Thesis for the Degree of Doctor of Philosophy

On the Role of Actin in Yeast Protein Quality Control

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Cover picture:

Fluorescent microscopy images of yeast cells at different stages of the cell cycle expressing the aggregation-prone disease protein Htt103QP-GFP. F-actin structures are visualized by Rhodamine-phalloidin staining.

Picture taken and edited by: Lisa Larsson Berglund

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To my family and friends

Abstract

Every cell is equipped with a protein quality control system to ensure the proper function of proteins. This is essential for both cell maintenance and the generation of new and healthy cells. In this thesis, the budding yeast *Saccharomyces cerevisiae* is used as a model to study both spatial quality control and the management of the protein involved in Huntington's disease. The role of the actin cytoskeleton in both these processes has been the special focus of the thesis.

Earlier studies established a role for the histone deacetylase Sir2 and the actin cytoskeleton in the asymmetrical inheritance of damaged proteins by the mother cell, as cells either lacking *SIR2* or subjected to a transient collapse of the actin cytoskeleton, fail in this segregation process. In this thesis the protein disaggregase Hsp104, the polarisome complex, and the molecular chaperone CCT were identified as additional factors having important functions in the asymmetric segregation of damaged proteins. CCT is an essential, cytosolic folding machine, vital for the production of native actin. The actin folding capacity of CCT appears to be regulated by Sir2. Without this regulation the cell suffers from a reduction in native actin molecules, which could affect the integrity of actin cytoskeletal structures. The polarisome complex ensures actin polymerization at the bud tip and the establishment of a retrograde actin cable flow from the bud to the mother. Our data show that the presence of a functional actin cytoskeleton allows for Hsp104, associated with protein aggregates, to use the actin cytoskeleton as a scaffold and prevent the inheritance of damaged and aggregated proteins by the daughter. The retention of damaged protein within the mother cell is important for the rejuvenation of the daughter cell, as a daughter being born with increased damage suffer from a reduced life span.

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Keywords: Protein quality control, protein aggregate, segregation, polarisome, CCT, Huntingtin, *text removed from public version*

Abbreviations

ARS	Autonomously replicating sequence
Cc	Critical concentration
CCT	Chaperonin containing TCP-1
CME	Clathrin-mediated endocytosis
DUB	De-ubiquitinating enzyme
ERC	Extra chromosomal rDNA circle
HD	Huntington's disease
HSP	Heat shock protein
Htt	Huntingtin
IB	Inclusion body
INQ	Intranuclear quality control compartment
IPOD	Insoluble protein deposit
JUNQ	Juxtanuclear quality control compartment
MTOC	Microtubule organizing center
NAC	Nascent chain-associated complex
NEF	Nucleotide exchange factor
NES	Nuclear exportation sequence
NPC	Nuclear pore complex
PrD	Prion domain
PRD	Proline-rich domain
PolyQ	Polyglutamine
RACF	Retrograde actin cable flow
ROS	Reactive oxygen species
PQC	Protein quality control
RAC	Ribosome-associated complex
sHsp	Small heat shock protein

SIM	Structured illumination microscopy
SPB	Spindle pole body
ts	Temperature sensitive
UPS	Ubiquitin proteasome system

Papers included in this thesis:

I. Erjavec N, **Larsson L**, Grantham J, Nyström T (2007) Accelerated aging and failure to segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein aggregation-remodeling factor Hsp104p. *Genes & Development* **21**: 2410-21.

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Introduction and aim of the thesis

Protein homeostasis refers to cellular processes that ensure a healthy and functional proteome. Within the cell, there is a highly interconnected network of components monitoring the proteome and promoting proper folding, translocation, and clearance of proteins. This network is called the protein quality control (PQC) system and includes molecular chaperones, proteasomes, and autophagic clearance mechanisms. The PQC system is designed to supervise proteins in the cellular environment and coordinate relief efforts upon conditions of stress. Too much stress will overwhelm the PQC systems and result in the accumulation of misfolded proteins and an increased risk of protein aggregation. Uncontrolled protein aggregation and misfolding have been linked to a family of diseases called protein misfolding or protein conformation diseases, which include Alzheimer's disease, Parkinson's disease, and Huntington's disease. To be able to treat these diseases it is important to understand the molecular mechanisms causing the disease. Yeast has proven to be an excellent model organism to study eukaryotic cellular mechanisms and even protein misfolding causing human protein conformational diseases. The aim of this thesis was to elucidate the role of the actin cytoskeleton in the cell's effort to manage protein damage. I have studied the process which promotes the progeny being born with an undamaged proteome, namely the asymmetrical inheritance of damaged proteins during cytokinesis. I have also studied the role of actin-dependent processes in the suppression of inherent toxicity of wild type huntingtin, a protein that in its mutant form is causing Huntington's disease.

Protein quality control

Protein folding

All living cells share the basic properties of keeping the outside out, importing and exporting substances, staying healthy, and reproducing. These processes are made possible by specialized functions performed by a wide repertoire of proteins encoded by genes within the DNA of the cell. The path from a gene within the genome to a functional protein includes various steps which are tightly regulated. The gene will first be transcribed into an RNA molecule which subsequently will be translated by a ribosome into a protein, a polypeptide chain consisting of different amino acids. For the protein to become functional it needs to fold

into a specific three-dimensional structure called its native state. The information for this fold is contained within the amino acid sequence, as certain amino acids are hydrophobic and others are hydrophilic. Since the environment of the interior of the cell is hydrophilic, hydrophobic modules of proteins will interact with each other and become buried inside the folded protein whereas the hydrophilic parts will locate on the surface, exposed to the cytosol of the cell. In a test tube, with appropriate folding conditions and no interfering components, this folding will occur spontaneously (Anfinsen 1973), at least for small globular proteins. However, the cytosol of the cell is very different from a test tube. In the cytosol a protein is exposed to an extremely crowded environment with a macromolecule concentration of 200-400 mg/ml (Ellis 1997). This will dramatically increase the risk of incorrect and potentially harmful hydrophobic interactions occurring within and between proteins as they emerge from the ribosome. To avoid this, the cell has specialized proteins called molecular chaperones that assist the nascent polypeptide on its way to the correct native fold. There are several types of molecular chaperones and they often work together to ensure proper protein folding (figure 1). Molecular chaperones not only aid newly translated proteins, they also assist in the re-folding of proteins that have lost their native state due to different types of stress, the assembly and disassembly of multimeric protein complexes, the translocation of proteins into various organelles, and the degradation of proteins. It is of great importance to understand the molecular mechanisms behind the maintenance system that keeps the proteome functional in order to design and develop medical treatments for diseases caused by protein misfolding and aggregation.

Chaperone systems

A molecular chaperone can be defined as a protein that interacts with, stabilizes or assists a non-native protein on the route towards the native structure whilst not being a part of the correctly folded end product (Hartl and Hayer-Hartl 2009). There is a wide repertoire of molecular chaperones within the cell and they are often categorized according to their molecular weight. Many of them are also called Heat Shock Proteins (HSPs) as their expression is upregulated when the cell is subjected to different stressors, such as heat. As soon as a polypeptide is emerging from the ribosome it encounters the risk of improper hydrophobic interactions. The cell has evolved a system where the ribosome-associated complex (RAC), a heterodimer of Hsp70 and Hsp40 family members, together with nascent chain-associated complex (NAC), bind co-translationally to hydrophobic-rich sequences of

the forming polypeptide to stabilize it. When translation is completed the protein is released from the ribosome and the NAC/RAC complex where after the classic, non-ribosome-coupled, cytosolic Hsp70 chaperones and co-chaperone Hsp40 take over in the folding process (figure 1) (Hartl and Hayer-Hartl 2002).

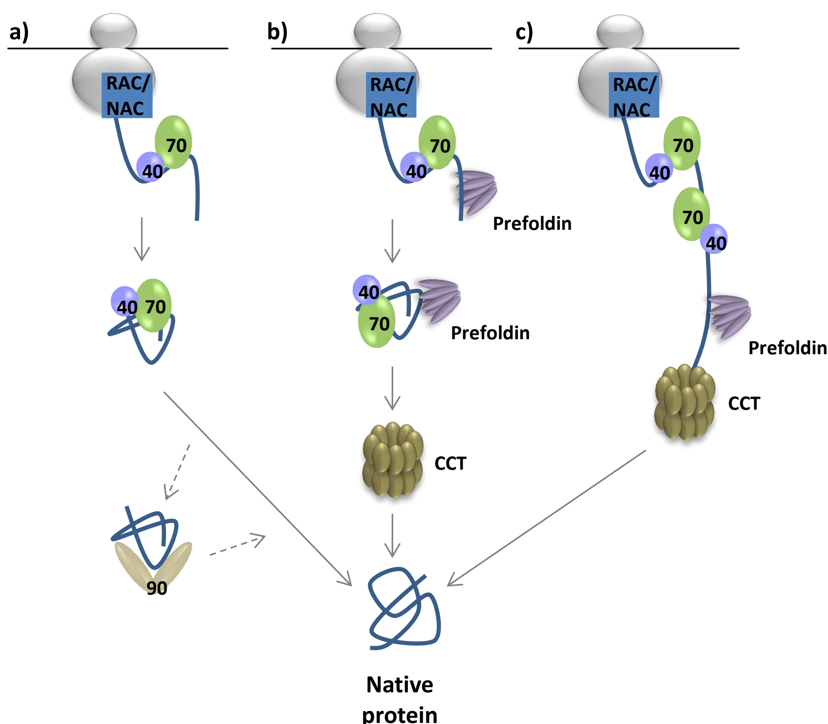

Figure 1. Chaperone-assisted protein folding in the cytosol of eukaryotic cells. The nascent polypeptide needs to fold into its native state to become a functional protein. Different molecular chaperones assist in this process either co- or post-translationally, indicated by the different folding pathways a), b) and c). The ribosome-bound chaperone complex RAC/NAC, binds and inhibits premature misfolding of the nascent chain as it emerges from the ribosome. When translation is completed, the cytosolic Hsp70/40 chaperone system takes over. A subset of proteins reach their native state by only interacting with Hsp70/40 chaperones, and a small portion needs the additional assistance from Hsp90 for correct folding (a). Some proteins need the help from the chaperonin CCT to become native. In this case, Hsp70/40 and the prefoldin complex deliver the non-native protein to CCT for final folding which can occur post-translationally (b) or co-translationally (c).

Hsp70 system

Almost all cells, with the exception of certain archaea, contain both constitutively expressed and stress induced Hsp70 chaperones. These Hsp70 chaperones consist of an N-terminal ATP binding domain and a C-terminal substrate binding domain (Zhu et al. 1996). Together with their co-chaperones Hsp40, also called J-domain proteins, and various nucleotide exchange factors (NEFs), they perform a wide variety of functions in the cell (Hartl and Hayer-Hartl 2002). These include the folding and assembly of newly synthesized proteins, re-folding of misfolded and aggregated proteins, membrane translocation of proteins destined for organelles or secretion, and regulation of protein activity (Mayer and Bukau 2005). It is with the help of a large subset of different Hsp40 co-chaperones that the Hsp70 proteins are guided to their different tasks. Different Hsp40 proteins can specifically bind different target proteins and in this way recruit Hsp70 to its proper destination (Kelley 1998). Hsp70 passively aids in the folding process by transiently binding hydrophobic segments of unfolded polypeptides to prevent unwanted interactions and aggregation of the substrate. Upon release from Hsp70, the substrate is allowed to try to fold. Several rounds of Hsp70 binding and release may be needed. This way of folding will work for proteins or protein domains that are so called "fast-folding". If the protein requires a longer time to fold, or if the protein needs help to overcome a high energetic barrier in its folding path, the Hsp70 chaperone can deliver a subset of substrates to another family of chaperones, the chaperonins (figure 1), which will assist the proteins to reach their native fold (Langer et al. 1992; Kerner et al. 2005).

Chaperonins

Chaperonins are essential folding machines (Fayet et al. 1989; Stoldt et al. 1996; McLennan and Masters 1998) that can be divided into type I and type II (Horwich et al. 2007). The type I chaperonins, also called Hsp60, are found in eubacteria and endosymbiotic organelles, of which GroEL is the most studied. Type II chaperonins exists in archaea and the eukaryotic cytosol and are named the thermosome and CCT (Chaperonin containing TCP-1, also called TriC) respectively. All chaperonins have the same basic architecture in that they are a protein oligomer with a total molecular weight of around 800 – 900 kDa, composed of two rings with seven to nine subunits per ring. The two rings are stacked in a back-to-back orientation creating two separate folding cavities. Each subunit can be subdivided into three different domains; the equatorial domain, that forms the binding surface between the two rings and is responsible for binding ATP, the apical domain, to which substrates bind, and a linking

intermediate domain (Hartl and Hayer-Hartl 2002). Upon substrate and ATP binding the cavity of the chaperonin is enclosed, either by a co-chaperone, GroES for GroEL, or by a conformational rearrangement of the helical protrusions present on the apical domain of the type II chaperonins (Roseman et al. 1996; Ditzel et al. 1998; Llorca et al. 1998; Sigler et al. 1998).

There are several differences between the two subclasses of chaperonins. The type I chaperonins consists of seven identical subunits per ring and substrate recognition occurs through hydrophobic surfaces in the apical domain and exposed hydrophobic segments of the substrate. Inside the enclosed cavity the substrate is released and allowed to passively fold into its native structure. Since the cavity is only closed for about 10 seconds, which is the time it takes before ATP is hydrolysed into ADP and the dissociation of the co-chaperone occurs, several rounds of folding within GroEL might be necessary before the substrate has reached its native state (Hartl et al. 2011). This is an example of a rather unspecific binding occurring via hydrophobic interactions that is consistent with GroEL being a general chaperone. In contrast, the type II chaperonin CCT consists of eight different subunits encoded by eight individual genes (Kubota et al. 1994), each having a fixed position within the ring (Kalisman et al. 2013). The apical domains of the different subunits within a species show a rather low level of similarity, while the same subunit across species has a high sequence homology (Kim et al. 1994). This, accompanied with data suggesting that the interaction between CCT and its major folding substrates actin and tubulin occurs via electrostatic rather than hydrophobic interactions (Hynes and Willison 2000; Pappenberger et al. 2002) reflects a much higher degree of substrate selectivity for CCT compared to GroEL. Furthermore, within the CCT cavity the substrates stay bound to the CCT subunits throughout the action of folding allowing for the conformational changes occurring from the ATP hydrolysis in the CCT subunits to actively force the substrates into their native states (Llorca et al. 1999; Llorca et al. 2000; Valpuesta et al. 2002). The obligate substrates of CCT, the cytoskeletal proteins actin and tubulin, need to be in a quasi-native state before they are recognized by CCT. Hsp70 and the CCT co-chaperone prefoldin may assist actin and tubulin in reaching this partially folded state as well as in the subsequent delivery to CCT (figure 1) (Geissler et al. 1998; Vainberg et al. 1998). Prefoldin is a hetero oligomeric protein complex composed of six subunits. The presence of a functional prefoldin complex ensures the efficient folding of actin, as cells with

one, two or three subunits of prefoldin deleted display a reduced rate of actin folding as well as lower cellular levels of native actin (Siegers et al. 1999).

Hsp90 system

Hsp90 is an essential ATP-dependent molecular chaperone in eukaryotes (Borkovich et al. 1989). In contrast to other molecular chaperones Hsp90 does not play a major role in the *de novo* protein folding (Nathan et al. 1997). Instead it has important functions downstream of Hsp70 where it facilitates the maturation and regulation of certain proteins called Hsp90 clients (figure 1) (Nathan et al. 1997; McClellan et al. 2007; Li et al. 2012). Over 200 proteins have been identified as Hsp90 clients with functions in signal transduction, cell cycle progression and transcriptional regulation (Li et al. 2012). Hsp90 is also involved in the degradation of proteins via a role in proteasome assembly (Imai et al. 2003). For Hsp90 to enable the conformational regulation, transportation and degradation of its client proteins, it needs help from several co-chaperones. The co-chaperones regulate the ATPase activity of Hsp90, link Hsp90 to the Hsp70 chaperone system, as well as recruit client proteins (Li et al. 2012). Many of the Hsp90 clients are kinases shown to be involved in tumor development. By selectively inhibiting Hsp90 with, for example, geldanamycin, a set of new drugs against certain forms of cancer are emerging (Neckers 2007).

Hsp104 system

Hsp104 is a non-essential protein belonging to the Clp/Hsp100 family of AAA+ ATPases (Parsell et al. 1991) found in the cytoplasm and nucleus of yeast cells (Kawai et al. 1999). Under normal conditions Hsp104 is expressed at low levels but after external stress, the protein levels are greatly increased (Parsell et al. 1994a). This is in agreement with the stress tolerance of the cell where Hsp104 levels have a major impact on the cells chance of survival (Parsell et al. 1994a). The role of Hsp104 in protein homeostasis is not to prevent protein aggregation, but instead to rescue trapped proteins by unfolding the aggregates (Parsell et al. 1994b). For aggregated proteins to be fully re-activated Hsp104 needs assistance from Hsp70 and Hsp40 (Glover and Lindquist 1998). In its active form, six Hsp104 molecules assemble into a ring structure creating a central pore (Parsell et al. 1994a; Glover and Lindquist 1998; Schirmer et al. 1998). Each Hsp104 contains two nucleotide binding domains, both of which are required for chaperone function (Parsell et al. 1991). There are currently two models for

how Hsp104 promotes disassembly and clearance of protein aggregates. Initially, a crow bar model was suggested where the aggregates are loosened up via Hsp104 ATPase induced conformational changes. This less compact state of the aggregate allows for Hsp70 and Hsp40 chaperones to further disassemble the aggregates followed by refolding of the substrate (Lee et al. ; Glover and Lindquist 1998). The second model is called the threading model in which polypeptides are extracted from the aggregate and translocated through the central pore of the Hsp104 hexamer via ATPase activity. Hsp70 and Hsp40 chaperones are then waiting on the other side to aid in the re-folding of the emerging polypeptides (Lum et al. 2004; Weibezahn et al. 2004; Weibezahn et al. 2005).

In bacteria the Clp/Hsp100 proteins can be divided into two subfamilies, ClpA and ClpB. The ClpA subfamily consists of ClpA, ClpC, ClpX and HslU, all having protein unfolding activities. Individually, these Clps form a complex together with a protease, such as ClpP or HslV, which promote ATP-dependent proteolysis of the substrate. ClpB is a homologue of yeast Hsp104. ClpB functions together with the bacterial Hsp70 protein DnaK to perform disaggregating and refolding activities in bacteria (Maurizi and Xia 2004; Kirstein et al. 2009). There is no Hsp104 homologue in metazoan cells. Instead, Hsp110 together with Hsp70 and Hsp40 possess limited disaggregating activity, acting on amorphous but not amyloidogenic aggregates. Addition of yeast Hsp104 was shown to further increase the disaggregating activity of Hsp110, Hsp70 and Hsp40 as wells as promote remodeling of amyloid conformers (Shorter 2011; Rampelt et al. 2012).

Small heat shock proteins

Small heat shock proteins (sHsp) are a diverse group of molecular chaperones present in all three kingdoms of life (de Jong et al. 1993). They are characterized by their rather small molecular weight ranging from 12 to 43 kDa, the presence of a conserved α-crystallin domain, and the property of forming large oligomeric structures (Haslbeck et al. 2005). There are only two sHsps found in yeast, called Hsp26 and Hsp42 (Haslbeck et al. 2004), whereas mammals have ten, named HspB1-HspB10 (Kappe et al. 2003). The sHsp are ATP-independent chaperones that prevent protein aggregation by tightly binding non-native proteins, creating stable sHsp-substrate complexes functioning as a reservoir for misfolded proteins during stress. The interaction with sHsp keeps the misfolded proteins in a folding competent state, awaiting stress relief and assistance from ATP-dependent chaperones such as Hsp70, either

alone or in combination with ClpB/Hsp104 to be refolded (Lee et al. 1997; Mogk et al. 2003a; Mogk et al. 2003b; Cashikar et al. 2005).

Protein degradation

Within cells there is a constant turnover of proteins, lipids and RNA. It is of great importance for the cell to be able to degrade molecules that are damaged and prevent their toxic accumulation. At the same time the cell needs to be able to rapidly degrade specific proteins involved in cell cycle progression, signal transduction, and developmental regulation. In order to degrade cellular components in a controlled and safe way it is essential that this process takes place in a protected environment to prevent unwanted hydrophobic interactions between degradation products and other cellular constituents. For this purpose the cell has evolved two proteolytical systems called autophagy and the proteasome (Ciechanover 2005; Huang and Figueiredo-Pereira 2010).

Autophagy

The degradation of cytosolic components performed within the lysosome (in metazoans) or the vacuole (in yeast) is called autophagy, or self-eating (Mizushima et al. 2008). Inside the lysosome there are several hydrolases capable of degrading proteins, lipids, glycosides, and nucleotides (De Duve and Wattiaux 1966). These hydrolases all have a high activity within the acidic environment of the lysosome. The degradation of proteins by the lysosomal proteases results in small peptides as well as free amino acids that are returned to the cytosol via permeases in the membrane (Yang et al. 2006; Chen and Klionsky 2011). Autophagy is involved in cellular quality control by clearing the cell of misfolded and aggregated proteins as well as providing a source of energy when the cell suffers from nutrient starvation. Autophagy is implicated in the remodeling of cells and tissues for example during development and is part of the cell's defense against bacteria, parasites and viruses (Mizushima et al. 2008). This illustrates the importance of a functional autophagic system for several housekeeping processes in the cell. Changes in autophagy have been associated with a growing number of protein conformational disorders and studies in fly and mouse models of Huntington's disease have shown that chemically increasing autophagic activities results in a slower disease progression (Ravikumar et al. 2004).

Ubiquitin proteasome system

The proteasome is a 2,5 MDa protein complex present in the cytosol, nucleus and ER of eukaryotic cells (Baumeister et al. 1998; Voges et al. 1999). It is responsible for the proteolytical degradation of proteins during cellular processes such as protein quality control, cell cycle control, apoptosis, inflammation, signal transduction, and transcription (Finley 2009). The 26S proteasome is made up of a 20S core and 19S regulatory particles (figure 2) (Voges et al. 1999; Finley 2009). The 20S core particle is a barrel shaped structure composed of four rings with seven subunits per ring. The two middle rings contain β-subunits, responsible for the proteolytic activity, whereas the two outer rings are made up of α-subunits, functioning as gates for the substrate destined to be degraded. The opening and closing of the gates is controlled by the regulatory 19S particles, binding to either side of the 20S core. The 19S also ensures substrate recognition, binding, and due its AAA ATPase activity it prepares the substrate for degradation by partly unfolding it, making it ready to be threaded into the active core and subsequently degraded (Finley 2009).

It is extremely important that only the appropriate proteins are degraded, a process controlled by the ubiquitin system. The proteasome recognizes proteins that are to be degraded by the presence of at least four molecules of ubiquitin, a 8,5 kDa protein conserved from yeast to mammals (Hershko and Ciechanover 1998; Miller and Gordon 2005). The ubiquitination process is controlled by the cell via enzymes named E1, E2, and E3 (figure 2). Initially, ubiquitin becomes activated by the ubiquitin activating enzyme E1 and transferred to the active site on ubiquitin conjugating enzyme E2. Thereafter, E2 interacts with the ubiquitin ligase enzyme E3, which in turn is responsible for the transfer of ubiquitin to the substrate. A second ubiquitin can now be added directly to the first one, and so on (Jung et al. 2009; Finley et al. 2012). To maintain a pool of free ubiquitin in the cell, there are different de-ubiquitinating (DUB) enzymes that remove the poly-ubiquitin chain from the substrate before the degradation process takes place. The 19S functions as a DUB at the entrance of the 20S core, but there are additional DUBs in the cell, some of which can rescue proteins from degradation by removing the ubiquitin chain and preventing the 19S from recognizing the

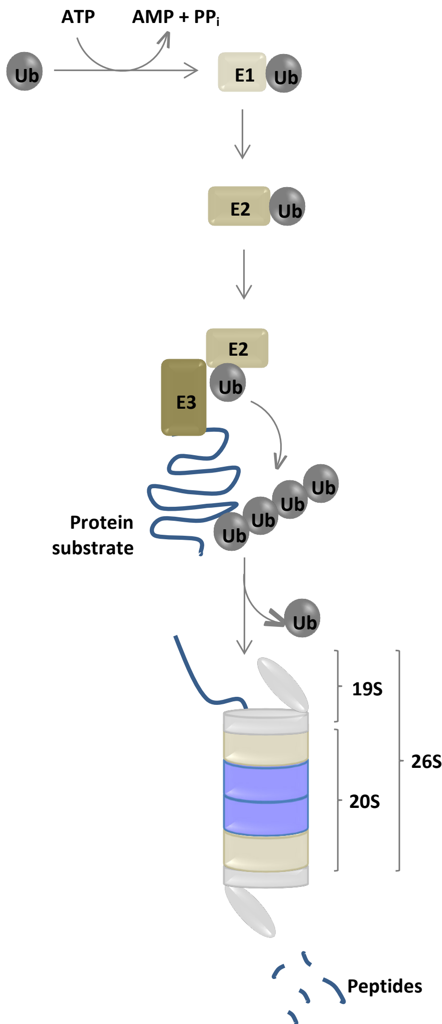

Figure 2. The ubiquitin proteasome system. Proteins destined for degradation via the ubiquitin proteasome system are recognized by the 26S proteasome through the presence of at least four molecules of ubiquitin. The conjugation of ubiquitin to the protein substrate is controlled by enzymes called E1, E2, and E3. Ubiqiutin gets activated by E1 and transferred to the active site on E2. E2 then interacts with E3, which conjugates the ubiquitin molecule to the substrate. Additional rounds of ubiquitination follow where after the 19S lid recognizes the substrates and delivers it to the 20S core for proteolytical degradation.

protein (Lam et al. 1997; Hanna et al. 2006). Proteasomal degradation is very important in maintaining cellular homeostasis and PQC. This becomes evident as impairment of the ubiquitin proteasome system (UPS) results in detrimental changes for the cell, and if unrestored, leads to cellular death. Failure in the regulation of proteasomal activity is linked to various diseases and aging (Huang and Figueiredo-Pereira 2010; Saez and Vilchez 2014; Schmidt and Finley 2014). For example, replicative old yeast mother cells, harboring an

increase in aggregated proteins, display a reduced UPS activity (Andersson et al. 2013), and protein aggregates present in several protein conformational disorders have been found to clog the entrance of the proteasome, causing a decrease in proteasomal activity (Keck et al. 2003; Landles and Bates 2004).

Protein damage

Eukaryotic cells living in aerobic environments produce reactive oxygen species (ROS) as a by-product from cellular metabolism. Under physiological conditions the cell contains a functional antioxidant defense system for prevention of molecular damage. This antioxidant system includes a primary defense which neutralizes ROS, and a secondary defense involving the repair and degradation of damaged molecules (Costa and Moradas-Ferreira 2001). The cell can sense increased levels of ROS which induces a defense response. An example of this is the activation of the yeast transcription factors Yap1 and Skn7 and the subsequent expression of proteins involved in oxidative stress resistance (Lee et al. 1999). If the cellular levels of ROS exceed the capacity of the antioxidant defense system the cell will suffer from oxidative stress. Too much ROS can be the result from either a decrease in antioxidants, caused by for example mutations, or an increase in the production of ROS, as a consequence of exposure to compounds generating ROS or the activation of cellular systems producing ROS (Costa and Moradas-Ferreira 2001). Under oxidative stress conditions proteins, lipids, and nucleic acids become oxidized. In the case of protein oxidation, the protein will undergo conformational changes resulting in partial unfolding and the exposure of hydrophobic amino acids. The partially unfolded protein would normally be refolded or sent for degradation by chaperones, but in the case of an overloaded chaperone system, the damaged proteins may form toxic aggregates within the cell (Costa et al. 2007). While some types of protein oxidation can be reversed by specific reductases, others are irreversible and are solely dependent on protein degradation for their removal. Carbonylation is an example of an irreversible oxidative damage found to increase with the age of cells, organelles and tissues in a variety of organisms (Aguilaniu et al. 2003; Nystrom 2005; Erjavec et al. 2007). Carbonylation is caused by a metal catalyzed oxidative attack on specific amino acid side chains which may lead to protein inactivation whilst at the same time the carbonyl group makes the protein more susceptible to proteolytic degradation (Nystrom 2005). Whereas proteins with a low carbonylation level are degraded, either by the lon protease or the

proteasome, highly carbonylated proteins tend to form large aggregates that escape degradation. Additionally, oxidized protein aggregates cannot efficiently be degraded by the proteasome, instead these aggregates have been found to inhibit proteasomal activity (Grune et al. 2004). There is an unequal distribution of carbonyls within the proteome, where glycolytic enzymes and molecular chaperones are major targets for carbonylation, both in cells subjected to oxidative stress by chemicals (Cabiscol et al. 2000) and in aged cells (Reverter-Branchat et al. 2004; Erjavec et al. 2007). Furthermore, carbonylated proteins are actively retained in the yeast mother cell compartment during cytokinesis (Aguilaniu et al. 2003; Tessarz et al. 2009).

Protein aggregation

Protein aggregation is an event occurring when the cellular PQC system becomes limiting. Due to the biochemical property of the polypeptide backbone, every protein has the potential to misfold but only a subset of proteins do (Dobson ; Carrell and Lomas 1997). Within the cell, soluble proteins are dynamic in their conformations. This means that native proteins may cycle between a fully folded and a partially folded, intermediate, state. For non-stressed cells, the proteins being in the non-native stage are quickly brought back to their correct native fold by the assistance from the PQC system. In contrast, cells experiencing cellular stress suffer from an overwhelmed quality control system causing a shift in the equilibriums towards proteins being in their partially folded state. This enhances the risk of unwanted protein-protein interactions and protein aggregation (Wolfe and Cyr 2011). Aggregates can be amorphous (disorganized) or they can be structured (ordered). The amorphous aggregates arise from proteins being in intermediate folding states having increased hydrophobicity and a tendency to self-associate, resulting in aggregation. If the PQC system is limiting in the cell, the amorphous aggregate will increase in size and eventually become large enough to form an insoluble precipitate (figure 3). The structured, highly organized aggregates on the other hand, form in a nucleation-dependent manner. Partially folded proteins associate to form a stable nucleus, functioning as a template for the addition of other partially folded intermediates. The rate-limiting step is the initial nucleation, which can be substantially accelerated by the addition of preformed fibrillary species, functioning as seeds. After further addition of intermediates, an insoluble amyloid fibril will eventually form (figure 3) (Chiti and Dobson 2006; Ecroyd and Carver 2008). True amyloid fibrils are defined by the International Society of Amyloidosis as ”extracellular depositions of protein fibrils with characteristic appearance

in electron microscope, typical X-ray diffraction pattern, and affinity for Congo red with concomitant green birefringence". This definition is mainly used for pathological diagnosis. Researchers focusing on the structure and the biophysical properties underlying the formation of amyloid fibrils use a more structure-based definition: polypeptides forming cross-β structures where the β-sheets run perpendicular to the fiber axis. This characteristic structure is shared by all amyloid fibrils (Chiti and Dobson 2006; Fändrich 2007; Wolfe and Cyr 2011). A common feature of all types of protein aggregates is that they are insoluble and metabolically stable under normal cellular conditions.

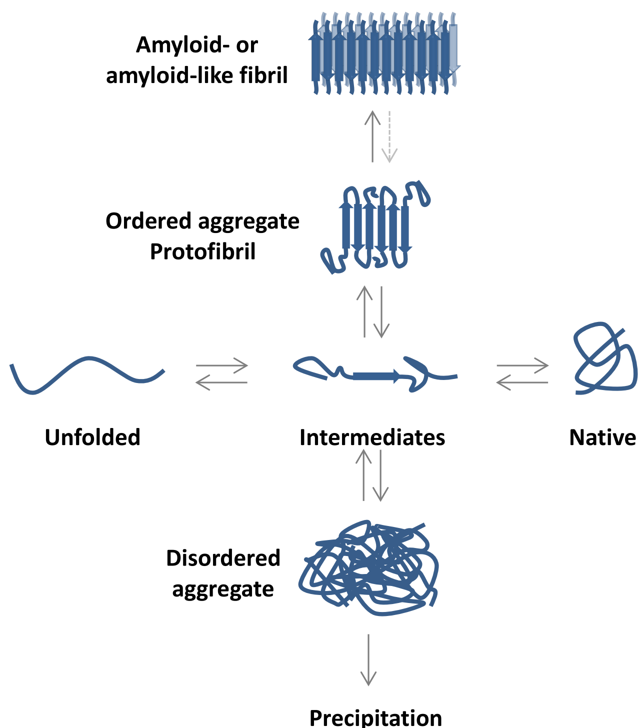

Figure 3. Protein aggregation. The unfolded protein reaches its native state through a partially folded, intermediate, state. Under non-stress conditions, the PQC system facilitates fast and proper folding into the native state. In contrast, during situations of stress or mutations, the protein exists for longer times in the intermediate state. This allows for aberrant protein interactions leading to either ordered or disordered mechanisms of protein aggregation and the formation of amyloid-like fibrils or amorphous aggregates, respectively.

Spatial protein quality control

The cell has evolved a way to spatially collect misfolded and aggregated proteins at cellular sites that also harbor components of the PQC system. This enables efficient management of aggregated proteins in terms of disaggregation and refolding or degradation. All cells display spatial sequestration of damaged proteins but the cellular localization of the deposition sites differs between organisms. Moreover, there are different aggregation sites within a cell, and where a misfolded protein is placed depends on the protein itself and what kind of stress the cell was subjected to. In bacteria, inclusion bodies of aggregated proteins tend to form at the cell poles (Lindner et al. 2008; Winkler et al. 2010). In yeast there are two distinct sites where aggregates accumulate. One is positioned at the periphery of the vacuole, called IPOD (insoluble protein deposit), and the other is found close to or inside the nucleus JUNQ/INQ (juxtanuclear quality control compartment/intra nuclear quality control compartment) (Kaganovich et al. 2008; Miller et al. 2015). Additionally, mammalian cells have a specialized form of cytoplasmic inclusion body called the aggresome (Johnston et al. 1998).

Aggresome

The aggresome can be found in mammalian cells and is defined as a microtubule-dependent cytoplasmic inclusion body present at the microtubule organizing center (MTOC) (Johnston et al. 1998). Smaller peripheral aggregates use dynein-based retrograde transport along polarized microtubule tracks to coalesce with the aggresome at the MTOC (Garcia-Mata et al. 1999). Apart from the major aggregated protein, the aggresome is enriched for proteasomes and molecular chaperones, such as Hsp40, Hsp70, and CCT, facilitating the refolding and/or degradation of misfolded proteins (Garcia-Mata et al. 2002). Additionally, the intermediate filament protein vimentin re-locates from the cytoplasm to form a cage-like structure surrounding the aggresome (Johnston et al. 1998). The presence of an aggresome has also been found to promote autophagic clearance (Garcia-Mata et al. 2002).

IPOD, JUNQ/INQ, and stress foci

In yeast there is no *bona fide* aggresome. The inclusion bodies found in yeast upon stress are not localized at the spindle pole body (SPB) (Kaganovich et al. 2008; Miller et al. 2015) which is the yeast equivalent of the mammalian MTOC. Further, the aggregation process of the aggresome is dependent on the microtubule network, while this is not the case for protein

deposits in yeast. Instead, an intact actin cytoskeleton is necessary for the formation of the yeast protein deposits (Specht et al. 2011). During heat stress in yeast, protein misfolding occurs and as a result there are many small aggregates rapidly forming in the cytosol (Spokoini et al. 2012) called peripheral aggregates, cytoQ, stress foci, and Q-bodies (Specht et al. 2011; Spokoini et al. 2012; Escusa-Toret et al. 2013; Miller et al. 2015). These structures, named differently by different research groups, may very well refer to similar or even the same cytosolic aggregates. In this thesis I will call these misfolded cytosolic protein structures stress foci. The stress foci are dissolved within less than a yeast cell cycle. If, on the other hand, the proteasome is inhibited there is no degradation of the stress foci and they will eventually end up in specific cellular deposit sites, through a process dependent on Hsp104 (Spokoini et al. 2012). Two deposit sites were identified; the JUNQ (juxtanuclear quality control compartment) and the IPOD (insoluble protein deposit) found in both yeast and mammalian cells (Kaganovich et al. 2008). The JUNQ compartment resides in close proximity to the nucleus and contains soluble proteins that can exchange with the surrounding, while the IPOD is found at the vacuole and contains terminally misfolded, insoluble proteins such as prions and amyloidogenic proteins (Kaganovich et al. 2008). Kaganovich and colleagues reported that ubiquitin functions as a sorting signal, where ubiquitinated proteins are directed to JUNQ and non-ubiquitinated proteins are sent to IPOD. Later studies by Miller and colleagues reported that JUNQ was positioned inside the nucleus and that ubiquitination was not necessary for the misfolded proteins to localize to this quality compartment, which was renamed INQ (intranuclear quality control compartment) by the authors (Miller et al. 2015). Although INQ is present inside the nucleus it still serves as a quality control compartment for both nuclear and cytosolic proteins. Sis1, an Hsp70 co-chaperone, helps cytosolic misfolded proteins to enter the nucleus via the nuclear pore (Park et al. 2013; Miller et al. 2015).

There is emerging evidence of important sorting factors for misfolded proteins. For example, Hsp42 is involved in the localization of amorphous, but not amyloidogenic, aggregates to peripheral deposit sites (Specht et al. 2011) and Sti1, a Hsp70/90 co-chaperone, plays a role in the sorting of misfolded proteins by directing substrates to JUNQ/INQ (Kaganovich et al. 2008; Miller et al. 2015). Furthermore, Btn2 is found to be involved in the formation of INQ, due to its function as a nuclear-specific aggregase (Miller et al. 2015). The exact molecular mechanism behind the formation and management of misfolded proteins is not yet fully

resolved, but it is evident that the cell uses specific factors to organize misfolded proteins. It appears to be a dynamic process where proteins get redirected in the cell's attempt to be as efficient as possible in maintaining essential cellular functions. Taken together, components of the PQC network are cooperating in binding and recruiting misfolded proteins to designated intracellular sites. This is valuable for the cell in several ways. It reduces harmful interactions between misfolded proteins and important cellular components, thus confining the damaged proteins until the PQC system is vacant. It also enables the cell to orchestrate the misfolded proteins to places where there are functional PQC systems available to reverse the damage, either by refolding or degradation via the 26S proteasome or autophagy. The ability to focus protein damage to a subset of locations also facilitates asymmetric segregation of the damaged components during cell division, an important process in cellular rejuvenation. In line with this, spatial quality control has been shown to be important for cellular homeostasis both during stress and aging (Rujano et al. 2006; Lindner et al. 2008; Liu et al. 2010).

Damage asymmetry and replicative rejuvenation

The presence of misfolded proteins, although in an aggregate, can attract and sequester components of the PQC system, thus having a negative impact on cellular fitness. Asymmetric cell division provides a way for complete removal of protein damage in that one of the two daughter cells inherits the damaged proteins whilst the other is born free of damage. Asymmetrical inheritance of protein aggregates has been identified in bacteria (Lindner et al. 2008; Winkler et al. 2010) and yeast (Aguilaniu et al. 2003; Erjavec et al. 2007; Liu et al. 2010) as well as in mammals (Rujano et al. 2006; Fuentealba et al. 2008; Bufalino et al. 2013; Ogrodnik et al. 2014) suggesting that protein damage asymmetry is a general, conserved mechanism. In yeast, the asymmetrical segregation of damaged macromolecules during cell division plays a role in cellular aging and rejuvenation. Yeast aging can be defined in two ways, chronological aging and replicative aging. Chronological aging can be described as the time a cell can survive in a non-dividing state and is used as a model for studying cellular aging in post mitotic, non-dividing cells (Fabrizio and Longo 2003). In contrast, replicative aging is defined as the number of daughters a mother cell can produce before it senesces, reflecting aging of dividing cells (Kaeberlein et al. 2007). The groundwork for replicative aging studies was established in the 1950's when it was discovered that yeast cells have a finite replicative capacity (Mortimer and Johnston 1959). The yeast *Saccharomyces cerevisiae* divides by budding, where a smaller daughter cell is

pinched off from the larger mother cell. While the mother cell progressively accumulates age related phenotypes, each daughter is born with a full replicative potential. This is called replicative rejuvenation. An exception to this is daughters produced from mothers very close to senescence, which have a shorter life span (Denoth Lippuner et al. 2014). For replicative rejuvenation to occur there must be a segregation of damaged components away from the daughter. By using yeast as a model organism, several factors have been identified as important players in the segregation of damage.

Players required for damage asymmetry

Sir2

In a study from our lab it was shown that the asymmetrical inheritance of oxidatively damaged proteins during cell division was dependent on Sir2, a highly conserved NAD^+-dependent histone deacetylase, as mutant cells lacking this gene had an equal distribution of damaged proteins between mother and daughter cells (Aguilaniu et al. 2003). Sir2 has been found to regulate the process of aging in a variety of organisms such as yeast, worms, flies, fish and mammals (reviewed by Nystrom 2011) and the role of Sir2 in yeast aging is linked to increased silencing at the rDNA locus. Sir2 performs genomic silencing at three genomic regions, the mating type loci, telomeres, and the rDNA locus. The rDNA locus is found on chromosome XII within a nuclear structure called the nucleolus. The rDNA locus consists of a 9,1 kb unit repeated between 100 and 200 times (Petes 1979) where each unit contains genes encoding the 35S and 5S rRNAs, an autonomous replicating sequence (ARS) responsible for the initiation of DNA replication, and a replication fork block site ensuring that the replication of this DNA is unidirectional (Philippsen et al. 1978; Brewer and Fangman 1988). The Fob1 dependent replication block may cause a DNA double strand break within the rDNA. This break can be repaired by homologous recombination, resulting in the formation of an extrachromosomal ribosomal DNA circle (Defossez et al. 1999). Since the excised ERC contains an ARS sequence it is replicated once every cell cycle round. The ERCs are asymmetrically inherited by the mother cell during cytokinesis (Sinclair and Guarente 1997), which is made possible by the anchorage of ERCs to nuclear pore complexes (NPCs) within the nuclear envelope and due to a lateral diffusion barrier, the NPCs with the bound ERCs are actively retained in the mother cell (Shcheprova et al. 2008). The combination of the traits of

self-replicating and accumulation in mother compartments leads to a massive increase in DNA within the nucleolus of the mother, eventually resulting in the fragmented nucleolus seen in aged mother cells. Yeast cells lacking Sir2 accumulate ERCs rapidly due to the loss of silencing at the rDNA locus and this has been linked to the short replicative life span of *sir2Δ* cells (Kaeberlein et al. 1999). The results showing that Sir2 also promotes the retention of damaged proteins highlight another role for Sir2 in aging and damage asymmetry. The deficient protein damage retention in cells lacking Sir2 seems to be independent from the accumulation of ERC seen in *sir2Δ* mutants, since the deletion of *FOB1* in a *sir2Δ* mutant, resulting in massive reduction of ERC levels, had no effect on the failure to retain damaged protein in the mother cell compartment (Erjavec et al. 2007). Thus, the question is raised how a nucleus-based deacetylase can regulate the distribution of damaged protein in the cytosol.

Actin

In addition to Sir2 having a role in the segregation of protein damage, it was also observed that an intact actin cytoskeleton was necessary for the mother-biased inheritance of damaged proteins (Aguilaniu et al. 2003; Erjavec et al. 2007).

Actin is a highly conserved and abundant protein within eukaryotes. It performs a wide variety of processes and is essential to all eukaryotic cells (Pollard et al. 2000). It is a 42 kDa protein folded into two domains with a stabilizing adenine nucleotide in between. Within the cell, actin exists either in a globular monomeric form (G-actin) or in a filamentous polymeric form (F-actin). The F-actin has a polarized, right handed double helix structure with a barbed end and a pointed end, also called the plus-end and the minus-end, respectively (figure 4). It is the concentration of actin monomers that determines if actin monomers will be added to the F-actin and the lowest concentration necessary for F-actin polymerization is called the critical concentration (Cc). The Cc is about 20 times lower for polymerization to occur at the barbed end compared to the pointed end due to the fact that the barbed end contains ATP-actin and the pointed end ADP-actin. The rapid hydrolysis of ATP into ADP after the addition of an actin monomer to the filament results in a conformational change in the actin, leading to a weaker interaction with neighboring subunits. If the cellular level of monomeric actin is between the Cc of the barbed and the pointed end, there will be a net assembly of actin at the barbed end and a net disassembly at the pointed end. An actin subunit added at the barbed end will slowly migrate within the polymer towards the pointed end where it ultimately

dissociates from the filament, a situation called tread milling. The cell contains many actin-binding proteins affecting polymerization and depolymerization of the F-actin. For example, the formation of a new filament requires three actin monomers functioning as a seed, a very unstable and unfavorable complex. The cell contains actin nucleators, such as Arp2/3 and formins, which stabilize the actin seed and allow for the elongation of the F-actin to occur. F-actin elongation is in turn promoted by profilin, an actin monomer binding protein, which exchanges ADP for ATP within actin monomers. Furthermore, profilin contains an SH3-like domain capable of binding the proline-rich region of formins and delivers ATP-actin to the formin, which in turn can add actin monomers to the growing filament (figure 4). In addition there are many more actin binding proteins encompassing functions such as stabilizing, capping, severing, and cross-linking actin filaments. Thus, the dynamic nature and extensive control via a network of regulatory proteins is essential for the role of actin in cellular movement, endocytosis, intracellular transportation of various molecules, and the separation of cells during mitosis (Pollard and Earnshaw 2007; Grantham et al. 2012). A comparison between metazoan and yeast actin cytoskeletons reveals many similarities as well as some differences. While actins in metazoan cells are encoded by six genes, yeast only contains one actin gene (Moseley and Goode 2006; Chhabra and Higgs 2007). Metazoan cells also have

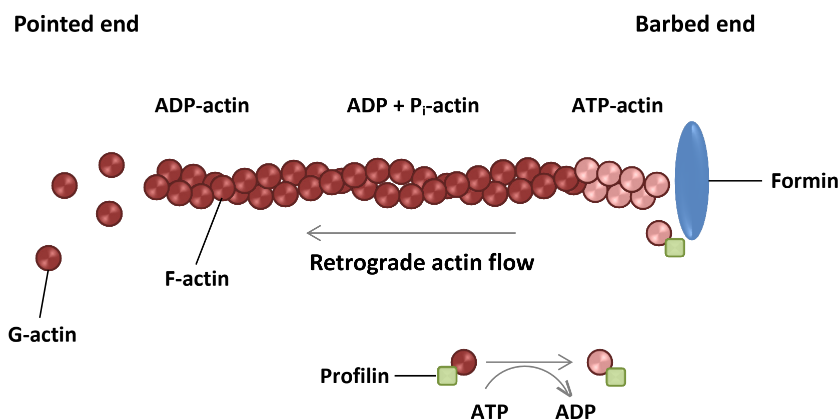

Figure 4. Actin dynamics. ADP-actin binds profilin, which exchange ADP for ATP and delivers the ATP-actin to a formin present at the pointed end of an actin filament. The formin polymerizes the F-actin by the continuous addition of actin monomers, making the actin monomers flow retrograde within the filament. Shortly after incorporation into the filament, ATP is hydrolysed into ADP, and eventually the ADP-actin will dissociate from the filament. Profilin then acts as a nucleotide exchange factor to produce ATP-actin which can then be re-incorporated.

more actin regulatory proteins as compared to yeast cells. Despite this, many actin-dependent processes are preserved between the two species. For example the process of F-actin polymerization and depolymerization, along with the Arp2/3- and formin-dependent actin assembly is conserved between yeast and metazoans. Further, they both use actin based processes for clathrin-mediated endocytosis (CME), involving many homologues proteins, and they both form bundles of F-actin inside the cell (Girao et al. 2008; Rohn and Baum 2010).

Yeast actin

Yeast actin, encoded by *ACT1*, gives rise to three actin structures: cortical actin patches, polarized actin cables, and the cytokinetic actin ring. The cytokinetic ring is only present just before and during cytokinesis as this structure is involved in the contraction of the cell membrane resulting in separation between mother and daughter (Moseley and Goode 2006). The actin patches, present on the plasma membrane, are composed of a dense network of branched F-actin, nucleated by the Arp 2/3 complex. The patches localize to sites of polarized growth and are involved in CME (Mishra et al. 2014). Actin cables are bundles of short, polarized F-actin extending along the mother-bud axis in yeast cells, involved in establishing and maintaining polarity. The actin cables in yeast serve as polarized tracks for motor-driven transportation and delivery of cargo, a function primarily involving microtubules in metazoan cells. In yeast, the type-V myosins Myo2 and Myo4 are responsible for the translocation of various organelles and mRNA along actin cables, needed to build up the daughter cell (figure 5) (Pruyne et al. 2004b; Moseley and Goode 2006; Mishra et al. 2014). Yeast also contain two formins, Bni1 and Bnr1, which are responsible for the polymerization of F-actin structures at the bud tip and bud neck, respectively (Pruyne et al. 2004a). Bnr1 is localized to the bud neck via interactions with septins and Bni1 is recruited to the bud tip via Spa2. Spa2, together with Bud6 and Pea2, is part of the polarisome complex (figure 5) (Sheu et al. 1998). Together with other proteins, such as Cdc42, Cdc24, Bem1, and Rho-GTPases, the polarisome complex regulates the polarized cell growth (Pruyne et al. 2004b).

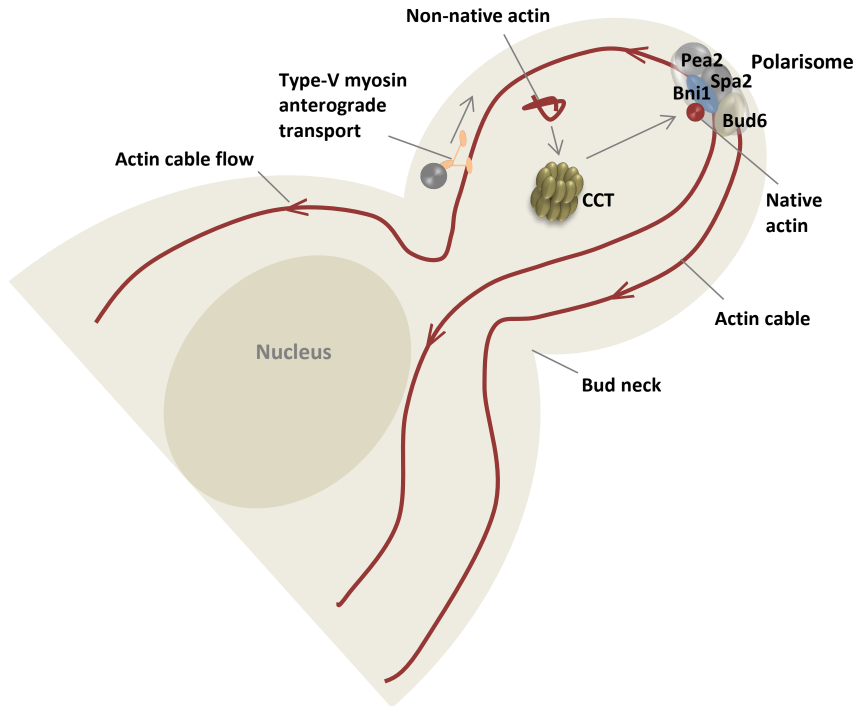

Figure 5. Regulation of yeast actin cables at the polarisome. Spa2, Pea2, Bud6, and Bni1 are part of the polarisome complex. Native actin monomers, produced by CCT, is added to the growing filament (actin cable) by the formin Bni1, creating a retrograde actin cable flow from the bud tip into the mother cell. Type-V myosins walk upstream, anterograde, on the polarized actin cables to deliver cargo needed to build up the new daughter cell.

Links between Sir2 and actin

Since old *sir2Δ* cells display aberrant F-actin staining (Erjavec and Nystrom 2007), a possible explanation for the failure in damage segregation in cells lacking Sir2 could be the presence of a compromised actin cytoskeleton. Further studies revealed that *sir2Δ* cells have a decrease in the actin cable abundance, are more sensitive to the actin monomer sequestering drug Latrunculin A, and show a depolarized actin patch phenotype (Liu et al. 2010; Higuchi et al. 2013). It was also demonstrated that actin cables in cells lacking Sir2 have a decreased rate of retrograde actin cable flow (RACF) (Higuchi et al. 2013). Together, this reveals that cells

lacking Sir2 suffer from a compromised actin cytoskeleton. A mechanistic link between Sir2 and the actin cytoskeleton is presented in the section results and discussion.

Yeast as a model system for protein conformational disorders

The budding yeast *Saccharomyces cerevisiae* is a eukaryotic unicellular organism. It has become a widely used model for studying cellular processes that can be used in the hunt to understand the molecular mechanisms behind human diseases. Budding yeast has many advantages. For example, it is relatively inexpensive to culture, it has a short life cycle (~1-2 h) and it has the ability to be genetically modified which gives the opportunity to delete or alter genes to study their role in cellular processes. The complete genome of *Saccharomyces cerevisiae* was fully sequenced in 1996 which enabled extensive experimental studies. Later, when the sequence of the human genome also became available it revealed that about 31% of yeast genes have mammalian homologues and another 30% show domain similarities (Botstein et al. 1997). This has made yeast an excellent model to study fundamental molecular mechanisms within the eukaryotic cell. As many cellular processes are well conserved between yeast and humans it makes it possible to study proteins involved in human diseases even though they lack a yeast homologue. This can be done by ectopically expressing the disease protein in yeast. An example of this is the protein huntingtin, which in its mutant form, causes the neurodegenerative disorder Huntington's Disease (MacDonald et al. 1993), discussed further below.

Protein conformational disorders

Problems with PQC have been linked to various protein conformational disorders also called proteopathies. The common feature for these disorders is that there is a conformational change in the 3D-structure of the disease protein, increasing the protein's propensity to bind to itself (Carrell and Lomas 1997). The structural change is often a switch to a β-sheet secondary structure. Several β-sheet structures can easily bind to each other, forming highly stable ordered conformations called amyloid-like inclusions (Fändrich 2007). Examples of protein conformational disorders are Prion diseases and the neurodegenerative disorders Alzheimer's disease, Parkinson's disease, and Huntington's disease (HD). The exact link between amyloid-like inclusions and the toxicity observed in conformational disorders is not fully established. There are studies suggesting that the inclusions themselves are harmful

(Yang et al. 2002) at the same time as other reports show a negative correlation between inclusions and toxicity (Saudou et al. 1998; Kuemmerle et al. 1999). Further, several recent studies show that the inclusions are in fact cytoprotective and that the more soluble oligomers are the toxic species (Walsh et al. 2002; Arrasate et al. 2004; Douglas et al. 2008). This is explained by the model where the toxic oligomers can be sequestered into an inclusion thus reducing harmful interactions between the oligomers and important cellular components. Studies have shown that polyglutamine-expanded and other amyloid-like disease proteins bind certain transcription factors and alter transcriptional regulation (Schaffar et al. 2004; Riley and Orr 2006). The disease proteins have also been found to inhibit both proteasomal degradation (Bence et al. 2001) and ER associated degradation (Duennwald and Lindquist 2008; Leitman et al. 2013). These disease proteins also promote abnormal protein-protein interactions where they sequester and titrate out certain glutamine-rich or glutamine/asparagine-rich proteins (Furukawa et al. 2009; Park et al. 2013; Ripaud et al. 2014). Even though the protein causing the disease is present in many different cell types throughout the body, it is only a subset of cells that are affected. An explanation for this could be that the proteome of a cell has important implications on the toxicity caused by a specific disease protein, as well as the levels of certain important components could be an essential factor for the cell's capacity to manage the disease protein (Wolfe and Cyr 2011). Studies have suggested that even small variations in the expression pattern of general PQC components can have severe effects on the fate of the disease proteins which can explain the selective vulnerability seen in specific cell types (Gidalevitz et al. 2006; Balch et al. 2008).

Huntingtin and Huntington's disease

Even though the mutation causing HD has been identified as far back as 1993, the complete function of the normal huntingtin protein is yet not fully known. Despite the fact that huntingtin is a very large protein, 348 kDa, it is, in contrast to many other proteins of similar size, completely soluble. It has no sequence homology with other proteins and is ubiquitously expressed, with the highest levels in testes and neurons in the central nervous system. Huntingtin is essential during embryogenesis since knock-out mice lacking the *HTT* gene die before day E8.5. It has been shown that the mutant form of huntingtin can compensate for the lack of wild type huntingtin during development as expression of a mutant version of human huntingtin in *HTT* null mice rescues them from embryonic lethality. In addition, human HD

patients, homozygous for the mutant allele, are born without any noticeable defects (Cattaneo et al. 2005). Extensive efforts have been made to decipher the function of huntingtin, finding that huntingtin appears to play a role in several diverse processes within the cell, for example in vesicular transport, apoptosis, transcriptional regulation, cell signaling, and clathrin-mediated endocytosis (CME) (Harjes and Wanker 2003). The huntingtin protein consists of several different protein binding domains, illustrated in figure 6. The Nt17 domain can interact with the nuclear pore protein TPR (Cornett et al. 2005) and due to its amphipathic α-helix structure the Nt17 domain has the ability to bind lipid membranes (Arndt et al. 2015). The polyglutamine (polyQ) sequence has been found to stabilize protein-protein interactions (Schaefer et al. 2012). The following Proline-rich domain (PRD) can interact with proteins having an SH3- or a WW domain (Kay et al. 2000; Gao et al. 2006) and the several HEAT repeats located downstream are involved in protein-protein interactions (Andrade and Bork 1995).

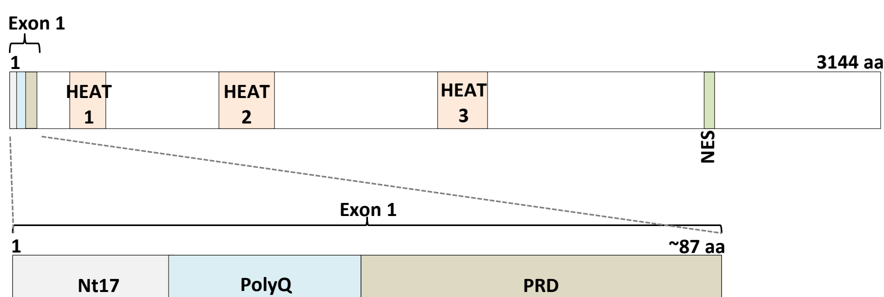

Figure 6. Schematic picture of huntingtin showing specific protein domains. Huntingtin has an N-terminal domain composed of 17 amino acids (Nt17), a polyQ repeat sequence, a proline-rich domain (PRD) and several HEAT repeats. All of these domains are implicated in interactions with other proteins, giving huntingtin the property of a scaffold protein. The C-terminal part of the protein contains a nuclear export sequence (NES).

Huntingtin has the ability to function as a scaffold protein, serving as a platform for other proteins and facilitate their interactions. This can be of importance within the crowded environment of the cell to ensure that components performing essential functions as a complex have the chance to interact. Depending on the spatio-temporal localization of huntingtin it can affect diverse cellular processes.

HD is the most common inherited neurodegenerative disorder (Finkbeiner 2011) affecting 1 in ~10.000 individuals (Harjes and Wanker 2003). It is an autosomal dominant inherited disease, meaning that a child of an affected parent has a 50% risk of developing the disease (MacDonald et al. 1993; Landles and Bates 2004). HD patients suffer from a selective neuronal death, with neurons in the cerebral cortex and striatum as primary targets, resulting in chronic and progressive symptoms such as involuntary chorea, cognitive impairment, mood disorders, and behavioral changes (Harjes and Wanker 2003; Myers 2004). At present there is no cure for HD, and patients will eventually die, not from the disease itself, but from complications related to the disease including pneumonia, cardiovascular diseases, and suicide (Roos 2010; Heemskerk and Roos 2012). HD is caused by a pathogenic expansion of the CAG trinucleotide repeat of exon-1 in the *HTT* gene (figure 6) (MacDonald et al. 1993). CAG encodes glutamine (Q) and expression of the disease gene results in a huntingtin protein harboring an abnormally long polyQ stretch, a property making HD part of a family of neurodegenerative diseases called polyQ diseases (table 1).

Disease	**Protein**	**Pathological repeat length**	**Brain region affected**
DRPLA	Atropin-1	49-88	Cerebral cortex
HD	Huntingtin	41-121	Striatum and cortex
MJD	Ataxin-3	61-84	Ventral pons and substantia nigra
SBMA	Androgen receptor	38-62	Motor neurons, brain stem, and spinal cord
SCA1	Ataxin-1	39-82	Cerebellum
SCA2	Ataxin-2	32-200	Cerebellar Purkinje cells
SCA6	CACNA1A	10-33	Cerebellar Purkinje cells
SCA7	Ataxin-7	37-306	Cerebellar Purkinje cells, brain stem and spinal cord
SCA12	PPP2R2B	66-78	Cerebral and cerebellar cortex
SCA17	TATA-binding protein	47-63	Cerebellar Purkinje cells

Table 1. PolyQ diseases. There are currently ten known polyQ diseases. Their name, disease causing protein, pathological polyQ length, and the region of the brain being affected are described. DRPLA: dentatorubropallidoluysian atrophy, SBMA: spinal and bulbular muscular atrophy, SCA: spinocerebellar ataxia, MJD: Machado-Joseph disease. Adapted from (Trepte et al. 2014).

The length of the CAG repeat determines if a person will develop HD or not. Unaffected, healthy, individuals have an *HTT* allele with <35 repeats, whereas an allele with >40 repeats ultimately will result in HD. Individuals carrying an allele with 35-40 repeats may or may not develop HD (Finkbeiner 2011). In general there is a negative relationship between the length of the CAG expansion and age of disease onset. Moreover, it is evident that the inverted correlation is strongest for the longer expansions, those with 60 or more repeats, while 40-55 repeats is a poor determinant for predicting age of disease onset (Myers 2004). Over all, the mean age for developing HD is 35 years. Interestingly, the life expectancy after disease onset is relatively constant with an average of between 15 and 20 years. In other words, the duration of the disease shows no correlation with the length of the CAG expansion (Landles and Bates 2004; Cattaneo et al. 2005; Finkbeiner 2011).

It is the presence of mutant huntingtin protein that will lead to the development of HD, as the corresponding DNA in an un-induced mouse model did not result in symptoms characteristic of HD. In addition, the symptoms can be reversed by switching off mutant huntingtin expression (Yamamoto et al. 2000). Mutant huntingtin forms inclusion bodies (IBs) both in the cytosol and the nucleus of affected cells. Apart from huntingtin, the IBs also contain molecular chaperones, ubiquitin, and proteasomal subunits, illuminating the involvement of the PQC system in the cellular management of mutant huntingtin (Finkbeiner 2011). A screen in yeast identified proteins involved in protein folding, stress response, and ubiquitin-dependent protein catabolism as modifiers of mutant huntingtin toxicity (Willingham et al. 2003). By using ubiquitinated model proteins containing either 25 (normal) or 103 (mutant) glutamines it could be demonstrated that the proteasome degrades both versions equally well, suggesting that polyQ disease proteins can be degraded by the proteasome (Michalik and Van Broeckhoven 2004). Moreover, when the proteasome is inhibited there is an increase in IB formation (Wyttenbach et al. 2000), and protein aggregation, caused by a mutant huntingtin fragment, has been found to inhibit proteasome function (Bence et al. 2001) further linking the proteasome to HD.

The effect of molecular chaperones on polyQ aggregation and toxicity has been studied in many model systems. Hsp70, Hsp40, and CCT affect the aggregation state as well as toxicity of mutant huntingtin. For example overexpression of Hsp40 and Hsp70, alone or in combinations, suppress both aggregation and toxicity in cell- and mouse models of HD (Jana et al. 2000) and overexpression of either Hsp70 or Hsp40 together with the mutant form of huntingtin in yeast, altered the aggregation state of huntingtin from a large detergent-insoluble

IB to smaller detergent-soluble inclusions (Muchowski et al. 2000). Moreover, a screen in *Caenorhabditis elegans* identified Hsp70, Hsp40, and CCT subunits as important factors for suppressing premature polyQ aggregation (Nollen et al. 2004) and CCT has further been shown to reduce polyQ toxicity and alter aggregation (Kitamura et al. 2006; Tam et al. 2006). Collectively, molecular chaperones seem to affect the conformation of mutant huntingtin and thereby influence the aggregation process. Expression of mutant huntingtin leads to the upregulation of autophagy and it has been shown that polyQ expanded proteins can be degraded by autophagic clearance. Increased autophagy has also been associated with reduced IB formation, but it is not clear if the entire IB is engulfed or if it is IB precursors that are degraded by autophagy and thereby decreasing IBs (Finkbeiner 2011).

Toxic species of mutant huntingtin

Initially, the IB was suggested to be the cause of HD, but more recent studies have suggested that oligomers are the toxic species, and that IB formation is a mechanism for the cell to cope with misfolded proteins (Arrasate et al. 2004; Arrasate and Finkbeiner 2005). The present view is that systems of chaperones, proteasomes and autophagy are linked and together guide misfolded proteins through interconnected pathways for efficient refolding, clearance and/or sequestration to maintain a healthy proteome. This has been illustrated by two studies. First, in a cell model of HD, the level of a proteasome substrate increased shortly before an IB was formed, while the levels decreased again shortly after the formation of the IB (Mitra et al. 2009). This demonstrates that the workload of the proteasome changes during the formation of the IB, possibly because the proteasome is occupied either with the mutant huntingtin protein itself, or with increased amounts of misfolded proteins occurring as a consequence of a widespread cellular stress caused by the mutant huntingtin. As the mutant huntingtin is sequestered into an IB it either directly or indirectly lowers the pressure on the proteasomal system which now can continue to process the substrate (Mitra et al. 2009). In the second study the authors expressed proteins with temperature sensitive (ts) mutations in *Caenorhabditis elegans*. Under non-stress conditions, these proteins were correctly folded and fully functional. When the worms were subjected to a low heat stress, the ts protein started to misfold and form inclusions, a condition also occurring when expanded versions of polyQ proteins was expressed already under non-stress conditions (Gidalevitz et al. 2006). Thus it seems like metastable proteins and polyQ expanded proteins compete for assistance from the

chaperone system (Park et al. 2013). Too many unfolded proteins in the cell will overwhelm the PQC system. This can further be applied to the observation that relatively small differences in protein sequence can cause dramatic effects in toxicity. If the sequence difference influences protein stability it could be "the straw that broke the camel's back", especially if the protein is expressed at high levels, and/or when cells age and suffer from a decline in the PQC system. Since molecular chaperones recognize non-native substrates, proteins that are the most dependent upon chaperone assistance would be a substrate exposing the most unfolded regions, having the largest amount of chaperone binding sites. An obvious solution for reducing the workload for chaperones would be to hide the binding sites inside an IB. This has proven efficient since aggregation in some cases can mitigate toxicity (Arrasate et al. 2004; Kayatekin et al. 2014; paper V). If chaperones are busy assisting misfolded proteins, for example mutant huntingtin, other metastable proteins also requiring the chaperone system would fail in reaching their active native fold resulting in a loss of function for the cell and eventually even death and neurodegeneration.

Huntingtin exon-1 as a model protein

It has been shown in mice that expression of mutant exon-1 of huntingtin leads to intracellular aggregates and that the animals develop symptoms similar to those of patients with HD (Mangiarini et al. 1996; Davies et al. 1997). Further, inclusions detected in brains of deceased HD patients show fragments of huntingtin very similar to exon-1. These fragments of huntingtin are generated through proteolytic cleavage of the full-length protein (Landles et al. 2010). Another study found that there is aberrant splicing of the huntingtin mRNA that is translated into an Htt exon-1 fragment. This splicing of the mRNA is dependent on the length of the CAG repeat (Sathasivam et al. 2013). In yeast the aggregation pattern of huntingtin exon-1 follows that of mammalian cells. A glutamine stretch of 25 repeats is diffusely distributed (soluble) while repeat lengths of 47 or more leads to protein aggregation (Krobitsch and Lindquist 2000).

PolyQ flanking sequences' effect on toxicity

The polyQ expanded disease proteins resulting in neurodegeneration show both similarities and differences. They are all characterized by a critical length of the polyQ repeat associated with protein aggregation and disease manifestation. These disease proteins are also expressed

in several tissues but only causing death in a subset of cell types (Petrakis et al. 2013). They differ between each other in that their toxicity affects different neurons (table 1), and that their polyQ sequences are surrounded by different compositions of amino acids (Duennwald et al. 2006a; Duennwald et al. 2006b). Thus, the molecular mechanism behind the toxicity of the polyQ proteins causing neurodegeneration is not only due to the polyQ tract itself, but may be linked to sequences flanking the polyQ stretch.

During certain circumstances, and in special environments, the secondary structure of a protein can change, resulting in the protein becoming toxic. For example, protein misfolding diseases often occur due to changes in amino acid sequences, causing altered intra- and intermolecular interactions, giving rise to a disturbance of cellular homeostasis. The importance of adjacent sequences becomes evident when huntingtin exon-1 fragments are expressed in yeast. The polyQ length-dependent aggregation of huntingtin exon-1 seen in mammalian cells is recapitulated in yeast, but the link between toxicity and aggregation observed in mammals is, for yeast, dependent on the removal of the proline-rich domain (PRD) flanking the polyQ stretch (figure 6) (Dehay and Bertolotti 2006; Duennwald et al. 2006b). An investigation using yeast revealed that addition of an acidic FLAG tag can convert a benign mutant exon-1 fragment into a toxic version, whereas the endogenous PRD, direct C-terminal of the polyQ stretch, can transform a toxic version into a benign species. The aggregate morphology seems to influence the toxicity as one to two tight inclusions, with a small surface area, are benign, while several smaller inclusions, with a large surface area, can be both toxic and benign (Duennwald et al. 2006b).

The polyQ region of huntingtin exon-1 is suggested to be able to adopt several different secondary structures. This will have implications on what proteins the polyQ stretch can interact with (Kim et al. 2009). Expansion of the polyQ region adds another level to the alterations in binding properties and might promote abnormal and harmful interactions leading to aggregation and toxicity. Furthermore, the PRD, flanking the polyQ stretch, influences the structure of the polyQ stretch (Kim et al. 2009) as well as the aggregation properties of huntingtin exon-1 (Dehay and Bertolotti 2006). Proline-rich sequences have the property to bind specific protein domains, for example SH3- and WW domains (Kay et al. 2000). The protective property of the PRD in huntingtin might come from the recruitment of other proteins having either a direct or indirect protective influence on the structural conformation of the huntingtin protein as well as its interactome.

The 17 amino acid long N-terminal domain (Nt17) of huntingtin, directly N-terminal of the polyQ stretch, has been found to influence huntingtin aggregation and aggregate stability. The Nt17 domain can self-assemble and form α-helix rich oligomers. As oligomers are considered the toxic species of mutant huntingtin, the promotion of oligomerization by Nt17 may influence huntingtin toxicity. It has been demonstrated that synthetic polyQ peptides lacking the Nt17 domain directly form fibrillary structures rather than oligomeric intermediates (Arndt et al. 2015). Another study used synthetic polyQ peptides with various combinations of the huntingtin polyQ flanking sequences and showed that the Nt17 domain is necessary for the polyQ peptide to bind to lipid bilayers and that a C-terminal P_{10} sequence enhance this interaction (Burke et al. 2013).

Proteome alterations and huntingtin toxicity

Changing the proteome composition by deleting genes or over expressing proteins has been found to have large effects on whether mutant huntingtin is benign or toxic (Duennwald et al. 2006a; Kayatekin et al. 2014; Ripaud et al. 2014) and in yeast, the protein Rnq1 needs to be in its prion form [$RNQ1^{+}$] for proline-less huntingtin exon-1 to cause toxicity (Meriin et al. 2002). Increasing cellular levels of certain Q-rich proteins was found to suppress huntingtin toxicity by releasing important proteins normally bound to huntingtin. This competitive binding of the Q-rich proteins also led to a change in the physical property of the huntingtin aggregate, as determined by an increase in SDS solubility (Ripaud et al. 2014). Another study in yeast reported that overexpression of Q-rich prion domain (PrD) proteins suppressed mutant huntingtin toxicity. The PrD promoted sequestration of huntingtin oligomers into a benign IB, the IPOD (Kayatekin et al. 2014). A combination of protein sequences and their ability to interact with other cellular components influences cellular toxicity. Different cell types harboring different proteomes will have an important impact on the interaction possibilities for the disease proteins and consequently lead to different outcomes regarding toxicity.

Results and discussions

Paper I - Accelerated aging and failure to segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein aggregation-remodeling factor Hsp104p

In an earlier study it was shown that oxidatively damaged, carbonylated, proteins are asymmetrically inherited by the mother cell during yeast cytokinesis (Aguilaniu et al. 2003). This segregation of damaged proteins was demonstrated to be dependent on the histone deacetylase Sir2, since mutants lacking this gene displayed an equal distribution of damaged proteins between mother and daughter cell (Aguilaniu et al. 2003). By analyzing specific proteins targeted for carbonylation, it became clear that, under normal conditions, the daughter cell primarily inherited proteins from the un-damaged pool, whereas the mother cell inherited the damaged part of the proteome. This was the case for all proteins analyzed, suggesting that it is the damage *per se* that is key for the selective and asymmetrical inheritance of damaged proteins. Other studies demonstrated that proteins present in the plasma membrane are also prevented from being inherited by the daughter due to a diffusion barrier created by the septin ring (Dobbelaere and Barral 2004). Furthermore, ERCs accumulate in the nucleus of the mother cell since they bind pre-existing nuclear pores that are prevented from translocation into the bud compartment by a septin-dependent diffusion barrier formed in the nuclear envelope (Shcheprova et al. 2008). No such barrier appears to exist for cytoplasmic proteins, since native cytoplasmic GFP-tagged proteins diffuse freely between mother and bud during cell division (Dobbelaere and Barral 2004). The fact that damaged cytoplasmic proteins, in contrast, are prevented from leaking over to the daughter cell during cytokinesis indicates that different mechanisms exist for the asymmetrical segregation of damaged cytosolic proteins and plasma membrane proteins. The actin cytoskeleton was identified as an important component in establishing asymmetrical inheritance of damaged proteins and is therefore a likely component in an active retention process acting on cytosolic proteins (Aguilaniu et al. 2003). Further, it has been demonstrated that cells lacking Sir2 display an aberrant actin cytoskeleton (Erjavec and Nystrom 2007; Liu et al. 2010; Higuchi et al. 2013), creating a link between the absence of Sir2, a compromised actin cytoskeleton, and the failure in retaining damaged protein within the mother cell. By searching for damaged proteins within *sir2Δ* cells, additional components necessary for maintaining damage asymmetry may be identified.

In **paper I** we showed that aged *sir2Δ* cells display a two-fold higher degree of total protein carbonylation as compared to wild type cells of similar replicative age. We further established that not all proteins are equally subjected to carbonylation and that in *sir2Δ* cells, molecular chaperones suffer from a higher load of carbonyl modifications when compared to the average carbonylation level for the entire proteome and the relative abundance of the proteins analyzed. Why certain proteins are more prone to carbonylation is not fully understood but it has been demonstrated that aberrant proteins show a higher risk of being carbonylated compared to native species (Dukan et al. 2000).

Protein carbonylation is an irreversible oxidative modification, giving rise to conformational changes of the protein. This may lead to loss of enzymatic activity of the damaged protein and exposure of hydrophobic regions, which will increase the risk of aberrant protein interactions further promoting cellular injury. In young wild type cells the PQC system ensures efficient management of damaged proteins, and in the case of carbonylated proteins, sends them for proteolytical degradation (Nystrom 2005). In contrast, aged cells suffer from a limitation in the PQC system resulting in the accumulation of protein aggregates. Since it has been shown that proteins oxidized *in vitro* give rise to severely carbonylated proteins forming high-molecular weight aggregates (Bota and Davies 2002), together with the observation that the *in situ* carbonylation signal in aged cells becomes speckled, it raised the question if carbonylated proteins form aggregates *in vivo*. Indeed, we could show that both stress- and age-induced carbonyl signals are speckled rather than diffuse and to a large extent co-localize with the aggregate remodeling factor Hsp104 and that Hsp104 and carbonylated proteins co-purify. This suggests that carbonylated proteins can form aggregates within cells and that the cell uses Hsp104 to manage these aggregates. It also allows for the use of Hsp104 as a marker for detecting protein aggregates, a method used in subsequent studies (see below).

The breakdown of asymmetric inheritance of damaged proteins, together with the premature aging phenotype of *sir2Δ* cells could partly be due to the increased damage observed for the PQC components of *sir2Δ*. The levels of Hsp104 are similar between wild type and *sir2Δ* cells but Hsp104 in *sir2Δ* cells displayed a nearly two fold increase in carbonylation levels, already in an exponentially growing culture, consisting of 50% newly born daughter cells. The increased carbonylation of Hsp104 from *sir2Δ* cells was accompanied by a reduced activity of Hsp104 since cells lacking Sir2 were strikingly slower in dissociating stress-induced Hsp104-containing aggregates. In addition, overproduction of Hsp104 in cells lacking Sir2 partially suppressed both the loss of retention of damaged proteins as well as the

reduced replicative life span observed for *sir2Δ* cells. This was not seen for wild type cells, demonstrating that Hsp104 functions are not limiting in wild type cells but appears to be so in *sir2*Δ cells. Hsp104 function is vital for the retention of damaged proteins by the mother cell, since mutant cells lacking Hsp104 displayed an equal distribution of carbonylated proteins as well as a reduced replicative life span. A possible mechanism of action for Hsp104 in the segregation of carbonylated proteins is its association with carbonylated proteins together with either a direct or indirect interaction with the actin cytoskeleton, thereby anchoring the damaged pool of proteins to structures within the mother cell. This is supported by findings from another study demonstrating that the actin cytoskeleton can function as a scaffold for protein aggregates (Ganusova et al. 2006). Further discussion on this subject will follow in the subsequent sections.

The potential role of the actin cytoskeleton in the rejuvenation of yeast daughter cells was investigated further using Latrunculin A, a drug causing depolymerization of the actin cytoskeleton. An aged and budding wild type mother cell was subjected to a transient Latrunculin A treatment, resulting in the daughter inheriting a high load of damaged proteins. The drug was subsequently removed whereby the actin cytoskeleton re-formed and the mother cell initiated a new budding event. The second daughter, produced under conditions retaining an intact actin cytoskeleton, was born free of damaged proteins and harbored a full replicative capacity. In contrast, the first daughter, born with increased damaged proteins, displayed a 20% reduction in life span. This demonstrates that a proper actin cytoskeleton structure and function is required for generating daughter cells with a full replicative potential, possibly by ensuring that damaged proteins, and/or other aging factors, are not entering the daughter cell.

Apart from displaying increased levels of damaged proteins, the short-lived *sir2Δ* mutant also suffers from early accumulation of ERCs, due to the loss of silencing at the rDNA locus. This rapid accumulation of ERC has been suggested to be the cause of the premature aging of *sir2Δ* cells (Sinclair and Guarente 1997). However, it is possible that damaged proteins and their segregation also contribute to accelerated aging in Sir2-deficient cells. A *fob1Δ* single mutant contains almost no ERCs and harbors an increased replicative life span compared to wild type cells. However, a *fob1*Δ *sir2*Δ double mutant has similar levels of ERCs as a *fob1*Δ single mutant yet has a life span similar to that of a wild type strain. This suggests that Sir2-deificeny affects aging by mechanisms unrelated to ERC accumulation. Such mechanisms

could include a failure to segregate damaged proteins. Taken together, this suggests that both ERCs and damaged proteins play important, but parallel, roles in the replicative life of yeast cells.

Main findings paper I

- Aged cells lacking Sir2 have a higher total load of carbonylated proteins compared to their wild type counterparts, with molecular chaperones displaying especially high levels of carbonylation.
- Sir2 is required for daughter cells to receive proteins from the damage-free pool of the proteome.
- Hsp104 is associated with aggregated carbonylated proteins.
- Hsp104 functions are limiting in cells lacking Sir2, since over production of Hsp104 partially restores damage asymmetry and the reduced replicative life span of *sir2Δ* cells.
- A fully functional actin cytoskeleton is essential for the daughter cell to obtain a full replicative capacity.

Paper II - The polarisome is required for segregation and retrograde transport of protein aggregates

In **paper II** we investigated further what molecular mechanisms and cellular machineries contribute to the segregation of damaged proteins during cytokinesis. An earlier study, using mathematical modeling, suggested that a failure to segregate damaged proteins may lead to a reduction in cellular fitness (Erjavec et al. 2008). With this in mind, we performed synthetic genetic array analysis to establishing the global genetic interactors of *SIR2*, displaying equal distribution of damaged protein, to identify genes involved in cellular pathways parallel to *SIR2*. Thus, components with important functions in damage segregation may be revealed. The screen resulted in the identification of 122 genes showing reduced fitness together with *sir2Δ*. Cellular function analysis of the hits showed that genes involved in genomic silencing, nuclear export/import processes, and actin cytoskeleton processes were enriched among the genetic interactors of *SIR2*. The group 'actin cytoskeleton processes' was of great interest to us since it was established earlier that the actin cytoskeleton is required for the retention of damaged proteins in the mother cell during cytokinesis (Aguilaniu et al. 2003; Erjavec et al. 2007). Several genes involved in establishing cell polarity via the polarisome complex, for example *BNI1*, *BUD6*, *MYO2*, *RHO3*, and *PEA2*, showed a genetic interaction with *SIR2*. To elucidate if it is the de-acetylating activity of Sir2 that is important for polarity functions, we used a catalytically inactive version of *SIR2*, *SIR2(H364Y)*, in parallel with the wild type version of *SIR2* to test for complementation of the increased sensitivity to Latrunculin B observed in the double mutant *sir2Δbni1Δ*. Only the wild type version could suppress the increased sensitivity to Latrunculin B observed for the double mutant.

An interesting question is how Sir2, a nuclear-localized histone deacetylase, can influence protein damage within the cytosol. Perrod and colleagues showed that increased levels of the cytosolic Sir2 homologue Hst2 resulted in altered Sir2-mediated DNA silencing although Hst2 remained in the cytosol. Based on these results, the authors suggest that Sir2 and Hst2 share a common ligand, which can be both nuclear and cytosolic, that becomes limiting for Sir2 when there is more Hst2 present (Perrod et al. 2001). In the absence of Sir2 such a ligand could potentially bind Hst2 and inhibit its activity. To test if Hst2 has a genetic interaction network similar to that of *SIR2*, SGA analysis was performed with *HST2* as query strain. Apart from three common genes, the genetic interaction networks of *SIR2* and *HST2* were completely different. *HST2* did not buffer for the loss of any of the genes involved in actin cytoskeleton processes or the genes involved in cell polarity identified in the *SIR2* screen.

This suggests that the failure of *sir2*Δ cells to asymmetrically segregate damaged proteins is not due to limiting Hst2 activity.

As discussed previously, it was observed that cells lacking Sir2 display an aberrant actin cytoskeleton (Erjavec and Nystrom 2007; Higuchi et al. 2013). Moreover, *SIR2* shows genetic interactions with the ts alleles *act1-133* and *cct6-18*, further indicating that actin functions are reduced in *sir2Δ* cells. Consistently, it was demonstrated that *sir2Δ* cells contain less native actin compared to wild type cells while the total levels of actin (native and non-native) were similar between the two strains. Actin is dependent upon the chaperonin CCT, which ensures that a quasi-native form of actin reaches its fully native state. Purified CCT from wild type and *sir2Δ* cells was analyzed to identify possible functional differences to account for the compromised actin cytoskeleton and the reduced pool of native actin in cells lacking Sir2. A folding assay using a non-native actin species produced in an *E. coli in vitro* translation system as substrate for CCT from wild type or *sir2Δ* cells, demonstrated that CCT from a *sir2Δ* strain produced native actin at a slower rate compared to CCT from a wild type strain.

CCT from cells lacking Sir2 displayed a higher level of lysine acetylation, linking de-acetylating activity (potentially performed by Sir2) to a reduced production rate of native actin. Lysine acetylation is a reversible post-translational modification, where the addition of an acetyl group to a lysine residue abolishes the positive charge of the lysine side chain. This alteration may induce a conformational change in protein as well as influence interactions between the protein and its substrates (Xiong and Guan 2012). Mass spectrometry data gave indications of a specific lysine residue, namely K493, being acetylated in CCT1 from *sir2Δ* cells, while the same lysine was un-modified in CCT1 from wild type cells (unpublished data). Lysine 493 resides in a hydrophilic stretch of amino acids, present within an exposed loop, not far from the ATP binding site, based on the crystal structure of the thermosome (Ditzel et al. 1998). It is possible that the presence of an acetyl group on K493 of CCT1 from *sir2Δ* cells may result in conformational changes within the CCT oligomer, which may have a negative effect on the folding efficiency of CCT. This could be tested by mutating K493 of CCT1 to amino acids either mimicking a non-acetylated lysine or a constitutively acetylated lysine, with subsequent analysis of the rate of actin folding by the CCT mutants. Earlier studies have demonstrated that subtle changes to CCT can have large effects on the actin cytoskeleton. It was shown in yeast that the ts allele *cct4-1*, containing a point mutation in the apical domain of CCT4 (Llorca et al. 1999), shows an abnormal actin cytoskeleton (Vinh and Drubin 1994) and a reduction in the capacity of CCT to fold actin *in vitro* (Shimon et al.

2008). An obvious question is if Sir2 directly de-acetylates CCT, which might be possible since Sir2 lacks a classical nuclear localization sequence and might therefore also reside the cytosol. Sir2 has also been found to de-acetylate the cytosolic protein Pck1, indicating that Sir2 can perform de-acetylating functions outside of the nucleus (Lin et al. 2009). At the same time, in yeast, CCT has been found to physically interact with nuclear pore proteins and nuclear proteins (Dekker et al. 2008), and in mouse embryonal carcinoma cell lines, certain CCT subunits have been found to localize inside the nucleus (Roobol and Carden 1999). Maybe CCT subunits can enter the nucleus, where regulation of post-translational modifications might occur (potentially involving Sir2) and re-locate back to the cytosol for folding cytosolic substrates. Initial protein interaction studies performed in vitro revealed that yeast Sir2 interacts with CCT from a rabbit reticulocyte lysate, highly enriched in CCT (unpublished data).

To elucidate if negative genetic interactors of *SIR2* also have a role in the segregation of damaged proteins, a selection of hits from the screen was analyzed with regards to their segregation of protein aggregates. In this paper we used a transient heat shock to induce protein aggregation and the aggregate remodeling factor Hsp104-GFP to study the behavior of the misfolded and aggregated proteins. The mutant strains *bni1Δ*, *bud6Δ*, and *rho1-1*, all affecting the establishment of polarity, displayed a reduced retention of aggregated proteins in the mother cell. Interestingly, the polarisome associated formin Bni1, but not the septin ring associated formin Bnr1, was important for the mother cell to retain damaged proteins. This implies that the polarisome functions in enabling proper segregation of protein aggregates during cell division. Heat-induced protein aggregates behaved similar to age-induced protein aggregates, illustrated by the reduced aggregate retention of aged mother cells lacking Bni1. Moreover, the ts allele *act1-133* showed aggregate segregation defects. Actin encoded by the *act1-133* allele has mutations in the myosin binding site resulting in reduced myosin binding to actin cables (Smith et al. 1995), suggesting a role of type-V myosins in damage segregation. Type-V myosins, Myo2 and Myo4, use actin cables for the transportation of cargo needed to build up the daughter cell. Myo2 delivers secretory vesicles, and whole organelles, such as vacuoles, peroxisomes, mitochondria, and late Golgi-elements via a tail region, absent in Myo4. Myo4 on the other hand, delivers mRNA to the bud (Schott et al. 1999). Mutants of Myo2, but not Myo4, showed a protein aggregate segregation defect. This

can potentially be the result of the polarisome protein Bud6 becoming localized to the bud via the Myo2-dependent secretory vesicle pathway (Jin and Amberg 2000).

Real-time imaging analysis of protein aggregate movements during cell division demonstrated that daughter cells could clear themselves from heat-induced aggregates by transporting the aggregates into the mother cell as long as the septum between the mother and daughter was open. This required *SIR2* and *BNI1*, but not *BNR1*. The aggregate clearance by daughter cells seemed to be dependent on linear actin filaments, since a *tpm1Δ* strain, showing a reduction in actin cables (Liu and Bretscher 1989), also showed reduced aggregate clearance by the daughter cell. In line with this, the Arp2/3 complex, responsible for the formation of branched actin filament network present at cortical actin patches, was dispensable for this clearance process. Hsp104-containing aggregates were found to localize to regions rich in actin cables rather than actin patches. Moreover, an *in situ* proximity ligation assay confirmed that after a heat shock, Hsp104 and actin were in close proximity to each other. The results from **paper II** allow for an expansion of the model where in addition to the requirement of Sir2, an intact actin cytoskeleton and the aggregate remodeling factor Hsp104, the polarisome and the molecular chaperone CCT are additional factors necessary for asymmetrical inheritance of aggregated proteins (figure 7). CCT provides native actin molecules, which are used by the formin Bni1 for the nucleation and polymerization of linear actin cables at the bud tip. In the case of aggregates present in the daughter, this allows for a retrograde transport of Hsp104-containing protein aggregates away from the bud and towards the mother by interactions of Hsp104 and actin molecules within the actin cable. In a study by Higuchi and colleagues the authors demonstrated that cells lacking Sir2 showed a reduced retrograde actin cable flow (Higuchi et al. 2013). This observation is supported by the reduced CCT folding rate of actin in a *sir2Δ* strain and the increased inheritance of protein aggregates by *sir2Δ* daughters. The actin retrograde movement is estimated to have a speed of 0,29 ± 0,08 µm/sec (Yang and Pon 2002), while the retrograde movement of Hsp104-containing aggregates appears to be slower. A possible explanation for the differences would be that the binding of Hsp104-associated aggregates to the actin cable is dynamic. If Hsp104 goes through cycles of binding and releasing the actin cable the net movement of the aggregate would be slower compared to actin molecules within the cable. Mutations affecting the actin cytoskeleton, for example mutants lacking Tpm1, Bni1 or Bud6, which all show fewer visible actin cables, could increase the risk of Hsp104 failing to bind actin cables and efficiently transport aggregates towards the mother cell. Furthermore, it is not known how large protein aggregates affect the

actin cable dynamics. The presence of a large aggregate could have a negative impact on the retrograde cable flow.

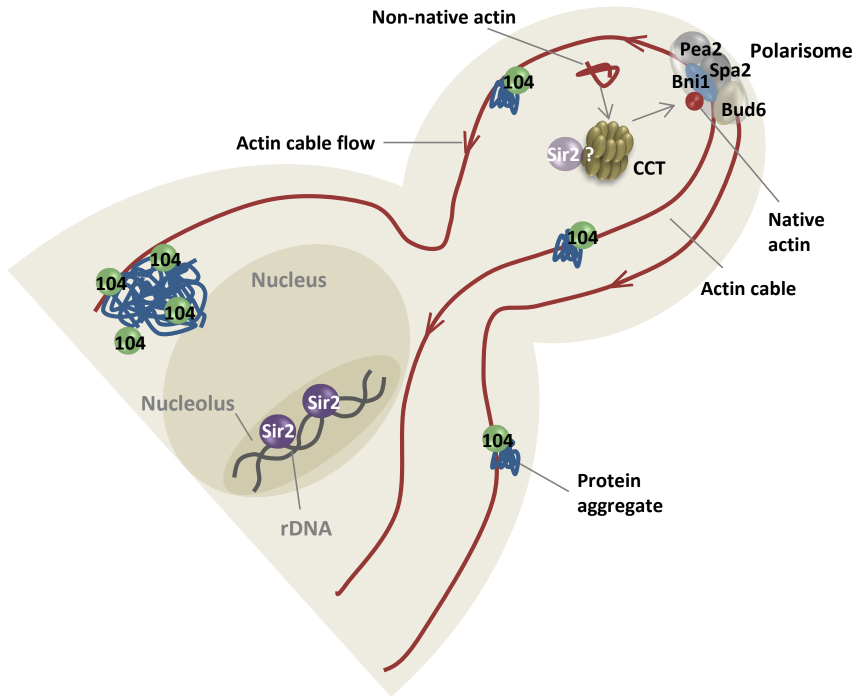

Figure 7. Factors needed for damage asymmetry. Sir2, an intact actin cytoskeleton, Hsp104, and the polarisome complex are important players in the asymmetrical inheritance of damaged proteins. We propose a model where CCT actin folding activity is either directly or indirectly regulated by the deacetylating function of Sir2. CCT provides native actin molecules to the polarisome formin Bni1, which polymerize linear actin filaments (cables) at the polarisome complex present at the bud tip. The retrograde flow of actin monomers within the cable enables the Hsp104-associated protein aggregates, tethered to the actin cable, to be retained within the mother cell, or promotes the retrograde transport of such aggregates from the bud into the mother.

Main findings paper II

- The global genetic interaction network of *SIR2* identified the polarisome, the formin Bni1 and the myosin motor protein Myo2 as important components of the machinery segregating protein aggregates during yeast cytokinesis.

- Daughter cells can clear themselves from protein aggregates by a polarisome- and tropomyosin-dependent mechanism.
- CCT in cells lacking Sir2 is less efficient in folding actin and shows a higher degree of lysine acetylation when compared to CCT from a wild type strain. Sir2-deficient cells display a reduced level of native actin monomers.

Paper III - Segregation of protein aggregates involves actin and the polarity machinery

Eukaryotic cells experiencing protein misfolding and aggregation can organize the damaged proteins into specific spatial quality control deposits; aggresomes, IPOD and JUNQ deposits in mammalian cells (Kopito 2000; Kaganovich et al. 2008) while yeast cells contain IPOD, JUNQ/INQ, and stress foci (Kaganovich et al. 2008; Specht et al. 2011; Spokoini et al. 2012) Within yeast cells, spatial sorting of misfolded proteins into such deposits as well as the asymmetrical segregation of such deposits/aggregates have, as described, been argued to involve the actin cytoskeleton, the polarisome, Sir2, and Hsp104 (Aguilaniu et al. 2003; Erjavec et al. 2007; Tessarz et al. 2009). However, the need for such factors has been questioned by Zhou et al 2011, and an alternative model was proposed in which asymmetry was argued to be entirely due to slow diffusion of aggregates and physical constraints of the bud-neck region. In **paper III**, we respond to this model.

In the study by Zhou et al 2011 the authors use computational modeling and particle tracking experiments to suggest that the motility of protein aggregates are completely random and un-directional. They also argue that the asymmetrical inheritance of protein aggregates during yeast cell division depends on passive diffusion of the aggregates, only prevented from entering the bud due to the transient opening of and the limiting diameter of the bud neck, and not through an active factor-dependent process (Zhou et al. 2011). In this model of passive diffusion of aggregates, such an aggregate is equally likely to move anterograde into the daughter and retrograde into the mother cell. Analyzing such cross-compartment movements of protein aggregates should only be made when such a mechanism is possible. This is when the polarisome is present at the bud tip and actin cables are extending into the mother compartment, which occurs during the S to early G2 phase of the cell cycle. Accordingly, we analyzed 393 wild type budding events for cross-compartment movements of aggregates. In 15% of the 393 budding events the protein aggregates showed cross-compartment movements. For 66.5% of these, the aggregates were moving from bud to mother, while only 25.4% showed aggregate movements in the opposite direction. The remaining budding events, 8.1%, showed aggregate movements in both directions. This clearly demonstrates a bias towards a retrograde movement of protein aggregates from bud to mother. In the study of Zhou et al 56-84 such budding events were analyzed.

The use of fluorescently tagged Hsp104^{Y662A}, without ATPase activity, allowed for visualization of stable protein aggregates. These aggregates formed a pattern of thread-like structures within the cell that showed a high degree of co-localization with fluorescently tagged Abp140, which binds actin cables. Since the actin cytoskeleton is not diffusing randomly within the cell, it seems highly unlikely that structures that align with actin cables would move randomly. These results, argue against the model that movement of protein aggregates is purely due to random diffusion.

We also used another model protein to analyze the inheritance of protein aggregates, the aggregation-prone disease protein Htt103Q-GFP. In yeast, expression of HTT103Q from a galactose inducible promoter results in the formation of several smaller aggregates without the need of preceding heat treatment. This allows for studying aggregate inheritance without increasing the temperature, which will cause a transient collapse of the actin cables. While wild type mothers actively retained the protein aggregates during cell division, mothers lacking the formin Bni1 displayed a reduction in the retention capacity.

It should be noted also that the factor-dependent model states that the key underlying mechanism for the establishment of asymmetric inheritance of protein aggregates is the prevention of the aggregates entering the daughter compartment in the first place, not retrograde movement. This active retention is suggested to depend on actin cytoskeleton structures functioning as a scaffold for Hsp104-containing aggregates. The observed retrograde movement of protein aggregates from bud to mother is therefore not the major contributor to the mother-biased segregation of damaged proteins.

Main findings paper III

- There is a bias towards the bud-to-mother movement of protein aggregates traversing the bud neck during yeast cytokinesis.
- Stable Hsp104-containing aggregates co-localize with actin cables.
- Proper polarisome function is required for the inheritance of Htt103Q aggregates by mother cells.

Paper IV - Essential genetic interactors of *SIR2* required for spatial sequestration and asymmetrical inheritance of protein aggregates

In **paper IV**, we tested if the asymmetrical inheritance of two reporters, the aggregation-prone disease protein Htt103Q and heat induced Hsp104-containing aggregates, during yeast cytokinesis was more likely to be the result of a passive diffusion (Zhou et al. 2011) or a factor-dependent mechanism (Liu et al. 2010; Liu et al. 2011). The model protein Htt103Q forms several very stable protein aggregates and since it is expressed from an inducible promotor it can be transiently expressed then aggregate inheritance analyzed. For wild type cells, most of the aggregates localized to the mother compartment during the following round of cytokinesis. In contrast, cells lacking Sir2 showed an almost two-fold increase in daughter cells containing aggregate(s). These results are comparable with the ones presented for cells lacking Bni1 in **paper III**. Two factors that could contribute to the asymmetrical inheritance of damage proteins proposed by the authors in the Zhou et al 2011 study, were the diameter of the bud neck and the time for free passage between the mother and daughter (generation time). These traits were measured in wild type cells and *sir2Δ* cells, displaying a reduced asymmetrical inheritance of protein aggregates. Results show clearly that there are no differences in the bud neck diameter, generation time, or number of aggregates per cell for the two strains. Thus, these traits cannot explain for the increased inheritance of damaged proteins by *sir2Δ* daughter cells.

An unbiased search for additional factors affecting the inheritance of damaged proteins was performed by extending the genetic interaction network of *SIR2* to also include essential genes. In addition to earlier identified genetic interactors of *SIR2*, such as genes involved in actin polarity, folding and nucleation (**paper II**), genes with a role in tubulin-dependent functions, ER-Golgi trafficking, chromatid segregation, and proteasomal degradation were discovered. Among the essential genes showing a negative genetic interaction with *SIR2*, genes involved in tubulin organization and ER/Golgi trafficking and functions also displayed a reduction in Hsp104-containing aggregate asymmetry. A number of mutants were also selected and analyzed for asymmetrical segregation of Htt103Q aggregates. All alleles tested, *cmd1-1*, *myo2-14*, *Sec53-6*, and *sec18-1*, displayed a reduction in Htt103Q aggregate asymmetry. The mutant allele of calmodulin, *cmd1-1*, had a large impact on the aggregate asymmetry. Calmodulin is normally involved in a wide variety of cellular processes, but not all are affecting damage asymmetry. Functions related to actin cable organization, vacuolar inheritance, and formation of the SPB were important for generating damage asymmetry,

while calmodulin-dependent microautophagy was not. This further demonstrates that specific cellular processes are involved in generating damage asymmetry, thus supporting the model where the asymmetrical inheritance of damaged proteins requires certain cellular factors.

A comparison between the behavior of heat-induced Hsp104 aggregates and Htt103Q aggregates revealed both common and specific traits. For example, Hsp104-containing aggregates need the assistance of the small heat shock protein Hsp42 to form stress foci (Specht et al. 2011) while Htt103Q aggregates form independent of Hsp42. The two types of aggregates co-localize immediately after heat shock, but while Htt103Q aggregates remain as smaller amorphous aggregates, Hsp104-containing aggregates are later directed to the deposit sites JUNQ/INQ and IPOD (Kaganovich et al. 2008). These large deposit sites are found to be tethered to the nucleus and the vacuole, ensuring their inheritance by the mother cell during the following cell division (Spokoini et al. 2012). Moreover, the factors Cmd1 and Myo2, both required for damage asymmetry, each co-localized with both Hsp104 and Htt103Q aggregates, whereas Sec53 only co-localized with Hsp104-associated aggregates. This demonstrates that, depending on the misfolded protein, there seem to be different cellular components involved in the formation and sorting of the forming aggregate. Although diversities exist between aggregates, the cell appears to contain certain basic mechanisms to prevent the inheritance of damaged and harmful components by the daughter. In **paper IV**, by using super-resolution microscopy, we could demonstrate that both heat-induced Hsp104-containing aggregates and Htt103Q aggregates align with actin cables and even seem to wrap around the cables. Supporting that in yeast, the actin cytoskeleton provides the means for the process of damage asymmetry (Aguilaniu et al. 2003; Erjavec et al. 2007; Tessarz et al. 2009; Liu et al. 2010; Liu et al. 2011). Taken together, results presented in **paper IV** strongly argues for the inheritance of protein damage during cell division being a factor-dependent process.

Main findings paper IV

- The asymmetrical inheritance of damaged proteins cannot be explained by the traits bud neck diameter, generation time, or number of aggregates.
- Additional factors, such as Cmd1, Myo2, Sec53, and Sec18, were identified in having a role in the establishment of damage asymmetry.
- Htt103Q and stable Hsp104-containing aggregates align with actin cables, visualized with super-resolution 3D SIM microscopy.

- The asymmetrical inheritance of aggregates during cell division most likely depends on a factor-dependent process.

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Main findings paper V

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Concluding remarks and future perspectives

In this thesis I have shown that in yeast, the actin cytoskeleton contributes to protein quality control at different levels. First, actin plays a major role in the asymmetrical inheritance of damaged proteins during yeast cytokinesis. The histone deacetylase Sir2 is affecting the integrity of the actin cytoskeleton via regulating the molecular chaperone CCT, required for the folding of actin. The polarisome complex, present at the bud tip, is dependent on the existence of native actin molecules for the polymerization of linear actin filaments and the establishment of a retrograde actin cable flow from bud to mother. The retrograde flow of actin may be utilized by Hsp104 in mediating interactions between protein aggregates and the actin cytoskeleton for both the retention of protein aggregates by the mother cell and, in the case of protein aggregates present in the bud, the retrograde transport of aggregates from the bud to the mother compartment (**paper II**). We could show that aggregates co-localize with actin cables and even seem to wrap around the cable (**paper III** and **IV**). The importance of the actin cytoskeleton in producing rejuvenated daughters, was demonstrated as a transient collapse of the actin cytoskeleton resulted in a daughter being born with increased damage accompanied by a reduction in the replicative life span (**paper I**).

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The asymmetrical inheritance of damaged and aggregated proteins is a general process present in bacteria (Lindner et al. 2008; Winkler et al. 2010), yeast (Aguilaniu et al. 2003; Erjavec et al. 2007; Liu et al. 2010; Spokoini et al. 2012), and mammals (Rujano et al. 2006; Bufalino et al. 2013; Ogrodnik et al. 2014). An arising question is how this segregation is carried out in higher eukaryotes. Is the segregation of damaged proteins in mammals dependent on similar factors as in yeast? Or, are there other systems responsible for this mechanism? It is known that the mammalian aggresome is formed from a microtubule-dependent transportation of smaller aggregates (Kopito 2000) while the formation of the

protein deposit sites in yeast requires the actin cytoskeleton (Specht et al. 2011). Further, a study by Ogrodnik et al 2014, using mammalian cell lines, could show that the mammalian IB JUNQ, along with the intermediate filament protein vimentin, were asymmetrically inherited by one daughter cell. As a result, the daughter free of JUNQ displayed a minor fitness advantage (Ogrodnik et al. 2014). Yeast cells lack intermediate filaments (Coulombe and Wong 2004) and it therefore appears to be some specific traits that are different between yeast and mammals in the segregation of damaged proteins. One such difference may depend on the use of polarized actin cables for long-range transportation of various cargo by yeast cells, while this role in mammalian cells is mainly fulfilled by microtubules (Moseley and Goode 2006; Girao et al. 2008). There is however still a requirement for the actin cytoskeleton for short-range transportation in mammalian cells, where for example early endocytic vesicles use myosin-dependent transportation on cortical actin structures before they are delivered to microtubule motors (Soldati and Schliwa 2006). It would therefore be interesting to elucidate the potential role of the actin cytoskeleton in the spatial sequestration and segregation of damaged proteins within higher eukaryotes. Potentially, the molecular chaperone CCT, which is essential for the production of both actin and tubulin, plays a role in the segregation of damaged proteins also in higher eukaryotes. A better knowledge about the molecular mechanisms behind the deposition and segregation of damaged proteins in mammalian cells might lead to a better understanding of the underlying causes of age-related proteopathies.

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