
- The HAND is retained in the mother during budding in a novel damage asymmetry pathway

Concluding remarks and discussion

Chaperone networks remain at the forefront of cellular self-protection, and need to be adaptable since different types of stress damage the cell in different ways. At the centre of these networks are the Hsp70 chaperones, who together with their many interaction partners can support proteostasis under a variety of conditions. In my work I have explored two levels of functional variability in the Hsp70 network mediated by interaction partners (**Paper I**) and by variability between Hsp70 isoforms (**Paper II**).

Chaperone pathways that promote fitness after oxidative stress and ageing are different from those that operate during heat stress. We have shown that the peroxiredoxin Tsa1 becomes essential in order for Hsp70s and Hsp104 to be recruited to misfolded proteins and inclusion bodies after hydrogen peroxide treatment and during ageing (**Paper I**). Increasing the levels of Tsa1 boosts proteostasis in aged cells and prolong yeast lifespan, in a manner that is dependent upon the

cytosolic Hsp70s Sssa1 and 2, but also on the proteostasis pathways supported by the sirtuin Sir2 (**Paper I**). Interestingly, other research into Tsa1-supported lifespan extension have revealed that Tsa1 also prolongs lifespan through mechanisms not directly related to proteostasis, but instead through H_2O_2-signalling in the Ras-PKA pathway (Roger et al., 2020), and Tsa1 is necessary for lifespan extension through caloric restriction (Molin et al., 2011). This ties multiple aging pathways together and demonstrates both how complex ageing is, but also how the pathways and tools that can prolong lifespan are equally complex.

While the short-lived phenotype of *ssa1*Δ *ssa2*Δ has been known for several decades, the reason why the two remaining Ssa proteins fail to support a full lifespan has not been understood. In **Paper II** we approached this question, and could demonstrate that Ssa4 can actually support a full lifespan, if the levels of the protein is increased. Not all Ssa1/2 functions could be restored through Ssa4 overproduction however, such as the recruitment of Hsp104 to inclusion bodies and their efficient resolution. This data suggests that the presence of protein inclusion bodies is not necessarily detrimental to a full lifespan (**Paper II**).

As Ssa4-mediated functions are enough to support a full lifespan, it would be interesting to see if Tsa1 can mediate lifespan extension in the *ssa1*Δ *ssa2*Δ strain with Ssa4 overproduction, or if Tsa1 exclusively supports Ssa1/2 functions (**Paper I and II**). In the same vein, it would be interesting to explore the larger chaperone networks that supports lifespan restoration by Ssa4 overproduction, in particular the NEF and JDP co-chaperones that regulate Hsp70 activities allosterically, since there are known differences in chaperone partners between the Ssa isoforms, and mammalian Hsp70 isoforms as well (Serlidaki et al., 2020, Gaur et al., 2020).

In **Paper III** I present work with the identification and initial characterisation of the nucleolar-associated site for misfolded

proteins, the HAND, which we identified as an Ssa1/2-dependent inclusion body in **Paper II**. Many interesting questions remain about this site, for example: what is the involvement of the nucleolus itself in the HAND formation and function? What Hsp70 chaperone partners supports its formation, and what is the fate of protein substrates that localise to the site? The nucleolus is an important PQC site in mammalian systems, and understanding the dynamics of this site in yeast might contribute to an increased understating of how fitness can be maintained in the face of proteotoxic stress sin humans and animals.

Furthermore, the asymmetric segregation of the HAND during cell division appears to be controlled by hitherto undefined asymmetry mechanisms. Hopefully the image analysis program developed in this project, coupled with genetic screening technologies will be a great help in further characterising the site, which can further our understanding in how cellular rejuvenation and ageing is maintained in yeast (**Paper III**).

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