Thesis for the degree of doctor of philosophy

On Aging and the Role of Ubp3 in Heterochromatic Gene Silencing and Protein Quality Control

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Cover picture: an old *ubp3*Δ mutant cell displaying protein aggregates in green (Ssa2-GFP), bud scars in red (WGA) and the nucleus in blue (DAPI). The image is presented as a maximum intensity projection of several Z-stack widefield fluorescence images.

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To Family and Friends

Abstract

Aging is characterized by a build-up of damage in organisms ranging from protists to multi-cellular species. This damage adversely affects core components such as DNA and proteins which are necessary to sustain life. Remarkably, as an old yeast cell divides, its daughter cell is fully rejuvenated suggesting that age-related damage can be asymmetrically inherited and/or completely ameliorated. This thesis approaches the central question of how cells combat such damage to allow longevity.

Specific interest was directed towards the deubiquitinating enzyme Ubp3 which had already been shown to be tightly linked to regulation of transcription and proteome surveillance, both of which are essential in cells adaptation to stress. In this thesis, I show that cells lacking Ubp3 are short-lived despite displaying decreased unequal recombination at rDNA and increased silencing at all three heterochromatic regions in *S. cerevisiae* subjected to transcriptional silencing. These findings are at odds with existing aging-models in yeast, highlighting that increased silencing at rDNA is associated with long lifespan. Instead, our data suggest that premature aging in cells devoid of *UBP3* is caused by a pathway other than rDNA recombination/silencing. Indeed, I found that Ubp3 has an important dual role in protein quality control by saving or destroying aberrant protein species depending on the stage at which the damaged protein is committed for proteasomal destruction. Furthermore, in virgin and young cells lacking *UBP3*, aggregated proteins accumulated prematurely at a juxta-nuclear position whereas wild-type cells showed no indication of protein damage. In middle-aged and older cells in the same mutant, more aggregates accumulated at a peripheral location. This accumulation of peripheral aggregates correlated, in time, with a decline in mutant cell survival.

Similar to Ubp3, the well-characterized silencing-factor Sir2 is known to regulate other aging-processes unlinked to silencing. We show that Sir2-deficient cells display increased daughter cell inheritance of stress and age-induced misfolded proteins deposited in aggregates and inclusion bodies. This asymmetric inheritance has been argued to take place in a passive manner due to slow and random diffusion of aggregates. We present evidence that this is not a plausible scenario. The control of damage inheritance is more likely mediated by Sir2-dependent regulation of the chaperonin CCT which is required for folding actin and feeding the polarisome with properly folded substrates. We discuss data underlying these conflicting models and seek to understand which model best explains how damage asymmetry is achieved.

Keywords: Aging, protein damage, segregation, transcriptional silencing, rDNA, *UBP3*, *SIR2*, protein aggregates

Abbreviations

ATP	Adenosine triphosphate
CPY	Carboxy peptidase Y
CVT	Cytoplasm to vacuole targeting
DNA	Deoxyribonucleic acid
DSB	Double strand break
DUB	Deubiquitinating enzyme
eccDNA	Extra chromosomal circular DNA
E-Pro	EXP-promoter
ER	Endoplasmatic reticulum
ERAD	Endoplasmatic reticulum associated degradation
ERC	Extra chromosomal circle
ERQC	Endoplasmatic reticulum quality control
GFP	Green fluorescent protein
Hsp	Heat shock protein
IPOD	Insoluble protein deposit
JUNQ	Juxta nuclear quality control compartment
MAT	Mating type
MTC	Mitochondrial translation control
NPC	Nuclear pore complex
ORC	Origin recognition complex
PQC	Protein quality control
rDNA	Ribosomal DNA
RENT	Regulator of nucleolar silencing and telophase exit
RFB	Replication fork block
RLS	Replicative lifespan
RNA	Ribonucleic acid
RNAPI	Ribonucleic acid polymerase I
RNAPII	Ribonucleic acid polymerase II

RNAPIII	Ribonucleic acid polymerase III
ROS	Reactive oxygen species
SGA	Synthetic genetic array
TF	Transcription factor
TFIID	Transcription factor II D
TFIIE	Transcription factor II E
TFIIH	Transcription factor II H
TOR	Target of rapamycin
ts	Temperature sensitive
Ub	Ubiquitin
UPS	Ubiquitin proteasome system

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Papers included in this thesis:

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II. Öling D, Eisele F, Kvint K, Nyström T (2014) Opposing roles of Ubp3-dependent deubiquitination regulate replicative lifespan and heat resistance. *The EMBO journal* **33:** 747-761

III. Liu B, Larsson L, Caballero A, Hao X, **Öling D**, Grantham J, Nyström T (2010) The polarisome is required for segregation and retrograde transport of protein aggregates. *Cell* **140:** 257-267

IV. Liu B, Larsson L, Franssens V, Hao X, Hill SM, Andersson V, Höglund D, Song J, Yang X, **Öling D**, Grantham J, Winderickx J, Nystrom T (2011) Segregation of protein aggregates involves actin and the polarity machinery. *Cell* **147:** 959-961

Other publications:

V. Caballero A, Ugidos A, Liu B, **Öling D**, Kvint K, Hao X, Mignat C, Nachin L, Molin M, Nyström T (2011) Absence of mitochondrial translation control proteins extends lifespan by activating sirtuin-dependent silencing. *Molecular Cell* **42:** 390-400

1. Introduction

1.1 Background

A consequence of modern medicine is that the quality and length of life has improved. One result of this is that there is a shift in the number of aged individuals in our population. A positive trend indeed, but this benefit comes with a cost as the primary source for many illnesses and diseases is old age. It is therefore of importance to understand the underlying mechanisms of aging in general, and age-related disorders in particular so that we can enjoy a full and healthy life.

1.2 Theories of aging

Aging is often defined as an organism's time-dependent loss of tissue and cellular function accompanied with decreased fertility and increased mortality (Kirkwood & Austad, 2000). However, there are organisms that do not follow the conventional pattern of changes in mortality and fertility usually accompanied by old age. One example is the mute swan (*Cygnus olor*) that shows, contrary to humans, an increase in fertility and near constant mortality rate during its lifespan (Jones et al, 2014). Such examples aside, there are several theories describing the aging process, none of which are mutually exclusive and should be viewed as complementary. However, most theories adopt well to the declaration by Dobzhansky, that "nothing in biology makes sense except in the light of evolution" (Ayala, 1977).

Weismann's theory of programmed death was one of the first aging hypotheses based on Darwinian evolutionary theory (Weismann, 1882). The

concept of programmed death implies that natural selection inheritably programs death to occur in order to limit the lifespan of an individual. As a consequence, old and new generations will not compete for the same resources and this will benefit the whole population (not only the individual). Other similar and more contemporary "evolvability" theories of aging have been proposed, highlighting that a programmed death aids a population by allocating resources to the younger members (Goldsmith, 2008; Skulachev, 2011). However, it should be noted that Weismann later discarded his theory in favor for the "germ-plasm" theory stating that the immortal "germ-line" transfers the hereditary material whereas the soma (somatoplasm) "ages" (Weismann, 1891). In this theory, aging of the soma occurs due to resource allocation to the maintenance of the germ-line.

Starting around 1950, the classical theories of aging arose as a response to the theory of "programmed death". The forerunners were Haldane, Medawar and Williams. Haldane studied the prevalence of Huntington's disease and was stunned by its frequency found in the English population. He suggested that the high frequency of this dominant, deadly neurological disease was due to the late onset of symptoms and that it therefore escaped natural selection. Medawar elaborated this idea in the theory of "mutation accumulation" (Medawar, 1952). As the name implies, it stipulates that detrimental mutations accumulate with old age. According to this theory, these mutations would not be under pressure of natural selection as it is rare to find old individuals in "the wild" due to predation and disease. Indeed, in 1966, Hamilton presented mathematical evidence coined "Hamilton's forces of natural selection" that supported the idea that natural selection declined with age (Hamilton, 1966).

Williams formed the first theory, coined *antagonistic pleitropy*, that implied certain "aging genes" (Williams, 1957). The name suggests that certain genes are beneficial early in life but at post-reproductive age, they may become

detrimental. Later, in 1977, Kirkwood presented a theory that was less based in population genetics than previous ideas. His idea of aging sought answers in the physiology of the body and reproduction. The theory, coined the "disposable soma" envisions the body as disposable and the germ-line as non-disposable (Kirkwood, 1977). According to this theory, the maintenance of cellular (soma) damage competes for energy with the reproductive system (germ-line). A prediction of this theory is that the soma will gradually deteriorate as energy is allocated from the soma to keep the germ-line intact.

The classical theories of aging has generated three major predictions. First, it is not likely that specific genes have been selected to promote aging. Second, aging is not programmed but rather a consequence of the accumulated somatic damage generated due to limited investments in maintenance and repair. Finally, there are genes whose actions may be adverse at old age (Kirkwood & Austad, 2000; Williams, 1957). These genes may simply have escaped natural selection (Huntington's disease), or they are pleiotropic in the sense that the organism enjoys positive effects of the allele at a young age but adverse effects at older age. More evidence and clear cut examples in support of the latter scenario is still pending. However, the target of rapamycin (TOR) drives growth and protein synthesis whereas its inhibition promotes aging. It has been suggested that these features make the TOR-encoding genes fall in to the category of antagonistic pleitropy (Blagosklonny, 2010). In this context it is interesting to note that most pathways regulating longevity are in some manner accompanied by an increased stress tolerance.

1.3 Yeast as a model system for studying aging

Research in mammalian cell systems is ideal to gather experimental evidence and draw conclusions relevant for humans. However, there is one big problem; their long lifespans. Rats and mice live 3-5 years and primates up to 40 years (Steinkraus et al, 2008). Nevertheless, research done in rodents has contributed greatly to our understanding of aging pathways. At the cellular level, human or mouse fibroblasts have traditionally been the preferred cell model as they have a limited proliferative capacity before they reach senescence - the Hayflick limit (Hayflick, 1965).

Invertebrate organisms have proven to be invaluable for aging-research because of their short lifespans and that they are easily manipulated genetically and environmentally. The most common invertebrate model systems are fruit flies (*Drosophila melanogaster*), worms (*Caenorhabditis elegans*) and yeast (*Saccharomyces cerevisiae*) with lifespans ranging from months to weeks, down to days. Yeast, in fact, may serve as a unicellular model to study both antagonistic pleiotropy and the disposable soma theory of aging since, like old animals, old yeast cells are rare in a population.

Yeast provides an exceptional model system when studying aging in general and cellular segregation in particular. Yeast divide asymmetrically by budding, leaving the larger mother with a bud scar (Seichertova et al, 1975) and a larger cell volume (Hartwell & Unger, 1977). Utilizing these characteristics of yeast, Robert Mortimer was the first to perform a replicative lifespan (RLS) analysis already in the late 1950s (Mortimer & Johnston, 1959). He did not, however, attempt to link replicative aging in yeast to higher multi-cellular organisms.

Is it feasible to utilize a single cell yeast species such as *Saccharomyces cerevisiae* to predict cellular mechanisms in multi-cellular

organisms? Yes, one hypothesis proposes that yeast can be used to predict replicative aging for mitotic or stem cell populations in complex organisms (Longo et al, 2012). Yeast has also been used extensively to study chronological aging which is thought to share features with non-dividing or post-mitotic mammalian cells (Longo et al, 2012). In addition, many pathways known to regulate aging such as calorie restriction is conserved in yeast, worms, flies and many more organisms (Masoro, 2005). Yeast has also been utilized to study *SGS1*, the yeast homologue of the human *WRN* gene which is implicated in Werner's syndrome, a rare genetic disorder characterized by premature aging in young adults (Yamagata et al, 1998). Another well characterized and conserved gerontogene is the silent information regulator *SIR2*, which together with ribosomal DNA (rDNA) was recently confirmed to be a major determinant of RLS (Kwan et al, 2013). This protein deacetylase is implicated in various aging pathways, a number of which are discussed in the subsequent chapters.

2. Aging factors and their inheritance

A yeast mother cell produces a finite number of daughter cells before it reaches a state of senescence. Remarkably, each daughter cell has a full replicative potential and may divide 20-30 times even when generated from an old mother cell. This implies that there are one or more senescence factors that are retained in the mother cell (Egilmez & Jazwinski, 1989; Henderson & Gottschling, 2008). There are at least four criteria that need to be met in order to classify as an "aging factor". First, the senescence factor should be more abundant in old cells. Second, introducing such factors in young cells should accelerate their aging. Third, reducing the progressive accumulation of a putative senescence factor should extend the lifespan. Last, the senescence factor should be retained in the mother cell during cytokinesis (Henderson & Gottschling, 2008). The last criteria of asymmetric segregation of aging factors may be viewed as the mother's sacrifice so that her progeny can start out in life free of damage. In yeast, at least four such factors have been characterized: malfunctioning mitochondria, vacuolar acidity, extrachromosomal ribosomal DNA circles (ERCs) and damaged proteins. The latter two are given special emphasis in this thesis.

2.1 Malfunctioning mitochondria

Damaged or faulty mitochondria is one putative senescence factor that accumulate with replicative age in yeast cells (Erjavec et al, 2013; Higuchi et al, 2013; Klinger et al, 2010; Scheckhuber et al, 2007; Veatch et al, 2009). It has been shown that mutations that reduce age-related mitochondrial fragmentation and dysfunction can extend the RLS of mother cells (Scheckhuber et al, 2007). Interestingly, mitochondria are segregated asymmetrically, with the healthier

mitochondria being primarily inherited by the daughter cell (Klinger et al, 2010; McFaline-Figueroa et al, 2011). Such a filtering process is intriguing and calls for a short summary of how mitochondria are currently thought to be inherited. Several reports suggest that mitochondria are transferred to the daughter cell via actin cables and the myosin V-type protein Myo2 (Vevea et al, 2014). Myo2 moves towards the daughter cell by its own motor-force, but against the actin cable flow. This is because actin monomers are inserted at the polarisome, a site at the tip of the daughter cell, which causes a flow backwards to the mother cell. This implies that factors entering the bud need, by necessity, to move faster than the backward cable flow. Evidently, cells utilize this feature to transport reduced and healthy mitochondria (to the daughter) which move faster against the flow than oxidized and malfunctioning mitochondria (Higuchi et al, 2013). Furthermore, manipulation of the cable counter-flow by mutations in the *MYO1* gene (reduced flow) resulted in more faulty mitochondria reaching the daughter cell whereas a *TPM2* mutation (enhanced flow) resulted in increased inheritance of healthy mitochondria. Importantly, increasing the inheritance of healthy mitochondria by enhancing the flow, prolonged the lifespan.

Segregation of mitochondria is also regulated by Sir2. This protein deacetylase is implicated in actin cable abundance and cytoskeletal functions - a process which is further addressed in ***paper III*****.** Removal of *SIR2* decreases the flow of actin cables and retrograde movement of mitochondria thereby allowing defect mitochondria to enter the daughter cell. Over-expression of *SIR2*, on the other hand, had the opposite effect on cable flow and mitochondrial inheritance (Higuchi et al, 2013). Intriguingly, deletion of *SOV1*, a member of the yeast mitochondrial translation module (MTC), increases Sir2 activity (**Paper V**). It has been speculated that such boost in activity, similar to Sir2 over-expression, provides an assurance mechanism whereby daughters receive the best

mitochondria as the mother cell ages and mitochondrial quality decline (Nystrom & Liu, 2014).

2.2 Vacuolar acidity

The sequence of events that occur during yeast aging is a topic of intensive research and has recently been traced back to an early functional decline of the vacuole. Gottschling and colleagues observed that mitochondrial inner-membrane potential was reduced by this functional decline manifested as an increase in vacuolar pH of the mother cell (Hughes & Gottschling, 2012). The mitochondrial membrane potential itself leads to further age associated problems such as genomic maintenance and loss of heterozygosity (Veatch et al, 2009). Vacuoles, like mitochondria, are transferred to the daughter as Myo2-cargo transported on actin cables (Hill et al, 1996). Intriguingly, the vacuole found in a mother cell displays erroneous pH control whereas the vacuole in a daughter cell regains its acidic pH (Hughes & Gottschling, 2012). This type of segregation control differs from the filtering of functional/dysfunctional mitochondria as it is the daughter specific environment that is important for a fully functional vacuole (Hughes & Gottschling, 2012).

The link between vacuole function and mitochondrial deficiency was proposed to be due to the reduced storage capacity for neutral amino acids which require proper acidification of the vacuole (Hughes & Gottschling, 2012). The authors speculated that, since mitochondria catabolize neutral amino acids, the excess of leaked amino acids in the cytoplasm could potentially interfere with proton-dependent mitochondrial carrier processes with subsequent failure to maintain the membrane potential. It is still obscure why vacuolar pH control initially fails and how daughter cells rejuvenate this control.

2.3 ERCs and transcriptional control

Another example of aging factors are ERCs (Sinclair & Guarente, 1997). In *S. cerevisiae*, rDNA consists of 100 to 200 tandem repeats (Fig. 1) and the ERCs may be formed by excision from these repeats. Subsequently, they can replicate via an autonomously replicating sequence (rARS) embedded in the sequence (Fig. 1). Formation of ERCs is tightly linked to transcriptional silencing and recombination frequency at the rDNA loci and has been observed to dramatically influence cellular aging (Sinclair & Guarente, 1997). In effect, silencing may be described as regions of poorly transcribed chromatin. In *Saccharomyces cerevisiae*, in addition to rDNA, silenced chromatin is found at the sub-telomeric regions and at the *HMR* and *HML* - cryptic mating type loci (Aparicio et al, 1991; Rusche et al, 2003). The Sir-complex establishes, maintains and spreads silent chromatin across these heterochromatin domains (Strahl-Bolsinger et al, 1997). In order to understand how ERCs are linked to silencing, it is important to understand the fundamental organization and structure of chromatin and rDNA.

2.3.1 Chromatin organization

DNA does not exist as a "naked" linear double-stranded helix but is instead organized in a complex of proteins and DNA which may be visualized as beads on a string. Each "bead" on the string represents a functional unit called a nucleosome. In yeast, this protein complex is represented by two copies each of the four core histones: H2A, H2B, H3 and H4 around which the DNA is wrapped. This compaction of DNA is called chromatin and is the constituent of chromosomes. The condensation enables the DNA to fit inside the nucleus, provides strengthening during mitosis and prevents DNA damage.

Furthermore, the condensation of chromatin is highly influential on transcription as it may facilitate or hinder access to genes by RNA polymerase and transcription factors. A region where chromatin is condensed is called *heterochromatin*. In contrast, a region where DNA is less condensed is called *euchromatin*. In order for genes to be accessed and transcription initiated, the chromatin needs to be "opened", or remodeled. The chromatin environment is determined by the joint actions of DNA methylation, ATP driven remodeling, incorporation of a histone variant (H2A.Z) and post transcriptional modifications (e.g. methylation, acetylation, phosphorylation and ubiquitination of histones) (Chen & Dent, 2014). Consequently, such modifications change the binding-affinity between histones and DNA (loosened/tightened) but can also promote recruitment of transcription factors. Thus, repositioning, modification or expelling of histones/nucleosomes is key in transcriptional, replicational and recombinatorial regulation (Rando & Winston, 2012).

2.3.2 Mating type loci

Yeast has the ability to switch sex (mating type) depending on what allele (*MAT*a or *MAT*α) is present at the *MAT* locus. This is possible because yeast has additional silent copies of each allele: *HMR* (hidden mating type right) and *HML* (hidden mating type left). The mechanism for establishing silent mating type at the *HML/HMR* loci relies on the Sir-complex which is recruited to flanking domains called *E* and *I* silencers. The silencer domains contain unique binding sites for DNA-binding proteins (Rap1 and Abf1) and origin recognition complex (ORC). Together these factors recruit the Sir-multi-protein-complex consisting of Sir1, Sir2, Sir3 and Sir4 (Ghidelli et al, 2001; Moretti et al, 1994). Subsequent to Sir-complex recruitment, Sir2 deacetylates a H3 and H4 tail residing on an adjacent nucleosome. The Sir-complex (except Sir1) then

proceeds to spread in between silencer domains to establish silent chromatin (Wierman & Smith, 2013).

2.3.3 Telomeres

Telomeric silencing is regulated by Sir2 and Sir4 in complex. This complex is recruited via Rap1 associated with certain terminal telomeric sequences. Bound Sir2/Sir4 pursues with histone deacetylation together with Sir3 at subtelomeric regions (Hecht et al, 1996; Hoppe et al, 2002; Tanny & Moazed, 2001). The H4 tail lysine K16 is particularly important since it has a dual role in silencing. Acetylated H4K16 recruits Sir2/Sir4 and repels Sir3, whereas deacetylation of H4K16 mediated by Sir2 promotes binding of the Sir-holocomplex (Sir2/3/4) (Oppikofer et al, 2011). This implies that acetylation/deacetylation of H4K16 mediates sequential binding of Sir-proteins to establish silent chromatin.

In higher eukaryotes it is well established that telomere length plays a key role in cellular senescence (Harley, 1991). Dividing cells not expressing telomerase, an enzyme able to "repair" shortened telomeres, will eventually reach a critical telomere length which is linked to the Hayflick limit. Consequently, cells reach senescence, or occasionally, restore length via a recombination event (Draskovic & Londono Vallejo, 2013). In yeast (*S. cerevisiae*) telomerase is constitutively expressed resulting in perpetual maintenance of telomere length in both mother and daughter cells (D'Mello & Jazwinski, 1991). However, mutations causing shortening of telomeres, do shorten RLS (Lundblad & Szostak, 1989).

2.3.4 rDNA

The mechanism of silencing at rDNA is different from that of telomeres and *MAT* since the deacetylase Sir2 is the only Sir-protein occupying this region. Instead, the scaffold for Sir2 recruitment constitutes the nucleolar protein Net1 and the phosphatase Cdc14, the so called RENT complex (Regulator of Nucleolar Silencing and Telophase exit) (Huang & Moazed, 2003). RENT localizes to two distinct regions in the rDNA locus (Fig. 1). First, RENT may be recruited near the 35S promoter. Divergently, RENT is also recruited to a place in the intragenic spacer region 1 (IGS1) where it mediates silencing of the RNAPII-dependent promoter E-pro. The recruitment of RENT to IGS1 is facilitated by Fob1 binding to the replication fork block (RFB, Fig. 1). In this process, Fob1 acts synergistically with a cohibin complex (Lrs4 and Csm1) to suppress rDNA recombination between rDNA repeats (Huang et al, 2006).

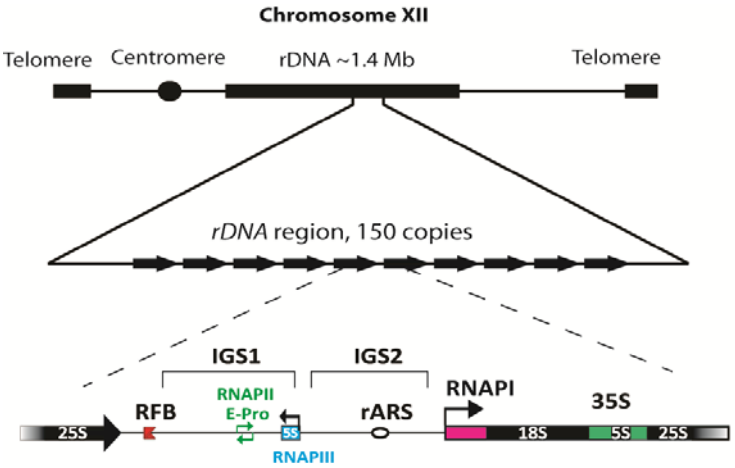

Figure 1. Schematic representation of chromosome XII and the rDNA repeats. Each repeat encodes a RNAPI-dependent 35S precursor rRNA and a 5S RNAPIII transcribed 5S rRNA. Each unit also contains two intragenic spacer regions (IGS1, 2). Other significant sites are the RFB, the autonomously replicating sequence-rARS and the bidirectional, RNAPII-dependent E-pro.

Maintenance of silencing of the highly repetitive rDNA is crucial for cell viability. Fob1 is primarily known for blocking replication at RFB in rDNA (Brewer & Fangman, 1988). Consequently, binding of Fob1 may cause double strand breaks (DSBs) of the DNA at stalled replication forks, which in turn could lead to unequal sister chromatid recombination. This mechanism is an important control mechanism for regulating rDNA copy number and variation. In agreement with this, in a *fob1*Δ mutant, unequal sister chromatid recombination is reduced and rDNA copy number is stabilized (Kobayashi et al, 1998). Another layer of rDNA copy number control is provided by Sir2 that silences an RNAPII-dependent promoter (E-Pro) at rDNA (Fig. 1). The transcriptional activity at E-Pro is thought to dissociate cohesin complexes responsible for binding sister chromatids together (Kobayashi & Ganley, 2005). Subsequently, a broken sister chromatid-end may recombine unequally during DSB repair thereby regulating copy number change, loss of genetic material, and ERC formation (Huang & Moazed, 2006; Kobayashi & Ganley, 2005). Consistent with this, a *sir2*Δ mutation prevents cohesin association thereby promoting unequal recombination and increased rDNA copy number. Taken together, Sir2 and Fob1 are key regulators of transcriptional silencing, recombination frequency, and stability at rDNA. These processes are further addressed in ***paper I***.

2.3.5 Segregation of ERCs

ERCs accumulate in aging cells and are distributed asymmetrically in a mother-cell biased manner (Sinclair & Guarente, 1997). One reason for this bias is that ERCs do not contain a centromeric sequence which is a prerequisite for equal distribution between mother and daughter cell (Murray & Szostak, 1983). Recently, two mechanisms further explaining this distribution have been

proposed: the tethering model and the diffusion model. The tethering model is explained by the attachment of ERCs to nuclear pore complexes (NPCs) which are themselves retained in the mother via a septin-dependent diffusion barrier (Shcheprova et al, 2008). The diffusion model, on the other hand, is founded on data showing that the NPCs are not blocked from moving into the daughter nucleus. Instead, the mother-biased segregation of ERCs is achieved by the geometry of the bud neck together with the short time of mitosis (Gehlen et al, 2011) which, would not allow diffusion of ERCs into daughters. To this end, it is noteworthy that the latter model does not exclude the possibility that ERCs are attached to NPCs.

2.4 Damaged proteins and protein quality control

Damaged and aggregated proteins have recently emerged as *bona fide* aging factors in yeast and the levels of these factors increase in aging mother cells (Aguilaniu et al, 2003; Erjavec et al, 2007; Hill et al, 2014). To understand how protein aggregates might lead to aging it is important to highlight the protein quality control (PQC) system aimed at avoiding the occurrence of such protein damage.

2.4.1 Protein misfolding and aggregation

PQC is a complex surveillance system evolved to combat proteomic aberrancies. Protein damage may be induced by exogenous or endogenous errors and stress causing misfolding. For instance, protein misfolding can occur as proteins are *de novo* synthesized, or more rarely, due to mutations. Misfolded or aberrant proteins are either refolded by molecular chaperones or cleared by proteolytic

degradation. Either way, these actions prevent proteins from aggregating. Understanding protein aggregation and its underlying mechanism is medically important since certain neurodegenerative diseases are associated with this feature.

Aggregation of a protein may occur when it is in an intermediate or misfolded state where it exposes hydrophobic residues. These residues are normally buried when the protein is in its native conformation. However, if exposed, they trigger aggregation (Hartl & Hayer-Hartl, 2009). Another cause of aggregate formation is mutations causing a protein to consistently misfold. Neurodegenerative diseases such as Huntington's, Parkinson's and Alzheimer's are represented by this type of inherent misfolding and subsequent aggregation (Chiti & Dobson, 2006; Powers et al, 2009; Ross & Poirier, 2004). Other, more frequent occurring aggregating scenarios include mis-incorporation of amino acids during translation or faulty assembly of protein complexes (Drummond & Wilke, 2008). Aggregation is also induced by environmental stressors such as heat and oxidative stress. Heat stress tends to cause widespread but reversible misfolding of proteins whereas oxidative stress is associated with both reversible and irreversible protein modifications (Parsell et al, 1994). Reactive oxygen species (ROS), may also induce widespread protein damage and misfolding by replacement of certain native side-chains of amino acids by carbonyl groups (Stadtman & Levine, 2000). These protein-modifications can lead to cross-linking with other proteins and subsequent aggregation.

Protein aggregation also occurs during aging. This is presumably a slower process than, for example heat shock, and the cause and effect of such aggregation is a topic of intensive research. It has been shown that oxidatively damaged and aggregated carbonylated proteins accumulate in cytoplasmic foci as a yeast cell age (Erjavec et al, 2007). We found support for a general and gradual decline in protein quality control as we observed a correlation with

certain types of cytoplasmic aggregates with replicative age (**Paper II**). These findings indicate that aged cells are less able to remove aberrant proteins and sequester them into specific inclusion bodies. Similarly, it has been suggested that such general decline in PQC is one reason for the late age onset of the aforementioned neuro-degenerative diseases (Tyedmers et al, 2010).

The term "protein aggregate" is a convenient terminology used to envision a state of accumulated misfolded proteins but perhaps too generic to describe the true nature of the complex conformation of misfolded proteins found in these structures. This complexity is exemplified by the observation that the same protein can yield different aggregate morphology depending on the type of denaturing agent used to induce misfolding (Wang et al, 2010). Nevertheless, aggregates may simplistically be divided into amorphous or amyloid-like. These both aggregate classes contain β-sheets to a varying degree and organization (Alberti et al, 2010). Most amorphous aggregates are extremely diverse in their structure, but it has been suggested that they consist of misfolded and aggregated proteins that are quite similar to their native conformation in solution (Qin et al, 2007). Amyloid fibrils, on the other hand, are represented by a higher amount of β-sheet content which form a densely packed core made up of a continuous sheet of β-strands arranged perpendicularly to the fibrillar axis (Kirschner et al, 1986). Amyloids grow at the fibrillar ends by incorporation of polypeptides with a similar primary sequence resulting in a homogenous aggregate composition. Moreover, the amyloid-like aggregates are considered as less soluble than amorphous aggregates based on their resilience to chemical perturbations that affect protein structure. The formation of this class of aggregates may be preceded by a form of prefibrillar aggregates which is highly reactive and toxic to the organism (Glabe, 2008). These prefibrillar aggregates as well as amyloid fibrils are associated with age-induced maladies such as Alzheimer's, Huntington's and Parkinson's disease. Whether age-induced

aggregates of non-disease proteins are amorphous or amyloid-like, or perhaps a mixture of both, is still an unresolved issue.

2.4.2 Chaperones and disaggregation

The first and most effective means for the cell to maintain proteostasis is to prevent proteins from misfolding. Molecular chaperones belong to a family of multi-domain proteins governing a wide array of tasks such as: protein folding, refolding/unfolding and protein remodeling. Chaperones in yeast are to a large extent represented by heat shock proteins (Hsp) including small Hsps, Hsp40s and Hsp70s. The core components of this machinery are the Hsp60s and Hsp70s which prevent the accumulation of misfolded proteins via ATP-dependent refolding (Hartl & Hayer-Hartl, 2009). Misfolded proteins which fail to refold to its native state are degraded by proteasomes or transported to vacuoles for degradation by acidic hydrolases (Goldberg, 2003; Kirkin et al, 2009). Failure in handling of corrupt proteins by any of these components results in protein aggregation.

Disaggregation or resolution of a protein aggregate requires a fine-tuned co-operation between several types of chaperones. Aggregate resolution by the oligomeric ring-forming AAA+ ATPase Hsp104 was first demonstrated in yeast (Parsell et al, 1994). However, Hsp104 alone has little effect on aggregate resolution but together with the Hsp40s and Hsp70s *e.g.* Ydj1 and Ssa1 respectively, it achieves its full potential as a disaggregase (Glover & Lindquist, 1998). The same is true for the Hsp40s and Hsp70s alone as they also show limited disaggregation capacity without each other.

This chaperone system works mechanistically by an initial binding of the Hsp70s together with J-proteins. The binding of these components is

assumed to prevent access of proteases to the aggregated protein (Zietkiewicz et al, 2004). The Hsp70-protein complex is then believed to present and mediate transfer of the complex to Hsp104 via its M-domain (Haslberger et al, 2007). Subsequently, Hsp104 is proposed to execute an ATP-dependent threading action leading to extraction of a misfolded protein from the aggregate (Lum et al, 2004). However, another model has also been proposed where Hsp104 acts as a molecular crowbar where an ATP-dependent conformational change in Hsp104 rips the aggregate open leading to disaggregation (Glover & Lindquist, 1998). Disaggregation is crucial for both proteasomal degradation or chaperone mediated refolding

2.4.3 The ubiquitin proteasome system

The ubiquitin proteasome system (UPS) is the principal pathway for degrading aberrant proteins in eukaryotic cells (Hershko et al, 2000). The degradation of a protein is specific and is conferred by the ubiquitin system, whereas the proteasome itself serves as a non-specific protease. The ubiquitination of a protein is governed by an enzymatic cascade tagging the protein with ubiquitin (Ub) which then serves as a signal for destruction by the proteasome. The degradation of most proteins is dependent on ATP-fueled proteases which was first described in the late 1970s (Etlinger & Goldberg, 1977). Prior to degradation, proteins are unfolded and prepared by AAA-ATPase complexes of homo- or heterohexameric ring structures for transport to the inner proteolytic compartment of the proteasome (Sauer & Baker, 2011). Degradation-products are by necessity transported inside the proteasome since the active sites are localized there (Lupas et al, 1997). The proteasome localizes to both the nucleus and cytoplasm (Tanaka, 2009).

The components of this degradation-complex consists of a cylinder-shaped proteolytic core, the 20S particle which is bound at one or both ends by the 19S regulatory particle (Lupas et al, 1993) (Fig. 2). These two subcomplexes together constitute the 26S proteasome (Fig. 2). The AAA-ATPase part of the 26S proteasome is represented by the subunits Rpt1-Rpt6 forming a heterohexamer which is proposed to have non-redundant functions (Bar-Nun & Glickman, 2012; Wollenberg & Swaffield, 2001). Other constituents of the 26S proteasome are the 13 Rpn-proteins which are not part of the ATPase family. The structure of the full 26S proteasome has remained elusive until recently when electron microscopy imaging shed a new light on the proteasomal composition (Lander et al, 2012; Lasker et al, 2012).

The proteasome cannot degrade aggregated proteins *in vitro* (Gregori et al, 1995). Furthermore, it has been shown that aggregates reduce proteasomal activity *in vivo* (Andersson et al, 2013; Bence et al, 2001; Verhoef et al, 2002). In addition, data also suggest that genetically manipulating proteasome levels can influence yeast replicative lifespan as boosting proteasome levels increased the lifespan whereas reducing proteasome levels shortened the lifespan (Kruegel et al, 2011). Kruegel and colleagues also observed a correlation with proteasome levels and aggregate management in young cells. Interestingly, a recent study found that over-expressing the yeast metacaspase Mca1, counter-acted accumulation of unfolded proteins/aggregates and prolonged lifespan. The lifespan extension was shown to be dependent on both Hsp104 and normal proteasome levels indicating that protein aggregates/inclusions are true aging factors in the yeast model system (Hill et al, 2014).

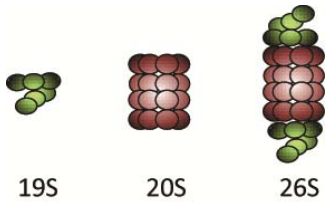

Figure 2. Schematic representation of the proteasomal subunits. The 19S can bind to one or both ends of the 20S subunit. Together they constitute the full, 2.5 MDa protease complex which is the 26S proteasome.

2.4.3.1 Ubiquitination

Ubiquitin is a small, ubiquitously expressed and highly conserved protein throughout the eukaryotic kingdom (Hershko & Ciechanover, 1998). Ubiquitin conjugated to a target protein can direct the substrate to a specific cellular location, trafficking route, modify the activity, recruit binding partners or present it to the 26S proteasome for destruction (Komander & Rape, 2012). Ubiquitination has also been shown to direct proteins to specific protein quality control compartments (Kaganovich et al, 2008). The ubiquitination of a protein requires three enzymes: E1 - the ubiquitin activating enzyme; E2 - the ubiquitin conjugating enzyme and E3 - the ubiquitin ligase (Pickart & Eddins, 2004). Efficient proteasomal targeting and degradation sometimes also requires a fourth, additional conjugation factor - E4, which facilitates multi-ubiquitination (Koegl et al, 1999). An additional level of regulation involves the usage of lysine residues on ubiquitin where the most common ones are K48 or K63 linkage-specific ubiquitination. A Ub-chain on K48 is sufficient to target a protein to the proteasome whereas a K63 chain has been associated with targeting membrane proteins for vacuolar degradation (Galan & Haguenauer-Tsapis, 1997; Springael et al, 1999). Moreover, these linkages of Ub may be branched differently although the function of these branches is still elusive.

The ubiquitination process is initiated by Ub-activation (E1) which is ATP-dependent (Fig. 3-1). The reaction involves the C-terminal domain of Ub (Gly76) attached via a thioester bond to a cysteine residue in the active center of the E1 (Sun et al, 2006). Next, in an intermediate step the activated Ub is transferred by the E1 to a ubiquitin- conjugating enzyme (E2) (Fig. 3-2). This step also requires the C-terminal domain of Ub (Gly76) which is attached to a cysteine residue of the E2 with a thioester bond. The last step is the transfer of Ub to the substrate mediated by an E3 enzyme (Fig. 3-3). The substrate protein itself is bound to the specific E3 as well as the Ub-charged E2. The substrate-ubiquitination can take place in two fashions: Ub may be transferred directly from the E2 to the substrate protein (RING E3); or from E2 to E3 and then subsequently from E3 to the substrate protein (HECT E3) (Hochstrasser, 2006). Subsequent rounds of ubiquitination may be facilitated by an E4 elongation factor (Fig. 3-4).

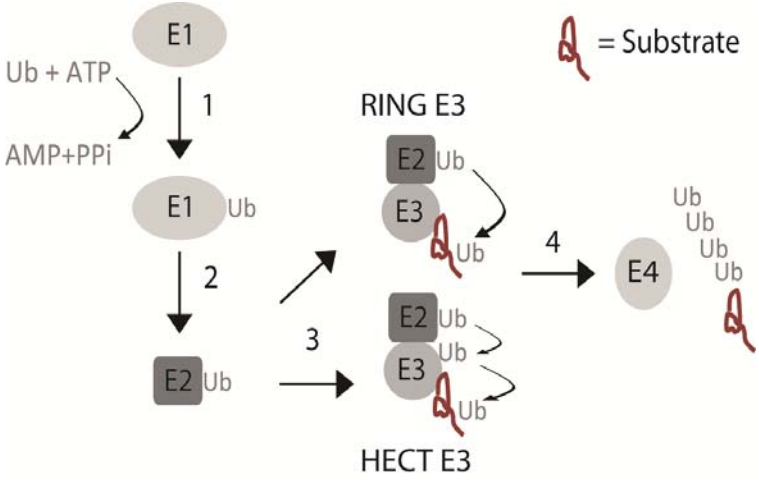

Figure 3. Ubiquitination. 1) ATP-dependent activation of Ub carried out by an E1. 2) Ub is transferred via an E2 Ub-carrier protein. 3) If the E3 is a RING domain ligase, the E2-Ub complex binds to the E3 carrying the substrate protein and transfers the activated Ub directly to the subsrate. If the E3 is a HECT domain ligase, Ub is transferred from the E2-Ub complex to an E3 and then to the substrate. 4) Polyubiquitin chain-elongation may be facilitated by E4 ligases.

Only one ubiquitin activating enzymes (E1) exist in yeast (Uba1) whereas E2s are more numerous (eleven enzymes) and most abundant is the E3 family where 42 ligases have been fully characterized so far (Lee et al, 2008) (database updated 2014). Ubiquitin ligases confer specificity to the ubiquitin proteasome system. Some ligases like Ubr1, Hul5 and Ltn1 act in the cytoplasm to clear aberrant proteins whereas others, Hrd1 and Doa10 act proximally to the ER to clear membrane proteins or substrates targeted for secretion (Theodoraki et al, 2012; Vembar & Brodsky, 2008). In addition, San1 a ligase acting as a nuclear PQC component has been reported to mediate destruction of misfolded proteins that are translocated to the nucleus (Gardner et al, 2005; Heck et al, 2010; Prasad et al, 2010). There is considerable redundancy in the PQC network of ligases although it has been shown specifically that Ubr1 promotes clearance of protein aggregates, particularly when the autophagic system is deactivated (Theodoraki et al, 2012).

2.4.3.2 Deubiquitination

Ubiquitin is long-lived and recycled which may seem surprising due to its attachment to substrates destined for degradation (Swaminathan et al, 1999). This is due to the action of deubiquitinating enzymes which cleave ubiquitin-protein bonds between Gly76 on ubiquitin and a Lys residue of the substrate protein or preceding ubiquitin. Deubiquitinating enzymes (DUBs) fall into a large family of cysteine proteases with four main subfamilies in yeast: the larger *UBP* family with 16 genes (ubiquitin-specific proteases) and the smaller Otu, Uch, and JAMM families containing one to two genes each. The Ubp enzymes vary in size, homology and structural complexity except for the conserved core catalytic domain which enables them to cleave ubiquitin from a wide range of substrates.

There are many major ubiquitin-controlled pathways in the cell such as cell-cycle control, DNA repair and vesicle trafficking (Kim et al, 2003). DUBs provide an extra layer of regulation by modifying the ubiquitin status of proteins involved in these pathways. DUBs act by executing various different actions. First, DUBs have the ability to process ubiquitin precursors. This biosynthetic processing is important because three of the four ubiquitin encoding genes *UBI1* - *UBI4* are translated as N-terminal fusions to ribosomal proteins (Finley et al, 1989). Ubiquitin is also translated as multiple linear Ub-fusions (*UBI4*). DUBs are responsible for the processing of these fusions to release and unblock the C-termini of ubiquitin (Fig. 4-1). Second, DUBs may edit a non-degradative Ub-signal by trimming the Ub-chain length (Fig. 4-2). This processing of the Ub-chain gives rise to a type of regulation where the Ub-linkage may be changed and thereby alter the fate of the ubiquitinated protein.

Another process carried out by DUBs is to detach protein-ubiquitin chains for substrates committed for proteasomal degradation (Fig. 4-3). Targets bound for proteasomal degradation may not enter correctly if the ubiquitin chain is still attached. Moreover, degradation of ubiquitin itself is energetically unfavorable. Last, DUBs can determine the fate of a protein by removing mono/poly-Ub chains. Moreover, these unattached Ub-chains are disassembled by DUBs which is important for the recycling of Ub (Fig. 4-3 and 4-4). This process prohibits abnormal accumulation of Ub-chains which have been shown to interfere with proteasomal degradation (Amerik et al, 1997).

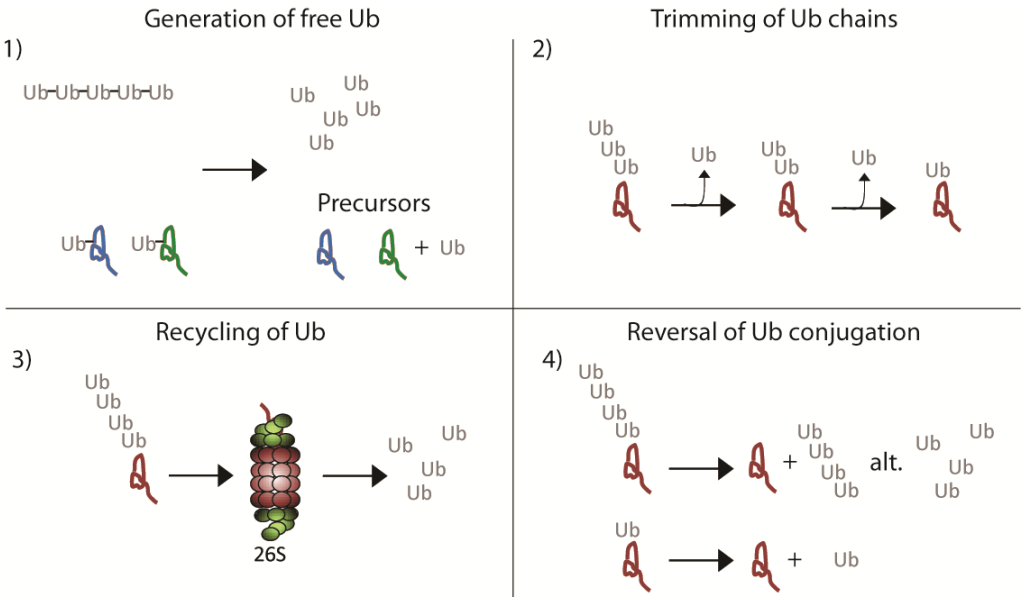

Figure 4. DUB functions. 1) DUBs cleave Ub-ribosomal hybrid fusions or polyubiquitin to generate free Ub. 2) A non-degradative Ub-chain may be edited by DUBs to change the Ub-signal. 3) Proteasomal degradation is associated with recycling of Ub which ensures homeostasis of the Ub-pool. 4) The reversal of ubiquitination determines the stability/fate of a protein. Moreover, DUBs disassemble Ub-chains to generate free Ub.

2.4.4 Spatial quality control

Chaperones have also been shown to be paramount for the spatial sequestration of misfolded proteins and aggregates into distinct "compartments". Several quality control compartments have been defined in yeast and organization of aggregates into these compartments depends on the type of protein species that was initially misfolded as well as protein damaging agent used. Spatial quality control and its terminology is a young field and these following sections aim at summarizing what is currently known about various aggregates and their compartmentalization.

2.4.4.1 Q-bodies/stress-foci/peripheral aggregates

An early stress responsive sequestration of ER-associated aggregates has recently been observed and was coined "Q-bodies" (Escusa-Toret et al, 2013). These Q-bodies form puncta that move and coalesce into larger structures which are speculated to be a transient stage prior to formation of other types of aggregates (Fig. 5) (Escusa-Toret et al, 2013). Similar to Q-bodies, there are reports that describe other transient aggregates such as "stress foci" and "peripheral aggregates" (Specht et al, 2011; Spokoini et al, 2012). These three "types" of small aggregates share many characteristics and may turn out to be the same. For example, all of them form upon heat-stress, associate with Hsp104 and are precursors for larger inclusion-body formation. Q-bodies, apparently also require ATP, sHSPs like Hsp42 and the cortical ER for maturation or formation (Sontag et al, 2014). Similarly, Hsp42 and the actin cytoskeleton are required for formation of peripheral aggregates (Specht et al, 2011). A recent study utilized the disease-associated protein Htt103Q which also form small Hsp104-associated foci similar to stress foci/Q-bodies/peripheral aggregates (Song et al, 2014). Intriguingly, the study found that Hsp42 is required for heat-induced Hsp104-associated foci but not for Htt103Q foci suggesting that the routes employed for foci formation are not identical (Song et al, 2014). We are only beginning to understand these PQC pathways and further experiments should provide insight into the requirements for these aggregation pathways.

2.4.4.2 JUNQ and IPOD

Soluble but misfolded proteins appear to accumulate in a juxta nuclear quality control compartment coined JUNQ - where proteasomes are concentrated (Fig. 5) (Kaganovich et al, 2008). Another compartment called IPOD, insoluble

protein deposit, sequesters less soluble proteins and is localized to the periphery of the cell in the proximity to the vacuole (Fig. 5) (Spokoini et al, 2012). These two compartments vary in what cellular components they require for formation and resolution.

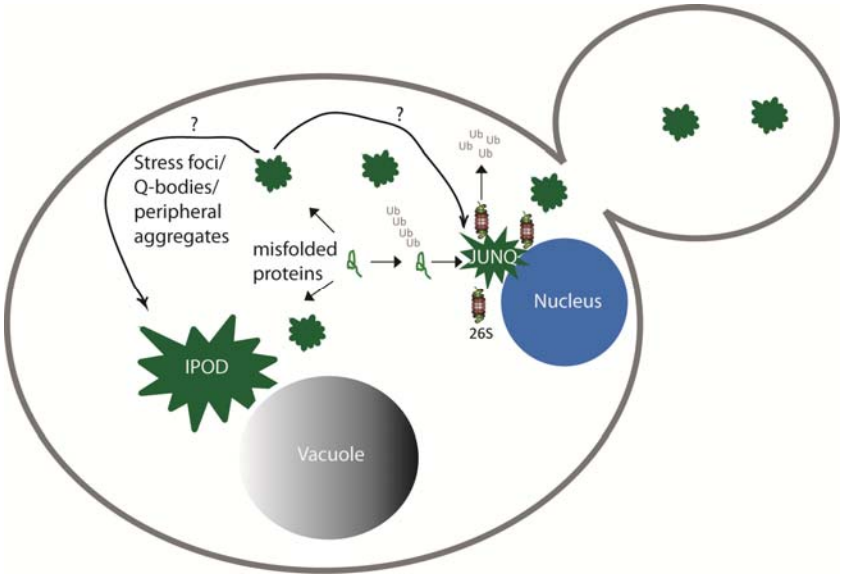

Figure 5. Model for management of misfolded proteins. Non-native proteins which are not refolded by chaperones are ubiquitinated and directed to proteasomes for destruction in the JUNQ compartment. Simultaneously (or prior to), misfolded proteins localize to cytosolic stress foci/Q-bodies/peripheral aggregates. These smaller aggregates are either cleared or may coalesce to larger inclusion bodies like JUNQ or IPOD.

Proteins destined to JUNQ co-localizes frequently with chaperones from the Hsp70 family, but less frequently with Hsp104 suggesting a more prominent role for Hsp70s in JUNQ management (**paper II**) (Malinovska et al, 2012; Weisberg et al, 2012). The formation of JUNQ requires the action of the ubiquitin conjugating enzymes Ubc4/Ubc5 (Kaganovich et al, 2008). The functional role of the JUNQ compartment has been suggested to be a storage depot for misfolded proteins keeping them in a folding-competent state for either refolding or destruction by the proteasome.

Proteins compartmentalized in the IPOD are represented by terminally misfolded proteins and amyloid-forming species such as prions or polyglutamine expanded Huntingtin. Factors co-localizing with the IPOD are Hsp104 and the autophagy marker Atg8 (Kaganovich et al, 2008). Autophagy is the vacuolar-dependent degradation of cellular material ranging from organelles and even protein aggregates (Lamark & Johansen, 2012). This process is usually mediated by a membrane-enclosed vesicle (autophagosome), which subsequently fuses with the vacuole (Reggiori & Klionsky, 2013). However, the precise role for Atg8 and autophagy in managing IPOD is still obscure. Hsp104 on the other hand, has been shown to be required for the maturation of smaller aggregates into both JUNQ and IPOD (Spokoini et al, 2012).

On a functional level, the IPOD is thought of as a compartment that provides suppression of cytotoxicity caused by aggregation-prone proteins (Kaganovich et al, 2008). In support of this notion, a toxic misfolded protein generated from a point mutation - *SOD1G93A*, accumulates in JUNQ, but directing it away from JUNQ to IPOD reduces the harmful effects on human cell viability (Weisberg et al, 2012). The toxicity of the Sod1-mutant was speculated to be due to sequestration of Hsp70s which thereby prevented the delivery of misfolded proteins to proteasomes (Weisberg et al, 2012).

Other quality control factors that regulate aggregate sorting during stress are the Hook family proteins Btn2 and Cur1. They physically interact with chaperones to provide a sorting pathway for misfolded proteins in the cytosol. It has been suggested that Btn2 together with Hsp42 promotes the accumulation of non-amyloid proteins to IPOD (Malinovska et al, 2012). In the same study, the authors also showed that Btn2 bound to the Hsp40 chaperone Sis1 promotes targeting of misfolded proteins to JUNQ. This duality in protein sorting is achieved via Cur1 which governs the sorting of Sis1 to the nucleus. Thus,

cytosolic concentration of chaperones determine the sorting of misfolded proteins.

2.4.4.3 Other quality control compartments

The earliest defined mammalian quality control compartment is ERQC - ER-derived quality control compartment, which is localized near the centriole and acts as a deposition-site for misfolded ER-derived secretory proteins (Kamhi-Nesher et al, 2001). The subsequent degradation of these proteins by the proteasome is called ERAD (ER-associated degradation). In yeast, prior to ERAD, proteins accumulate in a membrane bound deposition site termed ERAC (ER-associated compartment) (Huyer et al, 2004). Proteins can also be cleared from the ER by an autophagy pathway.

Mammalian cells and yeast show many similarities in spatial quality control as they both display JUNQ and IPOD-like compartments (Kaganovich et al, 2008). However, the utilization of cell components varies between the species. One striking example is the mammalian aggresome which share some features with the yeast IPOD. The mammalian aggresome is located at the microtubuli organizing centre and is encompassed by a vimentin cage as opposed to the yeast IPOD (Johnston et al, 1998). Targeting of proteins to the aggresome is directed by ubiquitin (in most cases), histone deacetylase 6 and dynein coupled with microtubuli (Kawaguchi et al, 2003). Furthermore, the aggresome has also been implicated in terminal sequestration of misfolded proteins, protein folding, clearance and aggregate retention (Sontag et al, 2014). Recent data also suggest that the mammalian JUNQ is surrounded by a vimentin cage, a finding which sheds new light on the definitions of PQC-compartments as well as serving as an example of how the same compartments may utilize different factors in different cell types (Ogrodnik et al, 2014).

2.4.5 Segregation of aggregates

Mother-cell biased segregation of damaged and aggregated proteins employ several cellular components so that the daughter cell may enjoy a damage free proteome. The disaggregase Hsp104 and Sir2 are required for this asymmetric segregation as well as components regulating the actin cytoskeleton (**paper III and IV**) (Erjavec et al, 2007; Song et al, 2014; Tessarz et al, 2009). Aggregates containing Hsp104 may be tethered to the actin cytoskeleton and thereby retained in the mother during budding (Fig. 6). A role for Sir2 in segregation appears to involve folding of actin mediated by the chaperonin CCT. The importance of the cytoskeletal network in aggregate segregation is evident also by the requirement of the polarisome, the formin Bni1, the myosin motor protein Myo2 and the actin organization protein calmodulin - Cmd1 (**paper III and IV**) (Song et al, 2014). By utilizing tropomyosin and formin-dependent actin nucleation at the polarisome, daughter cells can also clear themselves of aggregates by a retrograde flow back into the mother cell (**paper III**). An alternative idea is that aggregates are segregated due to slow diffusion rather than actin-dependent retention in the mother cell (Zhou et al, 2011). The models explaining how aggregate asymmetry is achieved is further addressed in the discussion of ***paper III*** and ***IV***

Segregation of aggregates and sequestration of proteins to quality control compartments are tightly linked. A yeast study showed that deleting Hsp104, or inhibiting its disaggregase activity, traps aggregates in small "stress foci" and a large portion of these aggregates were passed on to the daughter cell (Spokoini et al, 2012). However, if aggregates are allowed to mature into either JUNQ or IPOD, they are retained in the mother cell (Spokoini et al, 2012). Using VHL, a model misfolding and aggregating protein, it was suggested that JUNQ is attached to the nuclear surface whereas the IPOD is attached to the

vacuole (Kaganovich et al, 2008; Spokoini et al, 2012). The authors concluded that the confinement of aggregates to the surface of organelles exclude them from transfer into the daughter cell.

The formation and segregation of peripheral aggregates both require the actin cytoskeleton highlighting the importance of this system in asymmetrical inheritance. Interestingly, the disease protein Htt103Q which does not form either IPOD or JUNQ but rather smaller disperse aggregates reminiscent of Q-bodies, stress-foci and peripheral aggregates, also display polarity-dependent retention in the mother cell (**paper IV**). This evidence further strengthens the observation that smaller Hsp104-dependent aggregates as well as inclusion bodies are subjected to segregation quality control (Fig. 6) (Song et al, 2014).

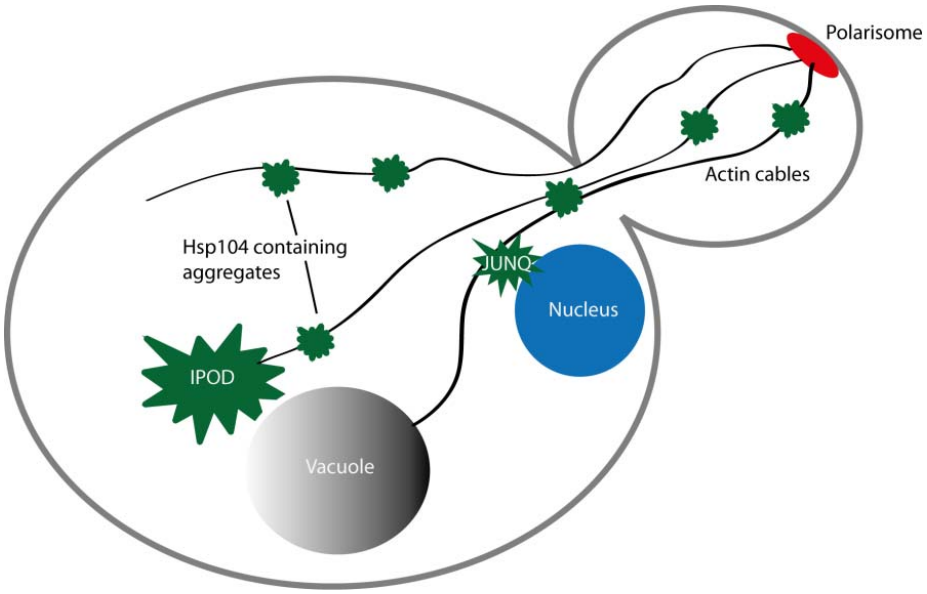

Figure 6. Model depicting segregation of protein aggregates. Various factors establish asymmetric segregation of protein aggregates in yeast. The polarisome, the actin cytoskeleton and Hsp104 are required for the retention of aggregates in the mother cell. In addition, mother-biased segregation is also mediated via sequestration of aggregates into JUNQ and IPOD. These compartments are themselves subjected to mother biased segregation due to their attachment to the nucleus and vacuole respectively.

3. Aim, results and discussion

The aim of this thesis was to elucidate the role of the deubiquitinating enzyme Ubp3 in aging. Specifically, I focused on heterochromatic silencing and protein quality control since Ubp3 had previously been identified as being linked to these processes.

3.1 Role of Ubp3 in genomic silencing

Ubp3 is a deubiquitinating enzyme with a human orthologue in Usp10. *UBP3* encodes a 101,9 kDa DUB which together with its cofactor Bre5, is involved in a number of cellular processes, some of which regulate transcription (non-transcription-related processes targeted by Ubp3 are discussed in ***paper II***). For example, it has been shown that Ubp3 positively activates osmoresponsive genes and is required for proper induction of *PHO5* (Kvint et al, 2008; Sole et al, 2011). In addition, Ubp3 interacts physically with key factors of the transcription machinery such as TFIID, TATA-binding protein (Tbp1) and RNAPII (Auty et al, 2004; Chew et al, 2010; Kvint et al, 2008). Moreover, the stability/function of both Tbp1 and RNAPII are regulated by Ubp3 (Chew et al, 2010; Kvint et al, 2008). Interestingly, loss of Ubp3 results in increased silencing at both mating-type loci and telomeres (Moazed & Johnson, 1996). Also, the authors found evidence for a physical interaction between Sir4 and Ubp3 suggesting that Ubp3 inhibits silencing via Sir4 or the Sir-complex (Moazed & Johnson, 1996). However, precisely how this works is not known.

In an attempt to understand how Ubp3 affects transcription in heterochromatic regions we discovered that, in addition to mating-type loci and telomeres, Ubp3 also acts as an anti-silencing factor at rDNA. There are many factors implicated in silencing but an exact explanation of the mechanism of

how RNAPII-dependent transcription is obstructed is still pending. One hypothesis is that the dense structure of heterochromatin prevents accessibility of the transcription machinery. However, one study presented data showing that general transcription factors (TF) such as TBP and RNAPII assembled at *HMR* without subsequent initiation (Sekinger & Gross, 2001). In addition, the same group found that TFIIH and a serine5-phosphorylated RNAPII could be detected at silent promoters strongly suggesting that Sir-mediated silencing suppresses transcription at a later step (Gao & Gross, 2008). However, conflicting data from another group suggested that neither TFIIB, TFIIE nor RNAPII were localized at silenced promoters but instead, an activator (Ppr1) was present (Chen & Widom, 2005). Our data supports those of Gross and colleagues as we observed that increased silencing by loss of *UBP3* correlated with lower levels of RNAPII at all silent loci in yeast suggesting that RNAPII is indeed active in wild-type cells in these regions (**paper I**).

In addition, in *ubp3*Δ mutants, relative Net1/RENT-occupancy at rDNA and Sir2/3 at *MAT*-loci respectively, is generally higher, suggesting that silent chromatin is not fully saturated with silencing factors in wild-type cells. Taken together, RNAPII seems to be present and active in heterochromatic regions in wild-type cells and this presence is dependent on Ubp3. However, the precise mechanism how Ubp3 alters silencing is still pending. In this context, it should be addressed whether congregation of silencing factors (*i.e.* denseness of chromatin), presence of general TFs or recruitment/modification of RNAPII is altered in Ubp3-deficient cells. It should also be noted that relative levels of H4K16ac/H4K16 and Sir2 occupancy differ between the three heterochromatic regions in *ubp3*Δ mutants (**paper I**). Thus, distinct mechanisms may operate to silence DNA at different regions.

Silencing and transcriptional regulation at the rDNA loci is intimately linked with replicative aging. One such link is ERCs which, when

they were first discovered, were believed to prevent cell division by titrating important factors for DNA replication and maintenance (Kaeberlein et al, 1999; Kwan et al, 2013). However, new data puts ERC formation secondary to rDNA stability as an aging factor as limited RLS has been observed regardless of the absolute number of ERCs (Ganley et al, 2009; Kwan et al, 2013). Ganley and colleagues presented evidence that a strain with reduced replication activity and low ERC-levels had a reduced lifespan due to compromised rDNA stability. No explanation for this instability was presented but the authors speculated that that non-functional DNA repair proteins are retained in the mother cell leading to accumulation of mother-specific rDNA damage. It was speculated that one possible outcome of this rDNA damage could be manifested as poor ribosome quality (Ganley et al, 2009).

The RNAPII and Sir2-dependent regulation of E-pro (driving expression of non-coding transcripts) is an integral part of the rDNA theory of aging as activity of this promoter directly affects stability at the rDNA via displacement of cohesin (Kobayashi, 2011). In support of this model, it was recently shown that Sir2's effect on lifespan is predominantly mediated by its action at the rDNA E-pro and it was suggested that the resulting rDNA instability is causative for aging rather than being a byproduct of it (Saka et al, 2013). We found that a *ubp3*Δ mutant, which has increased silencing (i.e. down-regulates E-pro activity) at rDNA, has a reduced RLS (**paper I**). In addition, cells lacking Ubp3 have very little unequal recombination at rDNA suggesting that rDNA instability is not a prerequisite for aging (in *ubp3*Δ mutants). However, *SIR2* is epistatic to *UBP3* with regard to unequal recombination at rDNA, whereas loss of Sir2 requires Ubp3 for full de-repression of a *URA3* allele at rDNA (**paper I**). These findings suggest that recombination is not directly proportional to levels of transcriptional activity at rDNA, as was previously proposed by Kobayashi and colleagues (Kobayashi & Ganley, 2005).

However, it is possible that Sir2 may have additional roles in inhibiting recombination that is partly independent of its role in silencing, but this has to be further studied.

We found evidence that the role of Ubp3 in aging may be unlinked to silencing/unequal rDNA recombination by combining mutations of *UBP3* with mutations in *SIR2* and *FOB1*. Deletion of *FOB1* somewhat suppressed the short lifespan of a *ubp3*Δ mutant, indicating that Fob1 either reduces recombination further, resulting in lifespan extension, or that Fob1 affects RLS extension via another pathway than rDNA recombination. Deleting *SIR2* in a *ubp3*Δ mutant reduced lifespan to that of a *sir2*Δ single mutant, suggesting that a short lifespan correlates with a high recombination frequency. Deletion of *UBP3* in the *sir2*Δ *fob1*Δ (same as wild-type lifespan) shortened the lifespan to that of a *ubp3*Δ single mutant, again suggesting a role for Ubp3 in RLS that is independent of silencing or unequal recombination in rDNA (Öling and Kvint, unpublished data). Similarly, the lifespan of *sir2*Δ *fob1*Δ double mutant calls for extra attention since deleting *FOB1* does not fully suppress the short lifespan of a *sir2*Δ mutant while fully suppressing ERC accumulation (Kaeberlein et al, 1999). These data, suggests that both Sir2 and Ubp3 are implicated in other functions related to aging that are distinct from transcription control at rDNA. For instance, proper clearance of protein aggregates have been shown in a number of studies to be associated with premature aging (Erjavec et al, 2007; Heeren et al, 2004; Hill et al, 2014; Kruegel et al, 2011). Such roles for both Ubp3 and Sir2 are discussed further in ***paper II, III*** and ***IV***.

3.2 Role of Ubp3 in protein quality control and aging

As described above, Ubp3 is involved in transcriptional regulation and silencing but there is also mounting evidence that this DUB is important for PQC. For example, nutrient signaling and protein quality control is mediated in part by Ubp3-dependent degradation of mature 60S ribosomes (ribophagy) during nitrogen starvation (Kraft et al, 2008). The E3 ligase Ltn1 was recently proposed to act as an antagonizer of Ubp3 in this process (Ossareh-Nazari et al, 2014). Furthermore, Ubp3 has been shown to regulate Ras/PKA signaling by interacting with Ira2 and regulating its activity, and level of ubiquitination (Li & Wang, 2013). These findings suggest that Ubp3 is controlling both the quantity and quality of diverse proteins.

Many additional processes are regulated by Ubp3 and its co-factor Bre5 during non-starvation conditions. Ubp3 regulates the degradation of Sec23, a component of the ER-Golgi transport related COPII vesicle (Cohen et al, 2003). It was later shown that the specific Sec23 degradation is regulated also by the AAA-ATPase Cdc48 and Rsp5, a ubiquitin ligase (Ossareh-Nazari et al, 2010). Ubp3 also reverses the ubiquitination of Atg19, a CVT pathway (cytoplasm to vacuole trafficking) receptor protein implicated in the vacuolar delivery of two enzymes, aminopeptidase I and alfa-mannosidase (Baxter et al, 2005). Two other seemingly unrelated processes regulated by Ubp3 include the microtubuli-system (Stu1) and DNA repair (Rad4) (Brew & Huffaker, 2002; Mao & Smerdon, 2010). The latter study showed that Ubp3 interacts physically with the 26S proteasome to facilitate destruction of Rad4. These studies point to a dual role for Ubp3 where it facilitates destruction of some proteins whereas it rescues others. This duality is addressed in ***paper II***.

To answer why cells lacking Ubp3 are short-lived even though they show increased silencing and decreased rDNA recombination, we hypothesized

that this might be linked to a role of Ubp3 in PQC. Craig and colleagues isolated Ubp3 as a high copy suppressor of a temperature sensitive strain deleted for the two major cytoplasmic yeast chaperones *SSA1/SSA2* (Baxter & Craig, 1998). The authors proposed a model where Ubp3 over-production rescued the double mutant by reversing ubiquitination of temporarily misfolded proteins, thereby preventing proteasomal degradation and allowing some residual activity of these proteins. However, no experimental evidence for this idea was provided. The chaperones Ssa1/Ssa2 belong to a subfamily of yeast Hsp70s which also include Ssa3 and Ssa4. *SSA1* and *SSA2* display roughly 98% sequence homology and are functionally redundant in many cellular processes whereas *SSA3* and *SSA4* are less (~80%) similar to *SSA1* or *SSA2*. There is little data on Ssa3 function and only slightly more on Ssa4. Though, one study showed that a *ssa1*Δ *ssa2*Δ mutant is synthetically dead if *SSA4* also is deleted (Werner-Washburne et al, 1987). The same study also showed that deletion of *SSA1/SSA2* induced expression of *SSA4*. Therefore, we speculated that the Ubp3-dependent suppression was achieved by up-regulating *SSA4* or possibly *SSA3*. However, this was not the case (**paper II**).

We next asked whether elevated Ubp3 levels provided enhanced folding capacity in the *ssa1*Δ *ssa2*Δ strain. Utilizing a luciferase refolding assay we observed no enhanced refolding activity by Ubp3 over-expression. Instead, we speculated that the suppression may be mediated by enhanced clearance of already misfolded and aggregated proteins. To address this question we used the well-characterized protein aggregate-reporter Hsp104-GFP, which is intimately connected with protein quality control and aggregate clearance (Glover & Lindquist, 1998). Hsp104 is dependent on Ssa1 or Ssa2 to find aggregates and over-production of Ubp3 did not suppress such requirement, suggesting that Ubp3-dependent suppression is mediated independently of Hsp104 (Winkler et al, 2012).

In the model proposed by Craig and colleagues, the loss of Ssa1/Ssa2-dependent folding activity is argued to be particularly detrimental at elevated temperatures as many proteins with residual activity are degraded rather than refolded (Baxter & Craig, 1998). Ubp3, in this model, removes ubiquitin-tags from the partly active proteins, rescues them from proteasomal degradation, and thereby allows better growth at high temperatures. We found experimental support for this model: first, we observed a Ubp3-dosage dependent suppression of Ssa1/Ssa2-deficiency where the highest levels of Ubp3 correlated with growth even at 37°C. Second, the misfolded model protein ΔssCPY* was stabilized by Ubp3-dependent deubiquitination in the absence of *SSA1/SSA2*. Last, we genetically altered proteasome levels by deleting *RPN4* (lower levels) and *UBR2* (higher levels) and crossed these mutants with a *ssa1*Δ *ssa2*Δ strain. In line with the proposed model, lowering proteasome levels in the *ssa1*Δ *ssa2*Δ allowed for better growth at high temperatures, whereas boosting proteasome levels (*ubr2*Δ) was detrimental for viability. Intriguingly, over producing Ubp3 in *ssa1*Δ *ssa2*Δ *ubr2*Δ restored viability (**paper II**).

Next, we also investigated whether the same mechanism held true for cells experiencing another kind of stress - aging. Loss of Ubp3 resulted in a shorter lifespan as did loss of *SSA1/SSA2*. However, over-expression of Ubp3 rescued the short lifespan of *ssa1*Δ *ssa2*Δ. In contrast to heat tolerance, this suppression was dependent on normal proteasome levels as seen by deleting *RPN4* in the Hsp70-deficient strain. In line with this, we also found that enhancing proteasome levels by deleting *UBR2* in a *ubp3*Δ mutant restored the lifespan back to wild-type levels (unpublished data). One interpretation of such results is that inefficient deubiquitination by Ubp3 can partially be overcome by boosting the end point of this pathway - proteasomal destruction. These observations point to an "aging related role" for Ubp3 mediating proteasomal destruction of proteins. Thus, we conclude that the Craig model explains the role

of Ubp3 in certain genetic and environmental contexts such as heat stress and Hsp70 deficiency, but fails to explain the role of Ubp3 in old cells.

Enhancing proteasome capacity extends lifespan and this extension has been suggested to be associated with reduced accumulation of aggregates (Kruegel et al, 2011). Similarly, aging is associated with a progressive decline in 26S proteasome activity, which could potentially redistribute misfolded proteins to specific protein aggregate compartments (Andersson et al, 2013; Kaganovich et al, 2008; Kruegel et al, 2011). Moreover, it was recently shown that the meta-caspase Mca1 acts as lifespan extending gene and that the extension required both Hsp104-dependent disaggregase activity and fully functional proteasomes which links this lifespan control to the removal of damaged and aggregated proteins (Hill et al, 2014). To this end, we sought to investigate a possible link between Ubp3, aggregate compartmentalization, and aging. To detect such a link we utilized the mother enrichment strain (Lindstrom & Gottschling, 2009) modified to C-terminally tag GFP on the endogenous Ssa2. Using this construct, we observed a premature JUNQ accumulation associated with loss of *UBP3* as compared to wild-type cells. In addition, formation of peripheral aggregates was drastically accelerated in the same mutant. We suggest that this phenotype is associated with the reduced capacity of *ubp3*Δ mutant cells to properly deubiquitinate proteasome substrates and allow entry to the 26S proteasome. It is possible that inefficient proteasome destruction of aberrant proteins could "overwhelm" the JUNQ compartment resulting in subsequent redistribution of aberrant proteins to peripheral sites in aging cells. Consistent with this, reducing proteasome levels by *RPN4* deletion displayed a similar phenotype as the *ubp3*Δ mutant (unpublished data). As the accumulation of peripheral aggregates coincides with accelerated aging in the absence of *UBP3*, it is tempting to speculate that these specific aggregates are indeed true aging factors.

Ubp3 can both stabilize and destabilize proteins according to our data. Rad4 has previously been shown to require Ubp3 for proper degradation (Mao & Smerdon, 2010). In contrast, Rpb1 is stabilized by Ubp3 (Kvint et al, 2008). These findings, together with data from this study on $Ubc9^{ts}$ and ΔssCPY* raises the question of how a DUB mechanistically can execute these two diametrically different actions on proteins. We envision deubiquitination to act at different stages towards destruction of proteins and that the timing determines the outcome. This notion is supported by the finding that ΔssCPY* was deubiquitinated and stabilized by Ubp3 over-production. This is consistent with deubiquitination occurring at a stage prior to proteasomal "commitment". In contrast, Ubp3-assisted proteasomal destruction of a substrate would not be detectable in a strain over-producing Ubp3 as this would rapidly degrade the substrate and escape analysis. However, loss of *UBP3*, we speculated, would result in increased ubiquitination and stabilization of such a substrate. Consistent with this, $Ubc9^{ts}$ exemplified this latter scenario (**paper II**). In conclusion, the data presented for ΔssCPY* and $Ubc9^{ts}$ are consistent with a scenario where Ubp3-dependent deubiquitination occurs at different stages toward commitment to proteasomal degradation (Fig. 7).

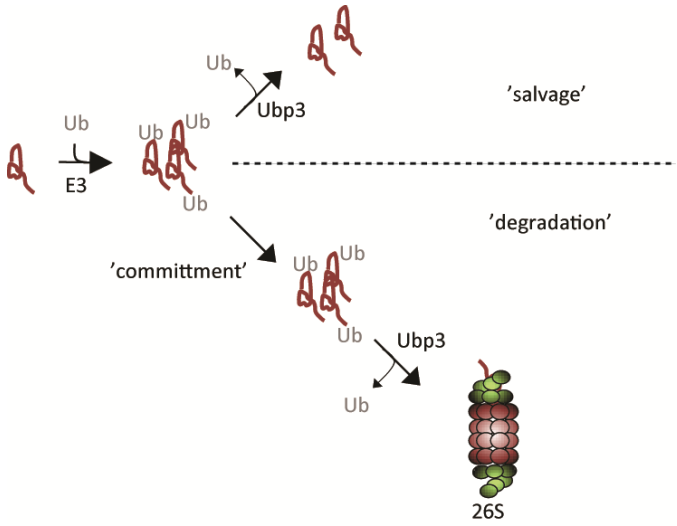

Figure 7. Schematic drawing of Ubp3-dependent deubiquitination. The salvage pathway (top half) depicts how a protein is rescued from destruction. The degradation pathway (lower part) depicts how a protein is committed to the proteasome for degradation.

3.3 Asymmetric inheritance of damaged proteins

Sir2 has been implicated as a regulator of aging and age-related maladies in a wide variety of organisms including yeast, worms, flies, fish and mammals (Galan & Haguenauer-Tsapis, 1997; Lindstrom & Gottschling, 2009; Masoro, 2005; Springael et al, 1999). As discussed previously, the yeast Sir2 accomplishes this regulation, in part, by histone-deacetylation resulting in increased silencing and decreased formation of ERCs (Kaeberlein et al, 1999). Just like ERCs, damaged and aggregated proteins accumulate in mother cells and are subjected to a mother-cell biased segregation (Aguilaniu et al, 2003; Erjavec et al, 2007). This damage asymmetry is dependent on Hsp104 and Sir2 where the latter is linked to actin cable-dependent processes and the polarisome (**paper III**).

The role of actin cables in damage retention has been suggested to be the result of the association of aggregates (including prions) to the actin

cytoskeleton thereby preventing their free diffusion into the daughter cell (Chernova et al, 2011; Tessarz et al, 2009). Sir2 deficiency reduces actin cable abundance and the velocity of retrograde actin flow from the polarisome (Erjavec & Nystrom, 2007; Higuchi et al, 2013). Apparently, in strains devoid of *HSP104*, specialized "compartments" such as IPOD and JUNQ are not properly maturing and aggregates are "trapped" in stress foci (Spokoini et al, 2012). These smaller foci are increasingly inherited by the daughter cell as opposed to IPOD and JUNQ which are sequestered by the mother cell. In addition, other small, stress foci-like aggregates such as those formed by Htt103Q are also asymmetrically inherited as shown in ***paper IV***. Taken together, these findings strongly suggest a model where asymmetric segregation of damaged proteins is dependent on various factors such as a functional actin cytoskeleton, as well as tethering of aggregates to organelles.

This model was challenged by Li and colleagues which suggested that asymmetric inheritance is a purely passive process due to the geometry of yeast cells and a slow random diffusion of aggregates (Zhou et al, 2011). This model views aggregate inheritance as regulated by the size of the bud neck and how long this channel is open for diffusion of aggregates. However, in this study, some aggregates appeared stationary whereas others where more mobile (Zhou et al, 2011). This diversity in aggregate population may not best be modeled by employing an average diffusion coefficient. In fact, this model was challenged in the Spokoini study due to the observation that the larger of these aggregates were IPOD or JUNQ inclusions. By definition, these aggregates cannot diffuse freely as they are attached to the vacuole and nuclear membrane respectively (Spokoini et al, 2012).

A recent study tested whether the passive diffusion model or the factor-dependent tethering model was more relevant to explain how asymmetrical inheritance of aggregates is achieved (Song et al, 2014). The

authors argued that increasing the time for completing cytokinesis would enhance aggregate inheritance of such aggregates moved by passive diffusion. However, this was not the case. Furthermore, mutants with increased daughter cell inheritance of aggregates did not show a larger bud-neck diameter, longer generation time or increased number of aggregates. If aggregates are segregated by random diffusion, these traits would be expected by mutants displaying increased inheritance. However, neither the *sir2*Δ mutant nor any of the other identified mutants displayed any of the aforementioned traits. Importantly, this study identified additional factors as essential for establishing damage asymmetry. These factors include the actin-associated proteins Cmd1 and Myo2 as well as ER- Golgi transport components. Moreover, both Huntingtin 103Q and heat-induced, Hsp104-associated stress-foci were found to co-localize with Cmd1- and Myo2-enriched structures and super-resolution 3-D microscopy showed that these aggregates co-localize with the actin cytoskeleton.

4. Concluding remarks

Faulty mitochondria, defective vacuoles, ERCs and/or rDNA instability, and damaged proteins all seem to be factors present in old yeast cells. The crucial question is: are the same factors dysfunctional in aged metazoans (animals) and can we transfer the lessons learned from yeast to therapeutically combat age-related disorders? Accumulating evidence suggest that we can answer cautiously with a "yes" to both parts of this question. For example, enrichment of dysfunctional mitochondria are causative for aging also in metazoans (Bratic & Larsson, 2013). One reason for this dysfunctionality in mice are mutations of the maternally originated mtDNA which, when transferred to the progeny, cause clonal expansion of these errors during development (Ross et al, 2013). Another mechanism, as described earlier, is dysfunctional mitochondria preceded by a collapse in vacuolar pH-control (Hughes & Gottschling, 2012). Whatever the cause for unhealthy mitochondria, keeping these organelles in good shape appears pivotal for a full lifespan. Lysosomes, similar to yeast vacuoles, are indispensable for autophagy and inhibiting this machinery triggers cellular degeneration (Rubinsztein et al, 2011). Importantly, reduced autophagic capacity is often accompanied by aging and such cellular degeneration. Therefore, targeting macro-autophagy seems like an interesting possibility for age-related therapeutic purposes.

Unlike ERCs in yeast, extra chromosomal circular DNA (eccDNA) in metazoans do not seem to be causative for aging but elevated levels of eccDNA have been observed in patients suffering from Werner's syndrome (described earlier) (Kunisada et al, 1985). However, the sirtuins, which are known to regulate ERC formation and rDNA stability, may still be targeted therapeutically as the mammalian sirtuins have been shown to play a key role in age-related diseases (Guarente, 2013). The fourth, and perhaps most intensely

studied aging factor in this thesis is damaged proteins and there is an increasing body of evidence suggesting that aberrant and aggregated protein-species affect also the rate of metazoan aging as well as trigger age-related neurological disease (Taylor & Dillin, 2011). For example, in *Caenorohabditis elegans*, protein-amyloids have been chemically targeted and this resulted in an extended lifespan suggesting that such targeting may prove useful for therapeutic purposes (Alavez et al, 2011).

Transcription control at the rDNA loci, which links aging to both rDNA instability and ERC formation, is influenced by *UBP3* in a manner that suggests that deletion of this gene should display an extended lifespan. However, we did not observe such extension of RLS, instead defects in PQC and proteasomal degradation with subsequent accumulation of aberrant proteins seems a more likely explanation to cause premature aging in the absence of *UBP3*. However, our data does not exclude the possibility that transcriptional regulation by Ubp3 is causing premature aging related to alterations of the proteome. Ubp3 was shown to divergently determine the fate of two model proteins but we know little of the native substrates regulated by Ubp3. It would therefore be interesting to compare the proteome of old and young cells in both a wild-type and *ubp3*Δ mutant. This is particularly interesting in light of the finding that levels of certain chaperones, such as Hsp70, decline with age in rats (Heydari et al, 1993). Whether or not *UBP3* (or *USP10* in mammals) is a good target for therapeutic purposes related to age-related disorders is too early to predict and more research on this subject is needed. To this end, it is interesting to note that *USP10* is required for efficient p53 activation in response to DNA damage and it suppresses tumor cell growth in RCC cells (Yuan et al, 2010). Moreover, several studies demonstrate that over-expression of *UBP3* is able to suppress the toxicity mediated by aggregation of the Parkinson's-related protein alfa-synuclein (Cooper et al, 2006; Tenreiro et al, 2014). The suppression of this

toxicity was suggested to be due to an Ubp3-dependent increase of forward ER-Golgi trafficking mediated via Sec23 stabilization and subsequent promotion of vesicle exit from the ER.

Recent insight of intracellular PQC-pathways has triggered my curiosity for the Hsp70-specific biochemical interaction enabling either Ssa1 or Ssa2 to present a substrate to Hsp104 for disaggregation. Further research should benefit from utilizing the *ssa1*Δ *ssa2*Δ mutant to create a cellular context where Hsp104 is largely absent from aggregates. In this environment it should be possible to screen for other factors that are important for Hsp104 in aggregate recognition. Initially, a plasmid collection containing sequences encoding the various domains of the Ssa-proteins should be generated to further study the Hsp70-Hsp104 interaction. These plasmids could also be utilized to study the effect of PQC in aging where Hsp104 is either present or absent from age-induced aggregates. The Hsp70-Hsp104 system is particularly interesting since it has been suggested to be required for formation of smaller aggregates such as stress foci, Q-bodies and peripheral aggregates. In addition, these aggregates have been speculated to be a transient stage to other types of larger aggregates/inclusion bodies (*i.e.* IPOD and JUNQ) (Escusa-Toret et al, 2013; Song et al, 2014; Spokoini et al, 2012). The smaller aggregates seemingly differ slightly in their nature. For example, $Ubc9^{ts}$ which is has been shown to form Q-bodies, move in an actin-independent (but energy-dependent) manner whereas peripheral aggregates and Htt103Q associate with actin cables (Escusa-Toret et al, 2013; Song et al, 2014; Specht et al, 2011). This discrepancy is interesting and may suggest that different proteins are directed to different spatial locations. However, another explanation is that different experimental protocols yield different results. This is exemplified by the fact that Htt103Q forms aggregates upon production whereas $Ubc9^{ts}$ misfold by elevated temperature, a procedure known to disrupt actin cables. In addition, some protocols utilize proteasome

inhibitors whereas others do not. This is important, especially as the JUNQ compartment readily forms upon inhibition of proteasomes (Kaganovich et al, 2008). Data from our group, and others, add to the complexity of this machinery since it implicates Ssa1 or Ssa2 as paramount for Hsp104 to find aggregates and perform any disaggregating activity (**paper II**) (Winkler et al, 2012). Yet, large IPOD like inclusions (GFP-Ubc9^{ts}) are frequently observed in the absence of these major Hsp70s (Escusa-Toret et al, 2013), indicating that at least IPOD can form regardless of Hsp70s and presumably also Hsp104. In addition, we also observed that ΔssCPY*-GFP localizes to both IPOD and JUNQ in the absence of *SSA1/SSA2* (unpublished data). However, we do not yet know if Hsp104 is present in these inclusions in this genetic context. It should also be noted that *SSA4* is highly induced in the absence of *SSA1* and *SSA2* which may aid a small fraction of Hsp104 to localize to (or form) IPOD. A further characterization of all these aggregate-types should benefit future research in the PQC-field.

PQC also encompasses asymmetrical segregation of protein aggregates. This phenomenon is analogous to the separation of germ-cell and somatic cell types in higher organisms (Guarente, 2010). Recently, it was shown that oxidatively damaged proteins segregate asymmetrically during cytokinesis of various types of stem-cells (Bufalino et al, 2013). Interestingly, these stem-cells employ different bias on the segregation of damaged components. Specifically, the cell-line that was on the receiving end of damaged proteins had in-fact always a shorter lifespan. Similar to yeast, this segregation was dependent on various factors (Ogrodnik et al, 2014). Finally, a deeper understanding of the underlying mechanisms of PQC will certainly help our understanding of why some proteins associated with neurological disorders and aging cause cytotoxicity, and how these may be therapeutically targeted.

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