Thesis for the Degree of Doctor of Philosophy

Damage Segregation and Cellular Rejuvenation in *Saccharomyces cerevisiae*

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Cover picture:
Fluorescent microscopy images of a dividing yeast cell
Top left: Vacuole inheritance visualized by a reporter protein in the vacuolar membrane (Vph1-mcherry). Top right: Bud scars of a replicatively old cell, stained with a WGA conjugate. Bottom left: Heat-shock induced aggregates, bound by Hsp104-GFP. Bottom right: Actin cables, stained using Rhodamine phalloidin.
Pictures taken and edited by: Sandra Malmgren Hill

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To my friends and family

Abstract

The process of aging is defined as a time-dependent decline in cellular functionality, and aging is thought to have evolved as organisms were optimized for reproduction, at the cost of an imperfect repair and maintenance system. As a consequence, different kinds of dysfunctional components and damage accumulate over time. Eventually these dysfunctional components, termed aging factors, reach critical levels at which they interfere with cellular systems, causing the age-related loss of function that ultimately leads to cell death.

The investment in propagation also encompasses the retention of aging factors within the progenitor cell, so that the progeny is born rejuvenated, free from damaging aging factors. The accumulation of oxidized and aggregated proteins has been established to act as aging factors in several organisms. These damaged proteins are asymmetrically distributed during cell division, a process that in yeast relies on the actin cytoskeleton and components of the cellular protein quality control (PQC) system. In my work, I have established that this asymmetric damage segregation is an active and factor-dependent process, accomplished through the actions of two interconnected systems. Mainly, sequestration of protein aggregates into certain quality control sites within the mother cell ensures the retention of damage, but cells have also evolved a process of aggregate removal so that any damage that accidentally leaks into the daughter cell is removed. This removal is achieved either by degradation or by retrograde transport of aggregates back into the mother cell.

In a genome-wide screen we identified functions that are required for this damage distribution, and could further pinpoint several of the asymmetry generating genes (AGGs) regulating this process. Through this approach, we found that the sequestration of aggregates into protective inclusions is dependent on vesicle transport and fusion to the vacuole, and we identified a novel role for the vacuole adaptor Vac17 in this process. Additionally, we found that the process of aggregate removal includes an unexpected role for the metacaspase Mca1, acting in conjunction with the proteasome and PQC system to degrade aggregated proteins. The link between protein aggregation and aging is further reinforced by our data demonstrating that altered levels of these identified AGGs affect cellular fitness and longevity.

Keywords: Aging, protein damage, segregation, quality control, metacaspase, Mca1, protein aggregates, vacuole, endocytosis, Vac17

Abbreviations

ROS	Reactive oxygen species
ERCs	Extra chromosomal rDNA circles
ARS	Autonomously replicating sequence
NPCs	Nuclear pore complexes
V-ATPase	Vacuolar ATPase
PQC	Protein quality control
LOH	Loss of heterozygosity
CR	Caloric restriction
TOR	Target of rapamycin
PKA	Protein kinase A
IGF-1	Insulin growth factor 1
ALS	Amyotrophic lateral sclerosis
HSPs	Heat shock proteins
NEFs	Nucleotide exchange factors
UPS	Ubiquitin-proteasome system
DUBs	De-ubiquitinating proteins
PAS	Pre-autophagosomal structure
JUNQ	Juxtanuclear quality control compartment
IPOD	Insoluble protein deposit
INQ	Intranuclear quality control compartment
PCD	Programmed cell death
SIM	Structured illumination microscopy
MSD	Mean square displacement
AGGs	Asymmetry generating genes
ts	Temperature sensitive

Papers included in this thesis:

I. 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

II. Song J, Yang Q, Yang J, Larsson L, Hao X, Xuefeng Z, **Malmgren-Hill S**, Cvijovic M, Fernandez-Rodriguez J, Grantham J, Gustafsson CM, Liu B, Nyström T (2014) Essential Genetic Interactors of *SIR2* Required for Spatial Sequestration and Asymmetrical Inheritance of Protein Aggregates. *PLoS Genetics* **10:** e1004539

III. Hill SM, Hao X, Grönvall J, Spikings-Nordby S, Widlund PO, Amen T, Jörhov A, Josefson R, Kaganovich D, Liu B, Nyström T. Vac17-dependent sequestration of aggregated proteins to the vacuolar surface extends lifespan. *Cell Reports* **16**: 826-838.

IV. Hill SM, Hao X, Liu B, Nyström T (2014) Life-span extension by a metacaspase in the yeast *Saccharomyces cerevisiae. Science* **344:** 1389-1392.

Other publications:

V. Hill SM and Nyström T (2015) The dual role of a yeast metacaspase: What doesn't kill you makes you stronger. *Bioessays*, **37**: 525–53

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1. Introduction

Thanks to the presence of stem cells, tissues within our bodies are continuously exchanged; so that damaged and worn out cells can be substituted with young and fresh ones. As an example, every skin cell in our body is replaced once every 20-30 days. This turnover is accomplished through the asymmetric division of epidermal stem cells, where each cell division generates one renewed stem cell and one daughter cell that can undergo terminal differentiation to produce a specialized skin cell (Weinstein and Van Scott, 1965, Zouboulis et al., 2008). However, this renewal process cannot withstand the pressure of time, and as we age, our skin starts to display characteristic signs of aging. The turnover rate of cells declines, and the amount of connective tissue decreases, causing our skin to become loose and wrinkled. This raises several questions: How is this renewal of stem cells accomplished in the first place, and what is it that occurs during aging that limits the rejuvenating capacity?

The cellular renewal, or rejuvenation, occurring in dividing stem cells as well as in microbial cells is accomplished by an asymmetric distribution of certain harmful factors that have accumulated throughout the cellular lifetime. Upon cell division, these so-called aging factors are restricted to one of the two forming cells, resulting in the other cell being born rejuvenated and damage-free (Aguilaniu et al., 2003, Bufalino et al., 2013, Erjavec et al., 2007, Liu et al., 2011). During aging, this process of damage asymmetry breaks down, leading to a downward spiral where both cells inherit aging factors and thus are born prematurely aged (Egilmez and Jazwinski, 1989, Hughes and Gottschling, 2012).

The aim of this thesis is to describe the nature of these aging factors, and elucidate the mechanisms underlying their asymmetric distribution during cell division. I will focus on both evolutionarily and mechanistic aspects of aging in

order to explain why asymmetric inheritance breaks down during aging and how such a seemingly suboptimal system has been allowed to evolve. My work is based on studies using the budding yeast *Saccharomyces cerevisiae* as a cellular model, where an age-related asymmetric segregation of aging factors occurs, similar to that reported for stem cells (Denoth Lippuner et al., 2014, Bufalino et al., 2013, Fuentealba et al., 2008). In yeast, these aging factors are retained in the mother cell to produce a rejuvenated daughter with a reset lifespan. While it has been established that many different aging factors are acting in parallel to affect cellular fitness and longevity, my work is focusing on the contribution of misfolded and aggregated proteins to the aging process. Through my work, I have identified several important genes and processes that contribute to the successful retention of protein aggregates, and further demonstrated how the action of these processes are linked to replicative aging. As many of these genes and processes are conserved throughout evolution, future studies might elucidate whether they are important also during aging in higher eukaryotes. The identification of such conserved mechanisms could be important for research on aggregation diseases and tissue rejuvenation.

2. Aging

Aging, or senescence, is defined as the time-dependent decline in biological functions accompanied by an increase in mortality, and it is a universal process that most living organisms experience (Rose, 1991). We are all well acquainted with the characteristic features of senescence, yet, despite its familiarity, it is still not known how this process came to be. As aging has its obvious drawbacks, why do we all age, and how could such a process have evolved throughout the history of life? Here I will describe the general theories on the evolution of aging, as well as the mechanisms that are thought to underlie this physiological decay.

2.1 Evolutionary theories of aging

The cause of aging has been widely debated throughout history, the biggest dispute, perhaps, being whether aging should be considered programmed or not. Initial theories, presented by August Weismann in 1891, regarded aging as beneficial to the species, where the death of weaker individuals provides space and allows for younger and healthier individuals to thrive (Weismann et al., 1891). Although Weismann later abandoned this theory, such ideas of group selection, along with the identification of several genes that affect longevity are used as arguments in favor of programmed aging (Longo et al., 2005).

There are however major flaws in this consideration: if aging had evolved as a genetically encoded program, with an intrinsic clock telling our bodies when to shut down, then one would expect the process of senescence to be very conserved. This is not the case, as aging manifests itself quite diversely, even when comparing individuals of the same genetic composition,

such as monozygotic twins (Kirkwood, 2005). Furthermore, due to extrinsic factors, animals in the wild rarely survive long enough to die from the physical deterioration of old age (Kirkwood and Austad, 2000). Thus, there could have been no force of natural selection to drive the evolution of programmed aging, as aging has not had any impact on mortality rate or reproduction. Consequently, theories have been developed to explain the evolution of aging as a consequence of living, rather than a programmed way of dying.

2.1.1 Mutation accumulation theory

The absence of selection pressure was in fact used by Peter Medawar in his mutation accumulation theory as an argument to explain how aging could have evolved (Medawar, 1952). As the force of natural selection declines with age, Medawar reasoned that any genetic changes, affecting fitness only later in life, would not be eliminated through evolutionary selection. Thus, an organism could accumulate numerous mutations throughout its lifetime, and as long as these mutations had no effect on early life and reproduction, they could spread in the population. A drawback of this theory is that it does not explain how aging could have evolved uniformly in all organisms. The theory only speculates that there is no selection *against* aging, and that mutated alleles of genes causing aging could as easily have been passed on as the "normal" allele. If this were the case, one would expect the inheritance rate of both kinds of alleles to be equal, and aging would only be seen in part of a population. In order to explain why aging is seen in *all* organisms of a population, one would have to include an argument for a positive force of natural selection.

2.1.2 Antagonistic pleiotropy

A problem with explaining the evolution of aging with the mutation accumulation theory is that it is based on random inheritance, and such genetic drift is considered to play only a minor role in evolution, especially for larger populations (Fisher, 1930, Orr, 2005). To account for this, George Williams expanded the theory and hypothesized that aging could have evolved due to a pleiotropic effect of some genes that are beneficial early in life, while having a negative effect on fitness later in post-reproduction life (Williams, 1957). Consequently, aging has evolved as a byproduct due to the selection of early reproduction and survival.

2.1.3 The disposable soma theory

Another contemporary theory of aging involves a similar trade-off between reproduction and survival. The disposable soma theory, presented by Thomas Kirkwood in 1977, postulates that aging is an evolved limitation in maintenance rather than a programmed decline (Kirkwood, 1977). This theory comprises the germ-plasm theory of heredity presented by August Weismann, proposing that each organism is composed of a body (soma) that is separable from the germ-plasm carrying the hereditary information (Weismann et al., 1893). Kirkwood based his theory on the premise that resources are limited, and that an organism has to balance resources going into repair and maintenance of its soma and investments going into reproduction (germ-line). The theory shares similarities with the theory of antagonistic pleiotropy in the sense that any genetic changes that increase the resources allocated for reproduction do so with the cost of less resources being available for repair (Hughes and Reynolds, 2005, Kirkwood and Holliday, 1979, Kirkwood and Rose, 1991). The major difference between the two theories is that the antagonistic pleiotropy theory visualized the same allele being both beneficial

and detrimental at different stages of life, whereas the disposable soma theory claims that the investment in reproduction, regulated by a specific set of genes, leads to less resources being available for maintenance, which is controlled by a different set of genes. Since repair and maintenance cannot be fully accomplished, damage will accumulate over time and eventually overwhelm the system, causing the functional decline that we associate with aging.

In addition to explaining the evolutionary occurrence of aging, the disposable soma theory also makes certain predictions regarding the biology of aging, many of which have support from experimental evidence (Holliday, 1997, Kirkwood and Austad, 2000, Kirkwood, 2005). However, there are examples from nature that cannot be easily explained with this theory, for example the occurrence of semelparous animals, which die while at the peak of reproductive capacity. A very drastic example of this, is that of the spider *Argiope aurantia*, which die shortly upon mating due to a programmed heart failure (Foellmer and Fairbairn, 2003). Despite the occurrence of such cases of seemingly programmed death, the disposable soma theory has become one of the most acknowledged and accepted theories for explaining how aging could have evolved.

2.2 Cellular mechanisms of aging

While the evolutionary theories explain why we age in the sense of how such a deleterious process can be allowed by nature, it still does not explain the proximate cause of aging: what is it that makes our cells weaken and eventually die of old age? The disposable soma theory explains that aging follows as a consequence of living, and that cells have evolved maintenance systems to be optimized for reproduction, rather than maximized for survival

(Zimniak, 2008, Kirkwood, 2005). The outcome of this inherent imperfection is that damage will accumulate, and it is this damage that eventually gives rise to aging characteristics. However, the questions remain: what kind of damage is accumulating, how does it occur and how is this damage affecting cellular fitness? By identifying the genes underlying these events researchers hope to understand and possibly even be able to modify the rate at which an organism ages. In this section I will summarize the main theories that have been presented to explain the molecular mechanisms of aging.

2.2.1 The free radical theory and damage accumulation

In 1956, Harman presented his free radical theory of aging, stipulating that oxygen radicals attack essential machineries in the cell and cause the functional deterioration associated with aging (Harman, 1956). As most of these reactive oxygen species (ROS) were shown to originate from the electron transport chain in energy producing mitochondria, the free radical theory was later extended into "the mitochondrial theory of aging" (Harman, 1972). Since the accumulation of ROS seemed to be a result of metabolism, the mitochondrial theory has been associated with the "rate of living" theory presented by Pearl in 1928, stating that a higher metabolic activity leads to an increased rate of aging (Pearl, 1928). While the rate of living theory has been questioned, the mitochondrial theory of aging has gained support from many studies. Aging is associated with increased levels of ROS in many organisms, and the harmfulness of these oxidative species has been related to protein oxidation and inactivation as well as to DNA damage (Stadtman, 2006, Finkel and Holbrook, 2000). Although the role of ROS is not exclusively harmful, as it has proven to be important for cellular signaling, the general consensus is that high levels of ROS are detrimental to cellular fitness (Finkel and Holbrook, 2000). Increased accumulation of oxidative damage is found to

cause both premature aging as well as many pathological conditions, and improving oxidative defense systems can have beneficial impacts on longevity, reinforcing the idea of ROS as an age initiator (Finkel and Holbrook, 2000, Knoefler et al., 2014, Molin et al., 2011).

2.2.2 Replication stress theories

As aging is not programmed, and rather happens stochastically as a result of extrinsic and intrinsic cues, it makes sense to think that cells can age differently depending on their way of life. Although many factors will be common for cellular aging, such as maintenance and repair pathways, the impact of each of these factors might vary depending on the different stresses a cell is exposed to during its lifetime. As an example of this, Kirkwood argues that a main cause of aging for constantly dividing cells will be linked to the risks of frequent replication (Kirkwood, 2005). Each round of DNA replication introduces a risk of error, and DNA repair and maintenance cannot be completely accomplished, as dictated by the disposable soma theory. Consequently, it is likely that DNA mutations will accrue over an increasing number of replications, and Leo Szilard hypothesized that it is the accumulation of such errors in somatic cells that causes aging (Szilard, 1959). An important aspect of his theory is that mutations can only be allowed to accumulate in the somatic cells of an organism, and not in the germ cells – as genetic changes in these cells would have a negative impact on the progeny. In line with Szilard's somatic mutation theory, studies have shown an increase in mutations and damage to both nuclear and mitochondrial DNA in aged animals and tissues (Kirkwood, 2005, Vijg, 2000, Barja and Herrero, 2000). Another alteration to DNA that has been connected to aging is the shortening of telomeres, located at the end of chromosomes. Telomere loss might function as an internal clock for a dividing cell, allowing only a limited

number of replications before chromosome stability collapses and the cell dies (Kirkwood, 2005). Telomere shortening is restricted to somatic cells, as germ cells and stem cells harbor an enzyme called telomerase that can elongate the chromosome ends, ensuring "immortality" of the germ line. Although both the somatic mutation theory and the telomere shortening theory have gained support from aging studies in metazoans, it cannot readily explain the aging process in unicellular systems such as yeast, where there is no division between the genome of the soma versus that of the germ line (Kaya et al., 2015).

2.3 Yeast as a model for aging

Unicellular species such as the budding yeast was once thought of as immortal, since a culture of these cells could propagate indefinitely. However, looking closer at individual cells, it was found that each distinct cell could only go through a limited number of cell divisions before it entered senescence and died (Mortimer and Johnston, 1959, Barton, 1950). This limitation in cell division is termed replicative lifespan, and yeast has an established replicative lifespan with a median of 25 divisions, and a maximum of around 40 divisions (Egilmez et al., 1989, Jazwinski et al., 1989, Egilmez and Jazwinski, 1989). In addition to having a limited replicative potential, yeast cells display age-associated alterations of cellular structure and functions; aging in yeast is accompanied by an increase in size, prolonged generation time, sterility, cell wall alterations and nuclear fragmentation (Mortimer and Johnston, 1959, Guarente, 1997, Muller et al., 1980, Smeal et al., 1996).

As yeast is fast growing and can easily be genetically manipulated, it has become a popular model for studying cellular aging. Many of the genes and pathways identified in replicatively aging yeast has been found to be

conserved in higher organisms, making it a relevant tool for identifying aging factors that could be potential therapeutic targets to treat age-related diseases and provide tissue rejuvenation (Nystrom, 2013, Denoth Lippuner et al., 2014).

Another way to study aging in yeast is to look at chronological lifespan, which is the survival of cells in a post-replicative phase. This type of aging is thought to be reminiscent to the aging occurring in post-mitotic cells such as neurons (Longo et al., 2012, Longo and Fabrizio, 2012). Although both types of yeast aging are of scientific interest, my work has been exclusively focused on replicative aging and thus this type of aging will be the main focus of this thesis.

3. Cellular rejuvenation

It might seem obvious that a fertile adult animal, no matter how old, will give rise to a young offspring with the potential to live a full life. However, this process of progeny rejuvenation gets more complicated when considering it on a cellular level. A cell divides, seemingly symmetrical, by splitting into two cells; so how is it possible for an aged cell to give rise to young and pristine daughter cells, which is the case when new tissue is produced from stem cells in our body? As postulated by the disposal soma theory, aging occurs due to accumulation of damage, left unrepaired as a part of the limited resources is allocated for reproduction (Kirkwood and Rose, 1991). The investments in reproduction are not only for producing progeny, but also to make sure that none of the accumulated damage in the aged individual is carried on to the next generation. As Kirkwood explains; “damage cannot be permitted to accumulate across generations without immediate risk of extinction” (Kirkwood and Austad, 2000). Thus, cell division has to be asymmetric with respect to age-related damage in order to produce a rejuvenated progeny. But what is this damage load that accumulates with age, and how can it be contained within the old progenitor cell during cell division? The damage load has been shown to comprise various non-functional cell components, so called aging factors, and several of these factors have been identified in yeast as well as in other organisms. Here, I will give a brief description of the major aging factors found in yeast, and how they contribute to aging and cellular rejuvenation.

3.1 Age asymmetry and progeny rejuvenation in yeast

Yeast cell division is asymmetric with respect to size; as a smaller daughter cell is budded off from the large mother cell (Hartwell and Unger, 1977). The replication process is also asymmetric regarding age; an aged and deteriorated

mother cell can give rise to a young and immaculate daughter cell, born with a full replicative potential (Aguilaniu et al., 2003, Kennedy et al., 1994, Jazwinski et al., 1989, Mortimer and Johnston, 1959). This suggests that there are some changes occurring in the mother cell as it ages, which are confined within the progenitor cell and prevented from being passed on to the next generation. Interestingly, this age asymmetry seems to break down during the final stages of life of the mother cell, and buds produced from these mothers are born "prematurely aged" and have a shorter lifespan (Kennedy et al., 1994, Egilmez and Jazwinski, 1989, Johnston, 1966). Furthermore, even though these late daughters are born with a reduced lifespan, the daughters and granddaughters of this cell displays a gradual restoration towards a normal lifespan – suggesting that the factors inherited by the prematurely aged bud are diluted in subsequent divisions (Kennedy et al., 1994). These data led to the theory that certain aging factors accumulate in a mother cell during replicative aging, and have to be retained within this cell to enable progeny rejuvenation. Similar events of asymmetric distribution of determining factors are seen in cells of higher eukaryotes, and are important for stem cell renewal and cell differentiation (Knoblich, 2008, Betschinger and Knoblich, 2004).

3.2 Aging factors

What are these aging factors that are asymmetrically segregated during cell division, why are they accumulating with age, and how can they induce aging? According to research on yeast, four criteria needs to be met in order for a factor to be classified as a true aging factor (Henderson and Gottschling, 2008, Denoth Lippuner et al., 2014): (1) This factor has to be more abundant in aged mother cells compared to daughter cells, and its levels should increase with age. (2) A putative aging factor should be asymmetrically confined in the

mother cell during cell divisions, so that none, or little, is transmitted to the daughter cell. (3) Reducing the levels of this factor in old cells should decrease age-related phenotypes and extend lifespan while (4) the introduction of higher levels in young cells should induce premature aging and shorten lifespan. Based on these criteria, several aging factors have been identified in yeast, and many of these seem to be conserved across taxonomic domains (Fig. 1) (Smith et al., 2008, Kaeberlein, 2010).

3.2.1 Extrachromosomal ribosomal DNA circles ERCs

Extrachromosomal ribosomal DNA circles (ERCs) are generated in yeast through homologous recombination of tandem repeats at rDNA loci. Replication fork stalling results in a double strand break followed by recombination and the subsequent excision of such ERCs from the chromosome. The ERCs can then self replicate due to an autonomously replicating sequence (ARS) that is contained within each rDNA repeat, and the accumulation of these circles is considered to cause age-induced fragmentation of the nucleolus (Guarente, 1997, Sinclair and Guarente, 1997, Steinkraus et al., 2008). ERCs are asymmetrically inherited, and thought to be retained in the mother cell through association with nuclear pore complexes (NPCs) and a diffusion barrier in the outer nuclear membrane (Fig. 1a) (Shcheprova et al., 2008). However, the exact mode of ERC retention has been debated, as another study showed that nuclear pore complexes are in fact inherited by daughter cells and therefore could not function as anchors to retain ERCs in the mother cell (Khmelinskii et al., 2010). An alternative explanation states that ERCs are passively diffusing, and inheritance is limited only by the geometrical boundaries formed by the shape of the nucleus and the rate of mitosis (Gehlen et al., 2011).

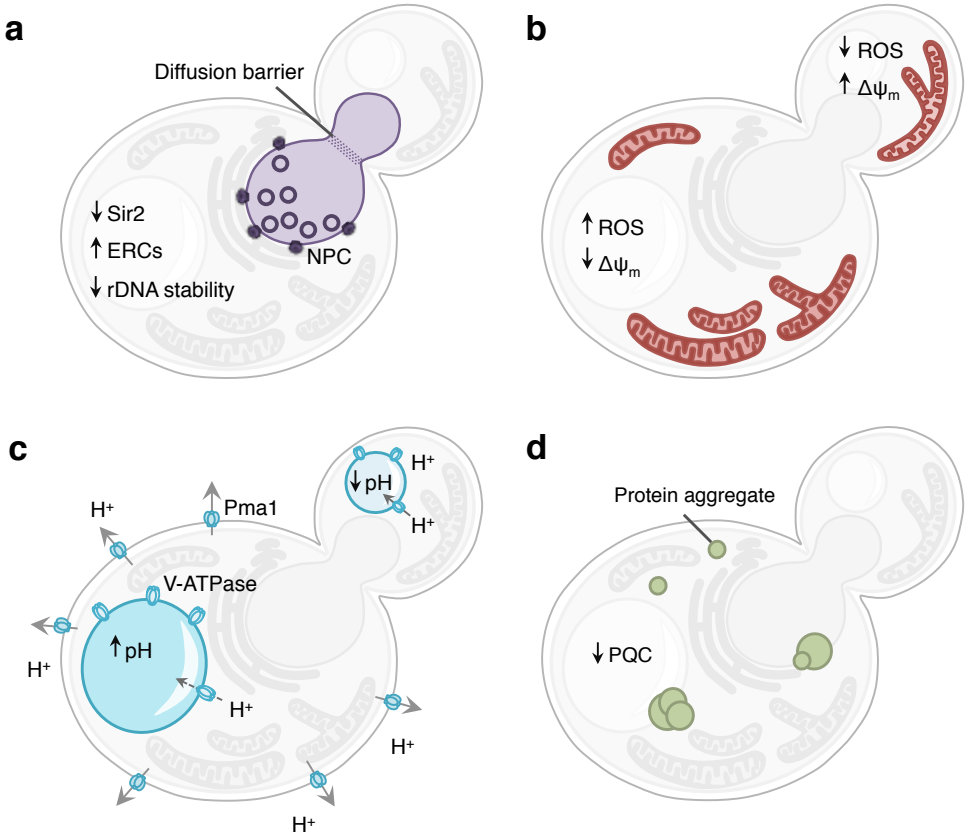


Figure 1. Aging factors that are inherited asymmetrically during cell division in yeast

An age-related decrease in Sir2 activity increases homologous recombination at rDNA, leading to an accumulation of ERCs in the nucleus. ERCs are retained within the mother cell during cell division, due to tethering to NPCs and/or a diffusion barrier at the nuclear membrane. **b)** Dysfunctional mitochondria, fragmented and with a decreased membrane potential ($\Delta\Psi_m$), are unequally distributed during budding, so that the daughter cell inherit the most functional organelles**. c)** Vacuolar acidity is lost during aging, but restored in daughter cells; due to the asymmetric distribution of the plasma membrane proton pump Pma1. Pma1 is retained in the mother cell and is pumping protons (H^+) out of the cell, leaving fewer protons available to be pumped into the vacuole by the V-ATPase. **d)** Oxidatively damaged and aggregated proteins accumulate with age, as levels of ROS increase and PQC function decline. These aggregates are asymmetrically inherited by the mother cell, contributing to the rejuvenation of the protruding daughter cell. Figure adapted with permission from Lippuner et al., 2014.

The accumulation of ERCs upon aging is connected to a decrease in the protein levels of the silence regulator Sir2, ortholog of the mammalian Sirt1 (Dang et al., 2009, Lesur and Campbell, 2004). Sir2 is a NAD+-dependent histone deacetylase suppressing the transcription of rDNA genes, as well as acting together with Sir3 and Sir4 to silence genes at telomeres and at the silent mating type loci (HML & HMR) (Moazed et al., 1997, Gottlieb and Esposito, 1989, Smith and Boeke, 1997). In accordance with the third and forth criteria for an aging factor, overexpression of *SIR2* increases silencing, resulting in less accumulation of ERCs and an extension of lifespan, whereas the deletion of *SIR2* increases levels of ERCs in aging cells and shortens lifespan (Kaeberlein et al., 1999). Similarly, altering the levels of the replication fork stalling protein Fob1 to either increase or decrease ERC formation displayed the same correlation between ERC levels and longevity, strengthening the evidence for ERCs acting as an aging factor in yeast (Defossez et al., 1999).

The effect of ERCs on cellular fitness, and how increased levels could induce senescence has been explained by a titration effect, where ERC titrate out essential DNA-binding proteins (Sinclair and Guarente, 1997). Another theory is that it is not the ERCs that are causing the cellular deterioration; rather it is the rDNA instability itself that is the aging determinant (Ganley et al., 2009, Merker and Klein, 2002). The role of ERCs in aging has been questioned further by accumulating evidence that aging still occurs even without the accumulation of ERCs, and that longevity can be manipulated without altering the levels of this proposed aging factor (Hoopes et al., 2002, Ashrafi et al., 1999).

3.2.2 Dysfunctional mitochondria

One hallmark of cellular aging is the breakdown of mitochondrial structure and function, as seen in several different organisms (Guarente, 2008, Wallace, 2005, Houtkooper et al., 2013, Boveris and Navarro, 2008). In yeast, the tubular network-structure of young cells is gradually lost, and old cells are found with small and fragmented mitochondria (Fehrmann et al., 2013, Scheckhuber et al., 2007). Mitochondria of old cells contain higher levels of ROS and exhibit an age-related loss of membrane potential accompanied with alterations of the mitochondrial DNA (Veatch et al., 2009, Laun et al., 2001, Lai et al., 2002).

Genes involved in mitochondrial inheritance were shown to be highly important for the establishment of age asymmetry and progeny rejuvenation, and deletion of such genes is detrimental to population fitness and results in clonal senescence (Lai et al., 2002, Piper et al., 2002). Additionally, several mitochondrial maintenance proteins, including the mitochondrial lon protease and the Aco1 acontinase, are less active in old mother cells when compared to their young daughter cells (Erjavec et al., 2013, Klinger et al., 2010). Together these results indicate that there is a difference between the mitochondria that are inherited by the daughter cell during cell division, and the ones that are retained in the mother cell. Evidence for such asymmetric distribution was obtained by the lab of Liza Pon, presenting evidence that the less fit mitochondria are retained within the mother cell (Fig. 1b) (McFaline-Figueroa et al., 2011, Nystrom, 2013).

Inheritance of mitochondria is enabled through interactions between an adaptor in the mitochondrial membrane and the myosin motor protein Myo2, allowing actin-based transport of the organelle towards the bud, opposing the direction of retrograde actin cable flow (Fig. 2) (Drubin et al., 1993, Simon et al., 1997, Westermann, 2014).

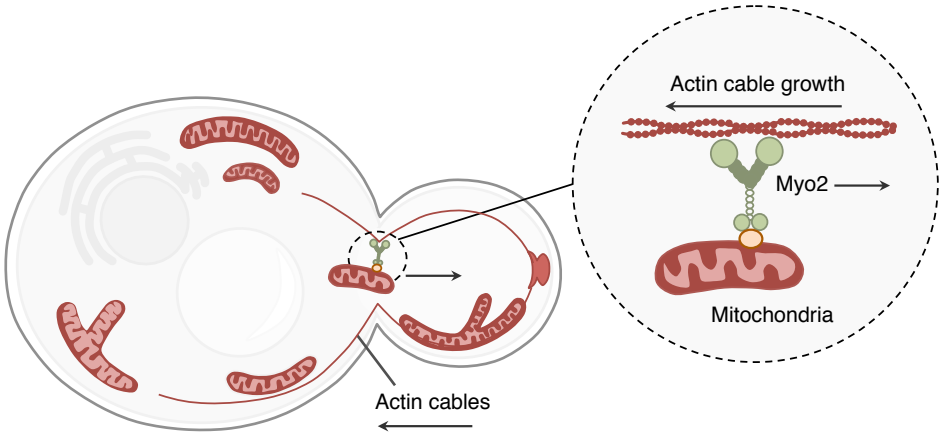


Figure 2. Mitochondrial inheritance

Mitochondria are attached to actin cables through binding of the Myo2 motor protein to a mitochondrial membrane adaptor. Myo2 transports mitochondria towards the bud, in an ATP driven process to overcome the opposing force of actin cable growth.

The asymmetric inheritance and segregation of mitochondria is linked to actin dynamics and the rate of such retrograde cable flow which have to be overcome in order for organelles to enter the bud (Higuchi et al., 2013, Vevea et al., 2014). As mitochondria with a reduced membrane potential are less motile, the retrograde cable flow will function as a quality control filter, retaining these dysfunctional mitochondria within the mother cell. In agreement with the criteria for an aging factor, genetic modifications that alter actin cable flow to affect mitochondrial inheritance also have an effect on longevity (Higuchi et al., 2013, McFaline-Figueroa et al., 2011). Asymmetrical apportioning of dysfunctional mitochondria has been reported also in mammalian stem cells, and has been found to be essential for rejuvenation and maintenance of stemness, further establishing the role of dysfunctional mitochondria as a true aging factor (Katajisto et al., 2015, Dalton and Carroll, 2013).

3.2.3 Vacuolar pH

The yeast vacuole is a lysosome-like compartment, an acidic organelle serving a central role in membrane trafficking as well as in the storage and turnover of macromolecules (Li and Kane, 2009, Armstrong, 2010). The area to volume ratio of vacuoles is dynamically regulated through fission and fusion events in response to environmental changes, and a functional vacuole is required for proper cell cycle progression (Wickner, 2002, Jin and Weisman, 2015).

Similar to other organelles, vacuolar function and morphology deteriorates during aging. Replicatively old cells (7-8 generations old) are found with enlarged vacuoles (Lee et al., 2012, Tang et al., 2008), and vacuolar acidity declines in a mother cell already after a few replications (Hughes and Gottschling, 2012). Intriguingly, both morphology and pH control are restored in the buds produced by these old mothers (Fig. 1c) (Hughes and Gottschling, 2012). Moreover, it was shown that improving vacuolar acidification, by overproducing the Vma1 subunit of the vacuolar proton pump (V-ATPase), increased cellular fitness and extended lifespan. Consistently, deletion of *VMA2*, encoding another of the V-ATPase subunits, caused a reduced lifespan, substantiating the importance of vacuolar acidification in the aging process (Hughes and Gottschling, 2012).

The unequal capacity of pH control between the mother and the daughter cell was further explained to be due to an asymmetric distribution of the plasma membrane proton pump Pma1 (Thayer et al., 2014, Henderson et al., 2014). During cells division, most of the Pma1 pool is retained within the mother cell, competing with the V-ATPase for protons. In the emerging daughter cell, the lack of Pma1 results in more protons being available in the cytosol that can be used for acidification of the vacuole (Fig 1c).

The early loss of pH control is considered an initiating event in the aging process, subsequently leading to mitochondrial dysfunction (Hughes and Gottschling, 2012). This inter-organelle communication is mediated through a decreased vacuolar storage capacity, where neutrally charged amino acids cannot be imported into the vacuole. These amino acids are instead transported into mitochondria at the cost of a reduction in membrane potential (Hughes and Gottschling, 2012).

In addition to the suggested downstream effect on mitochondrial fitness, vacuolar acidity is also required for vesicle transport and membrane fusion to the vacuole (Coonrod et al., 2013, Baars et al., 2007). Both endocytotic transport and vacuole fusion decline in old cells, and overexpression of genes in membrane fusion have been shown to extend lifespan (Tang et al., 2008, Gebre et al., 2012). Another vacuolar function that is dependent on luminal acidity is macromolecular degradation by autophagy, whereby a large portion of the cytoplasm is membrane-enclosed and transported to the vacuole for degradation by vacuolar proteases (Huang and Klionsky, 2002). Longevity manipulations such as caloric restriction is dependent on autophagy to mediate its lifespan extending effect, and autophagy also plays a major role in aging and stress resistance of higher organisms (Tang et al., 2008, Ruckenstuhl et al., 2014, Cuervo, 2008, Rubinsztein et al., 2011).

Hence, there is strong evidence that dysfunctional vacuoles act as aging factors during yeast replicative aging, although the exact mechanisms and downstream effects of a lost pH control that contributes to aging remains to be elucidated.

3.2.4 Damaged and misfolded proteins

Protein homeostasis is of great importance, and an intricate system of protein quality control (PQC) has evolved to assure proper function and structure of the protein pool. With aging, there is a decline in this quality control system, as well as an increased risk of protein damage. Decreased mitochondrial function increases the accumulation of ROS, which can cause oxidative modifications, such as irreversible carbonylation of proteins, thereby altering their structure and functionality (Stadtman, 2006). If these oxidatively damaged proteins are not degraded they can coalesce and form high-molecular-weight aggregates. Such aggregates of damaged proteins accumulate in old cells, and are retained in the mother cell during cell division in a process dependent on the actin cytoskeleton, the disaggregase Hsp104, Sir2 and the polarisome (Fig 1d) (Aguilaniu et al., 2003, Liu et al., 2010, Erjavec et al., 2007, Tessarz et al., 2009). In addition to aggregate retention in the mother cell, an asymmetric distribution of protective proteins, such as the catalase Ctt1, ensures that daughter cells are provided with a superior defense system and ROS scavenging capacity (Erjavec and Nystrom, 2007).

The major protein targets of age-related carbonylation include both cytosolic and mitochondrial chaperones (Reverter-Branchat et al., 2004). Additionally, accumulation of aggregated proteins obstructs proteasomal function and limits protein degradation (Andersson et al., 2013). Hence, the accumulation of protein damage impedes the PQC system, creating a downward spiral eventually leading to the collapsed proteostasis seen in senescent cells (Andersson et al., 2013).

Genetic perturbation of protein homeostasis leads to premature aging and a reduction in lifespan, as demonstrated for mutants deleted in genes encoding chaperones, ROS scavenging proteins, or subunits of the proteasome (Molin et al., 2011, Erjavec et al., 2007, Erjavec and Nystrom, 2007, Kruegel et al.,

2011, Erjavec et al., 2013). Correspondingly, genetic alterations that increase the levels or the activity of these PQC proteins improve cellular fitness and extend lifespan (Kruegel et al., 2011, Molin et al., 2011, Erjavec et al., 2007). The connection between PQC efficiency and longevity has been recognized in many organisms, including worms, flies, mice as well as humans (Denzel et al., 2014, Garigan et al., 2002, van der Goot et al., 2012, Verbeke et al., 2001, Rana et al., 2013, Min et al., 2008, Gutsmann-Conrad et al., 1998, Bonelli et al., 2001). Furthermore, asymmetric distribution of damaged and aggregated proteins during cell division also seem to be a conserved mechanism, as it has been found in species ranging from bacteria to mammalians and is also found in human stem cells (Ackermann et al., 2003, Stewart et al., 2005, Nyström, 2007, Lindner et al., 2008, Rujano et al., 2006, Bufalino et al., 2013, Fuentealba et al., 2008).

3.3 The actions of different aging factors are connected

The different aging factors presented in the previous sections, do not represent different theories of aging mechanisms, rather it is more likely that they are all contributing to the senescence phenotype. As many, if not all, cellular pathways are interconnected it is highly plausible that also the aging pathways are intertwined and affect one another (Dillin et al., 2014, Costanzo et al., 2010). Examples of this interconnectivity between aging factors have already been explained in the previous section where a loss of vacuolar pH control causes a subsequent loss of mitochondrial function (Hughes and Gottschling, 2012, Henderson et al., 2014). The loss of mitochondrial function has been further connected to a loss of DNA stability in the nucleus, causing increasing rates of recombination and loss of heterozygosity (LOH) (McMurray and Gottschling, 2003, Andersen et al., 2008).

Is this connectivity of aging pathways a sign of frailty within the system – where one subcellular system breaks down and takes another down with it? Evidence might point to this conclusion, but it could also be a sign of robustness; where connections between different pathways create a buffer system, more resistant to insults (Dillin et al., 2014). In this scenario the decline of function in one system could be compensated through the upregulation of another. Such a case has been observed in yeast, where a loss of protein quality control in mitochondria can be counteracted by increased levels of cytosolic chaperones (Erjavec et al., 2013). Another example of such a buffering system is the retrograde response where a loss of mitochondrial membrane potential signals to the nucleus and leads to an altered metabolism as well as upregulation of several stress response genes (Jazwinski, 2014, Jazwinski, 2013). Another consequence of mitochondrial breakdown and loss of mitochondrial translational control is the activation of sirtuin-dependent silencing that can extend cellular lifespan (Caballero et al., 2011).

Sirtuins, and specifically the histone deacetylase Sir2 plays a central role in many aging pathways and is further evidence for the complex network-characteristics of aging. In addition to its role in silencing and limiting ERC accumulation, Sir2 has been implicated in the segregation of other aging factors such as dysfunctional mitochondria and protein aggregates (McFaline-Figueroa et al., 2011, Kaeberlein et al., 1999, Aguilaniu et al., 2003). The role of Sir2 in all these processes might be through its effect on the actin folding chaperonin CCT, thereby regulating the cytoskeleton which is important for these segregation processes (Liu et al., 2011). A central role of the actin cytoskeleton is further strengthened by evidence that lifespan can be modified when genetically altering actin dynamics (Gourlay et al., 2004)

3.3.1 Caloric restriction

The complexity of aging is important to consider when studying interventions of the aging process or examining potential therapeutic drug targets, as an alteration in lifespan is often linked to global genetic changes rather than alterations to a single aging-pathway. One of the most studied means of longevity control is caloric restriction (CR) which has been shown to extend lifespan in many organisms, ranging from yeast to primates (Kenyon, 2010). The importance of metabolism and nutrient availability in aging is predicted by the disposal soma theory, explained in the previous chapter. When nutrients are sparse, energy is redistributed to maintenance, increasing the survival of the organism until times when conditions have improved and the probability of a successful reproduction has increased (Shanley and Kirkwood, 2000).

The downstream molecular mechanisms of CR have been widely studied in order to explain why this extends lifespan. The beneficial effects of CR are linked to nutrient sensoring kinase-pathways, such as target-of-rapamycin

(TOR), cAMP-dependent protein kinase A (PKA) and insulin/insulin-growth factor-like 1 (IGF-1) signaling, but the exact downstream events generating lifespan extension are still uncertain (Lin et al., 2000, Kenyon, 2010).

In addition, CR leads to activation of Sir2, as Sir2 activity is coupled to the cells nutrient state through the NAD+/NADH ratio (Smith et al., 2000). In low-glucose environment, yeast cells shift to respiration over fermentation and the levels of NADH decrease, while levels of Sir2-activating NAD+ increase (Smith et al., 2000, Guarente and Picard, 2005, Lin et al., 2000, Lin et al., 2004). As mentioned previously, Sir2 has a role in the regulation of many aging factors, and increasing its activity will certainly have a beneficial effect that could be mediated through all of these pathways. Consistently, the lifespan extension of CR was reported to be completely lost upon *SIR2* deletion (Lin et al., 2002), and the lifespan extending compound resveratrol was demonstrated to mediate its effect through Sir2 activation (Howitz et al., 2003). However, the role of Sir2 in CR has been debated, and another study reported that a different CR protocol extended lifespan even in the absence of Sir2 (Kaeberlein et al., 2004). Thus, Sir2 may act on another longevity pathway, parallel to the one promoting lifespan extension upon CR.

CR is also associated with an increased defense against oxidative stress, and decreased levels of ROS, an effect that in yeast is mediated through the activation of the peroxiredoxin Tsa1 (Reverter-Branchat et al., 2004, Molin et al., 2011). CR has also been revealed to prevent the age-related decline in proteasomal degradation (da Cunha et al., 2011). Thus, by decreasing levels of ROS and maintaining a high activity of the proteasome, the effects of CR will also decrease the risk of protein oxidation and aggregation, acting on yet another known aging factor. To further illustrate the biological complexity, the lifespan extending effect of CR has also been linked vacuolar fusion, pH

regulation and autophagy (Tang et al., 2008, Ruckenstuhl et al., 2014, Hughes and Gottschling, 2012).

The link between CR and longevity provides a good overview of the interconnectivity and complexity that lies within the process of aging. It is important to keep in mind that one process cannot fail or be altered without being buffered by, or affecting other cellular systems during the aging process. There is not one single key factor in aging, and the process itself is not a linear course of events where the accumulation of one aging factor leads to the onset of another. Many researchers have therefore started to study aging at network level using –omics approaches, trying to identify the most sensitive nodes in the aging network as well as their connection (Soltow et al., 2010). Interestingly, it was found that proteins associated with aging have significantly higher connectivity than expected by chance, a pattern not seen for many other datasets (Promislow, 2004).

Although many aging factors and pathways are simultaneously in play during the process of aging, my work is mostly focused on the processes connected to damaged and aggregated proteins. In the following chapters of this thesis, I will provide a more detailed description of the cellular quality control systems acting to prevent the accumulation of protein damage, and how the efficiency of these systems is affected during aging. Additionally, I will recapitulate the known mechanisms behind the asymmetric inheritance of this protein damage, as well as explain how my own work has contributed to this knowledge.

4. Proteostasis and aging

The maintenance of protein homeostasis, or proteostasis, is crucial for cell survival, and the collapse in proteostasis and accumulation of damaged and misfolded proteins is a hallmark of aging (Lopez-Otin et al., 2013). Misfolded and aggregated proteins are harmful to cells, not only due to the loss-of-function of the non-native proteins, but mostly due to the gain-of function of protein aggregates interacting with and impeding essential processes in the cell (Winklhofer et al., 2008). The importance of proteostasis is exemplified by the many diseases that are associated with protein misfolding and age-related deficiency in proteostasis; such as cystic fibrosis and amyotrophic lateral sclerosis (ALS), Alzheimer's and Parkinson's disease, where proteins of aberrant structure interfere with cellular function and eventually cause cell death (Balch et al., 2008, Hipp et al., 2014, Vilchez et al., 2014).

Proteostasis is maintained through an intricate system of temporal and spatial quality control. A complex set of chaperones work to prevent misfolding, aid in refolding as well as to assist in the degradation of terminally misfolded proteins, thereby regulating both quantitative and qualitative aspects of the proteome. Concurrently with the age-related decline in proteostasis, misfolded proteins start to accumulate within the cell. The spatial quality control system then works to sequester the damage to specific quality control sites, limiting cytotoxicity and enabling progeny rejuvenation by limiting damage inheritance during cell division. In this chapter I will review the components of the proteostasis system and what is known about the mechanisms behind the segregation and spatial deposition of protein aggregates.

4.1 Temporal protein quality control

Proteins are the functional components of the cell, responsible for structure, function, and regulation of all active processes. Proteins are comprised by a chain of amino acids and contain, within this sequence, the information required to reach its final three-dimensional structure (Anfinsen, 1973). Therefore, proteins can fold autonomously *in vitro*, attaining its native structure without any assisting chaperones (Dobson and Ellis, 1998).

Hydrophobic forces drive the process of folding where in a polar environment, such as the cytosol, non-polar amino acids are buried within the core of a protein to reach the most thermodynamically stable formation (Bartlett and Radford, 2009). Throughout this process, all possible intramolecular interactions are explored and a protein goes through a number of intermediary structures on its way to the native fold. In the cellular environment, the folding space is limited due to molecular crowdedness, and folding intermediates are at risk of harmful interactions that could trap the protein in any of these states or lead to aggregation (Hartl et al., 2011, Eichner et al., 2011, Ellis and Minton, 2006). Consequently, proteins are in need of assistance during the folding process *in vivo*, and a family of chaperones has evolved to protect newly synthesized proteins, restricting interactions with the environment while they acquire their native fold (Hartl, 1996).

4.1.1 The chaperone system

A molecular chaperone is defined as a protein that helps another protein to reach its functional conformation, without itself being part of the final structure (Hartl, 1996, Hartl and Hayer-Hartl, 2009). Some chaperones are called Heat shock proteins (HSPs) due to their upregulation upon stresses such as increased temperature, and are divided into subfamilies based on their

molecular weights. During de novo protein synthesis, Hsp70s bind to nascent polypeptides emerging from the ribosome (Fig. 3). Substrate binding is followed by cycles of binding and release, achieved through conformational changes linked to ATP hydrolysis, which is regulated by Hsp40 co-chaperones and nucleotide exchange factors (NEFs) (Rudiger et al., 1997, Kampinga and Craig, 2010, Rampelt et al., 2011). This cycle of substrate binding and release generates a kinetic partitioning that allows sequential folding of the emerging polypeptide without risking interaction and aggregation of folding intermediates (Hardy and Randall, 1991, Hartl, 1996).

Once released from the ribosome, a protein might need to undergo further Hsp70/Hsp40 binding cycles before reaching its native state. Additionally, some essential proteins such as actin and tubulin need further post-translational assistance from the chaperonin CCT/TriC (Fig. 3). Chaperonins (HSP60s) are barrel-shaped complexes that rely on ATP driven conformational changes to enclose substrates within a central cavity, to allow for their subsequent folding and maturation (Spiess et al., 2004). Moreover, many proteins involved in signaling require folding assistance from the Hsp90 family (Taipale et al., 2010).

Throughout its lifetime, a protein can be subjected to insults such as high temperature or oxidative stress that can disrupt its three-dimensional structure. A structure collapse, exposing hydrophobic residues, could drive aggregation or, for some proteins, the formation of cytotoxic fibrillar aggregates or amyloids (Chiti and Dobson, 2006). Binding of Hsp70s, small heat shock proteins and certain ‘holdases’ (for example the NEFs Sse1/2 of the Hsp110 subfamily) to exposed surfaces can help stabilize the protein and prevent further misfolding and aggregation while initiating refolding (Haslbeck and Vierling, 2015, Shaner et al., 2005).

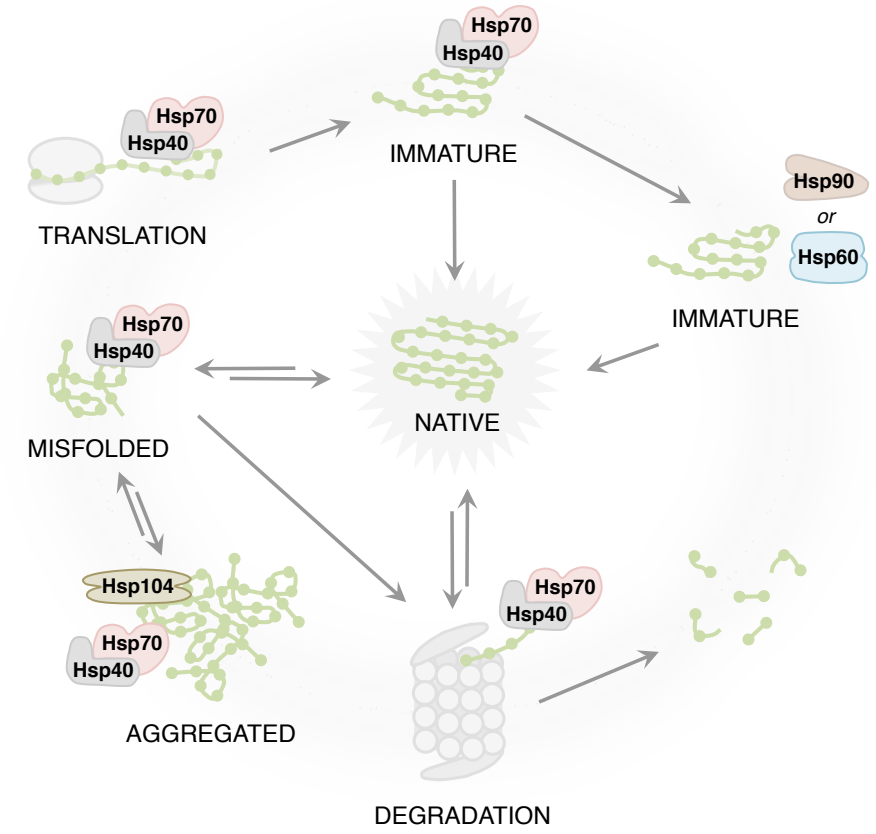


Figure 3. The cytosolic protein quality control (PQC) system

De novo synthesis of proteins occurs via translation, where Hsp70 and Hsp40 chaperones together with NEFs (not shown) aid in the progressive folding as the nascent polypeptide emerges from the ribosome. Newly synthesized and immature proteins need additional folding assistance from the cytosolic Hsp70/40 system. A subset of proteins must also interact with the Hsp60 chaperonins or the Hsp90 system to obtain their native conformation. Stress can cause proteins to misfold and aggregate, and cells rely on the disaggregase Hsp104 in concert with the Hsp70/40 chaperones to dissolve protein aggregates and aid in refolding. Terminally misfolded proteins, as well as native proteins can be destined for degradation by the 26S proteasome.

In addition, existing protein aggregates can be resolubilized by the AAA+ ATPase disaggregase of the Hsp100 family, existing in bacteria, plants and several unicellular eukaryotes (Neuwald et al., 1999). The yeast disaggregase Hsp104 consist of homohexameric rings and works in conjunction with the

Hsp70 system to extract misfolded peptides from a multiprotein aggregate, rendering them accessible for refolding or degradation (Fig. 3) (Glover and Lindquist, 1998, Parsell et al., 1994, Bosl et al., 2006). Because of its specific localization to protein aggregates, Hsp104 has been used as a marker to study cellular aggregation and disaggregation (Glover and Lindquist, 1998, Erjavec et al., 2007, Specht et al., 2011, Liu et al., 2010).

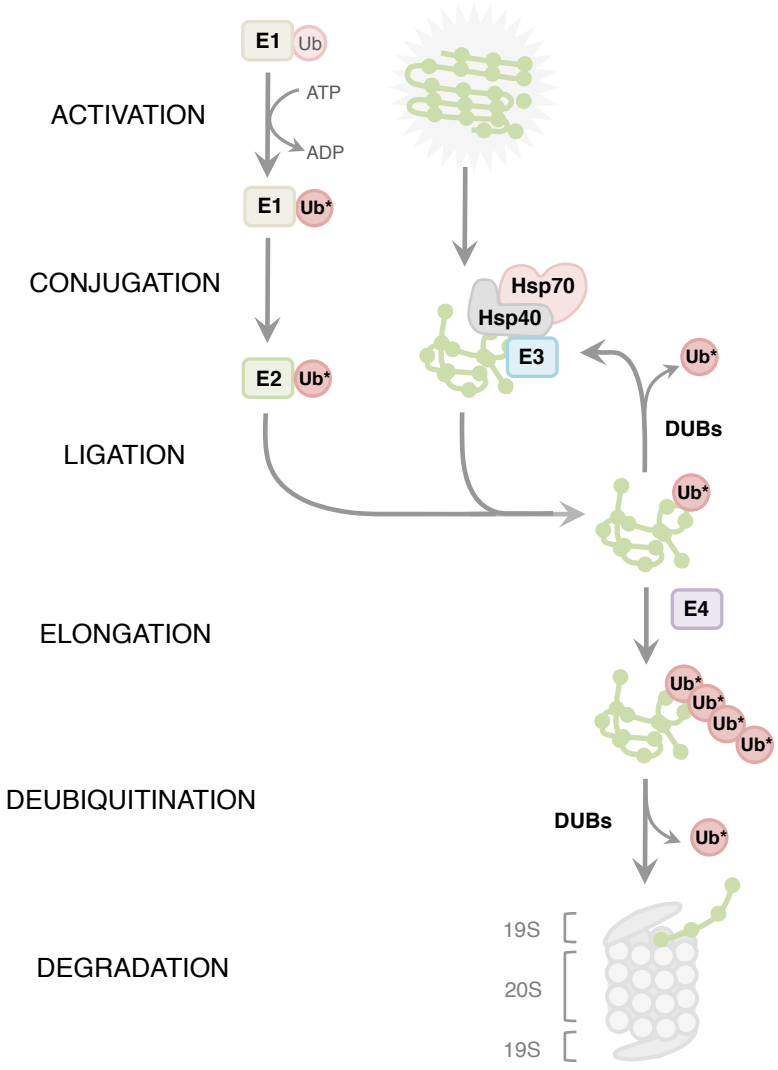
4.1.2 Proteasomal degradation

The quantity of a certain protein in the cell at any given time is regulated not only by levels of gene expression and protein synthesis rate, but also by its rate of degradation. The protein turnover varies greatly within the proteome, with some proteins being very long-lived whereas others need to be rapidly degraded, for example during the progress of the cell cycle (Belle et al., 2006, Thayer et al., 2014). In addition, misfolded proteins that cannot be rescued by the chaperone system have to be recognized and degraded in order to prevent the accumulation of cytotoxic damage. To accomplish this, cells have evolved an intricate system for the specific recognition, tagging and degradation of proteins, called the ubiquitin-proteasome system (UPS).

Proteins destined for degradation are attached to a chain of ubiquitin (Ub), through a series of steps involving four different enzymes: E1-E4 (Fig. 4) (Chau et al., 1989, Finley, 2009, Hwang et al., 2010). Ubiquitin is activated by an E1 enzyme, and then transferred to one of several E2 conjugating enzymes (Haas and Rose, 1982). The E2 enzyme then conjugates with an E3 ligase that can transfer the activated ubiquitin to the substrate protein. Subsequent polyubiquitination is achieved through cooperation of E2 and E3 with an E4 enzyme (Hwang et al., 2010).

The specificity of substrate recognition in the ubiquitination-process is conferred through the range of different E2 and, especially, E3 ligases. In yeast there exist only one E1 activating enzyme, whereas there are 11 and 42 E2s and E3s, respectively (Lee et al., 2008). The labeling of cytosolic misfolded proteins for degradation is mediated through specific E3 ligases (Ubr1, Ubr2, Rsp5 and Hul5 in yeast) that act in concert with the Hsp70 chaperone system (Park et al., 2007, Esser et al., 2004, Eisele and Wolf, 2008, Heck et al., 2010, Fang et al., 2014, Fang et al., 2011).

Figure 4. The Ubiquitin Proteasome system (UPS)



Ubiquitin (Ub) is activated by E1 in an ATP-driven process, and the activated molecule (Ub^{*}) is trans-ferred to the conjugating E2. Ub is then attached to the misfolded substrate that is bound by the E3 ligase and assisting chaperones. Polyubiquitination of the substrate is achieved through cooperation with an E4 enzyme, and is necessary for recognition by the 19S lid of the proteasome. DUBs remove the Ub chain before the substrate enters the 20S core of the proteasome for proteolytic degradation. DUBs can also act prior in the UPS chain, and rescue proteins from degradation.

Upon attachment of a chain consisting of at least four ubiquitin molecules, a protein marked for degradation can be recognized and degraded by the proteasome (Thrower et al., 2000). The 26S proteasome is a 2,5 MDa large complex, consisting of the catalytic 20S cylindrical core and one or two 19S regulatory lids (Fig. 4) (Beck et al., 2012, Finley, 2009). The 19S lid includes subunits that recognize ubiquinated proteins, remove the ubiquitin tag and unfold the protein for progressive entry into the 20S core that encompasses the proteolytic activity (Finley, 2009, Lam et al., 2002). Removal of the ubiquitin can also be accomplished by a number of deubiquitination enzymes (DUBs) that are not part of the proteasomal lid. Deubiquination enables recycling of ubiquitin, but also allows for an additional regulation point where proteins can be rescued from degradation and returned to the functional protein pool (Fig. 4) (Amerik and Hochstrasser, 2004, Oling et al., 2014).

4.1.3 Autophagy and the vacuole

Another route for protein degradation is mediated by autophagy and vacuolar/lysosomal proteases. In the process of macroautophagy, a large portion of the cytoplasm or even entire organelles are engulfed by a double membrane structure, followed by transport and fusion to the vacuolar membrane where the content of the autophagic vesicle is released into the vacuolar lumen (Nakatogawa et al., 2009). Autophagy is an essential process, allowing the cell to adapt to environmental changes such as nutrient depletion, but has also been presented to play a major role in the trafficking, recycling and degradation of many proteins. Induction of autophagy in response to starvation is mediated through TOR signaling (Reggiori and Klionsky, 2013). The nucleation site for the generation of autophagosome is called the pre-autophagosomal structure (PAS) and is located next to the vacuole in yeast.

The PAS generates an expanding membrane through the addition of phospholipids to enclose the cytosolic cargo and form the autophagosome (Suzuki and Ohsumi, 2007). Once the autophagosome has been completely sealed, it relies upon the regular vesicle trafficking machinery of the cell to be delivered to the vacuole, where fusion to the vacuolar membrane is mediated by the HOPS tethering complex and SNARE proteins (Nakatogawa et al., 2009, Nair et al., 2011).

Both macroautophagy as well as chaperone-mediated autophagy has been proclaimed to be major contributors in the clearance of misfolded and aggregated proteins in mammalian cells (Iwata et al., 2005, Cuervo and Wong, 2014). This system is interconnected with the UPS, and a decline in one system leads to compensatory responses by the other (Pandey et al., 2007, Korolchuk et al., 2009). In yeast, it has been reported that a mutated version of alpha synuclein is degraded through autophagy (Petroi et al., 2012), but the involvement of this process in the degradation of endogenous misfolded and aggregated proteins remains elusive. However, there are similarities between the yeast and the mammalian systems suggesting that autophagy-dependent degradation could occur also in yeast. In yeast, aggregated proteins are confined to a distinct deposit site (discussed in the next chapter), which is located at the vacuolar surface, and colocalize with many autophagic markers (Kaganovich et al., 2008). This deposit site displays resemblance to the mammalian aggresome, located in close connection to lysosomes and evidenced to be subject of autophagic degradation (Iwata et al., 2005, Fortun et al., 2003).

In addition, autophagy is essential for many longevity pathways in yeast, further implicating that autophagy is a cytoprotective function with an important role in preventing senescence (Ruckenstuhl et al., 2014, Tang et al., 2008, Morselli et al., 2009).

4.2 Spatial quality control

In bacteria, yeast, and specific stem/progenitor cells, the generation of rejuvenated progeny includes an asymmetrical distribution of oxidized and aggregated proteins (Aguilaniu et al., 2003, Rujano et al., 2006, Ogrodnik et al., 2014, Bufalino et al., 2013). This process is dependent on the cooperation of two intertwined systems; the temporal quality control system described in previous sections, working to sustain low levels of damage, as well as a spatial quality control system regulating the location of existing damage. The spatial quality control encompasses the process of limiting inheritance of aggregates during cell division by retention within the mother cell, together with a strategy of aggregate deposition at specific sites within the mother cell. In this section I will review the existing knowledge on damage segregation, and the regulation underlying this spatial quality control.

4.2.1 Models for aggregate segregation

Two models have been presented to explain the process in which damaged proteins are retained in the mother cell, and kept from being inherited by the progeny. One theory suggests that asymmetric inheritance is enabled by a purely passive process: where the slow diffusion of aggregates within a crowded cytoplasmic space and the spatial restraint by the bud neck keep the protein aggregates from entering the daughter cell (Fig. 5a) (Zhou et al., 2011). This theory was based on mathematical modeling and tracking of aggregate movements in time-lapse experiments.

The other model for asymmetric inheritance argues for a more active process in which specific factors prevent free diffusion of aggregates into the daughter cell (Fig 5b) (Aguilaniu et al., 2003, Erjavec et al., 2007, Specht et al., 2011, Tessarz et al., 2009). The histone deacetylase Sir2 and the

disaggregase Hsp104 were two factors identified as regulators of this asymmetry generating process (Erjavec et al., 2007, Ogrodnik et al., 2014, Specht et al., 2011, Tessarz et al., 2009), and a study based on the genetic interactions of *SIR2* further revealed an essential role for the actin cytoskeleton in the movement of aggregated proteins (Liu et al., 2010). The model presented for the active segregation involves tethering of aggregates to actin cables, causing retrograde movement of aggregates away from the bud as the actin cables grow. The growth of actin cables is mediated by actin nucleation at the bud tip, regulated by the polarisome (Fig 5b). Aggregates that already had been inherited by the daughter cell were even shown to move back into the mother cell in some cases, providing further evidence for a regulated process. Furthermore, the myosin motor protein Myo2, involved in transport along actin cables, was revealed to be important in this process (Liu et al., 2010).

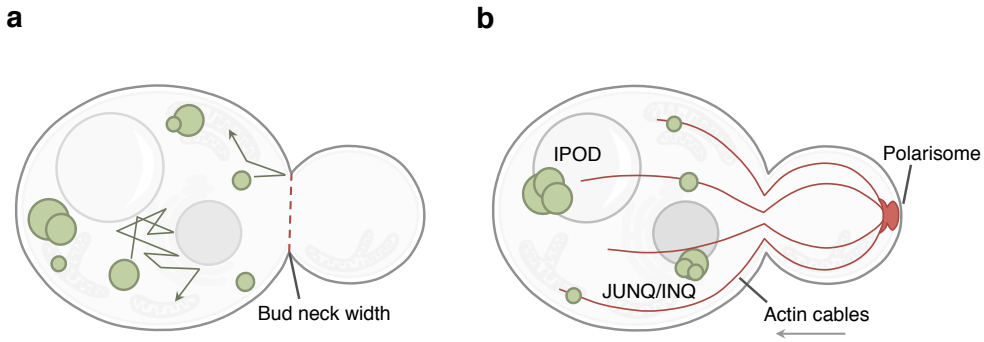


Figure 5. Models for damage asymmetry in budding yeast

a) The passive diffusion model hypothesizes that aggregates move freely within the cytosol, and are retained in the mother cell solely as a consequence of molecular crowdedness and the small size of the bud neck. b) The active segregation model explains that aggregates are transported along actin cables away from the bud. The actin cable flow is generated through actin nucleation by the polarisome. Aggregates are transported into distinct quality control sites within the mother cell; the Juxtanuclear, or intranuclear, quality control site (JUNQ/INQ) close to (or even inside) the nucleus, and the insoluble protein deposit (IPOD) located in close proximity to the vacuole.

The model presented by Liu et al also explained the previously identified role for Sir2 in aggregate segregation, where Sir2 affects the actin cytoskeleton, through its regulation of the chaperonin CCT (Erjavec et al., 2007, Liu et al., 2010). Sir2 affects CCT deacetylation, which controls the proficiency of this complex to fold actin. Additionally, aggregates have been shown to colocalize with membranes of the nucleus, vacuoles, mitochondria and ER, suggesting that aggregates could be tethered to organelles that are transported along actin cables (Escusa-Toret et al., 2013, Zhou et al., 2014, Kaganovich et al., 2008, Miller et al., 2015).

4.2.2 Quality control compartments

The control of damage inheritance is dependent on the retrograde actin cable flow, and the subsequent spatial deposition of damaged/unfolded proteins within the mother cell, into specific protein inclusions. Upon stress, misfolded and aggregated proteins can be found at multiple sites throughout the cytosol (termed stress foci, peripheral aggregates, Q-bodies or CytoQ; Fig. 6) (Escusa-Toret et al., 2013, Specht et al., 2011, Spokoini et al., 2012, Miller et al., 2015). Under prolonged stress, these aggregated proteins were reported to fuse and accumulate at two distinct locations within the cell (Kaganovich et al., 2008, Spokoini et al., 2012). One inclusion was seen in close proximity to the nucleus and was therefore named JUxta-Nuclear-Quality-control (JUNQ; Fig. 5b). Kinetic studies revealed that the JUNQ compartment is highly dynamic: its contents are rapidly exchanging with the cytosol, a process explained by chaperone-mediated refolding of aggregated proteins within the compartment. This was in contrast to the second observed inclusion site, which exhibited almost no dynamic exchange (Kaganovich et al., 2008, Specht et al., 2011). This more peripheral inclusion was therefore though to contain amyloid fibrils and terminally misfolded proteins, and was named

Insoluble-Protein-Deposit (IPOD; Fig.5b) (Kaganovich et al., 2008). This compartment was further shown to be located to the surface of the vacuolar membrane (Spokoini et al., 2012). Later reports questioned the localization of the juxtanuclear inclusion deposit, by providing evidence that this quality control site might actually reside *inside* the nucleus and the authors therefore suggested that this quality control site should be labeled the intranuclear quality control site (INQ) rather than JUNQ (Figure 5b) (Miller et al., 2015).

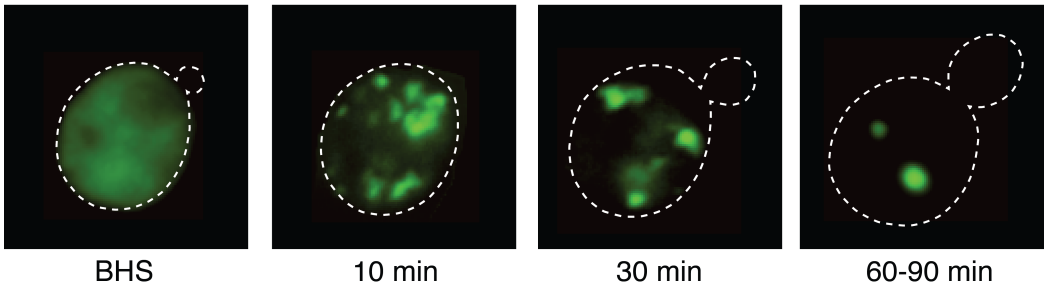


Figure 6. Aggregate fusion and deposition into quality control sites

Representative microscopy images of aggregate segregation, using the disaggregase Hsp104 fused to GFP as a reporter for protein aggregates. Before heat shock (BHS) Hsp104 is distributed evenly throughout the cytosol. Upon applied heat stress (10 min into heat shock), Hsp104 forms multiple foci throughout the cell, representing sites of protein aggregation. Following prolonged stress (30 min), these aggregates starts to fuse and at 60-90 min of continuous heat shock the aggregated proteins have been successfully sequestered into two distinct foci. Fusion and sequestration of aggregates occurs also upon stress relief, but is not as easily visualized as aggregate segregation occurs in parallel with degradation.

An inclusion site, named the "aggresome" is known to accumulate close to the nucleus in mammalian cell systems and sequestration of damage to this site is essential for asymmetric inheritance and progeny rejuvenation (Ogrodnik et al., 2014, Rujano et al., 2006, Johnston et al., 1998, Bufalino et al., 2013). Although functionally similar to the yeast JUNQ, aggresomes are

enclosed by a vimentin cage and are formed in a microtubule-dependent manner, as opposed to the actin dependence suggested for the yeast protein deposit (Ogrodnik et al., 2014, Specht et al., 2011). Thus, spatial quality control seems to be a conserved process, although the exact mechanisms for aggregate sequestration and retention might differ between cell systems.

4.2.3 Spatial sorting

The regulation and the requisites for protein sorting to quality control compartments is still not fully unraveled. When the IPOD and JUNQ compartments were first discovered, it was suggested that JUNQ harbored proteins that had the potential to be refolded or degraded, whereas the IPOD contained terminally damaged proteins and amyloids (Kaganovich et al., 2008). Although the perivacuolar IPOD site persists for a longer period after stress, this compartment is eventually cleared upon returning cells to permissive conditions. This clearance was shown to be dependent on the disaggregase activity of Hsp104, arguing against the notion that proteins within IPOD are terminally misfolded (Miller et al., 2015). Despite this, there is evidently a difference between the two compartments and effort has been put into identifying the regulation behind this spatial sorting.

The small heat shock protein Hsp42 has been identified as essential for the formation of IPOD, and deletion of this gene causes misfolded proteins to be redirected towards the JUNQ/INQ (Malinovska et al., 2012, Miller et al., 2015, Specht et al., 2011). For sequestration of proteins towards the nuclear control site, two members of the Hook family of proteins, Btn2 and Cur1, have been implicated to function together with the Hsp40 co-chaperone Sis1 (Malinovska et al., 2012). Furthermore, Sis1 was reported to enable the transport of misfolded proteins into the nucleus for degradation, reinforcing the hypothesis of an intranuclear quality control (INQ) site (Park et al., 2013,

Miller et al., 2015). Another protein associated with the formation of JUNQ/INQ is the Hsp90 co-chaperone Sti1. Although not essential to the process, deletion of either Sti1 or Sis1 dramatically impedes the transport of misfolded proteins into the nuclear deposit (Kaganovich et al., 2008, Miller et al., 2015).

The ubiquitination status of a misfolded protein has been suggested to function as a sorting signal, and increasing ubiquitination enhances the direction of proteins to the JUNQ (Kaganovich et al., 2008). Correspondingly, the amyloidgenic protein Rnq1 that exclusively localizes to IPOD could be partly redirected to JUNQ by the addition of a ubiquitination signal. However, as ubiquitinated proteins can be found in both JUNQ and IPOD (Miller et al., 2015), it is still unclear exactly which features of the misfolded proteins that enables interaction with certain sorting factors and directs them to either compartment.

5. Cell death and apoptosis

The damage accumulating in aging cells eventually interferes with essential cellular systems and reduces functionality, but how does this decline result in the death of an aging cell? Is cell death a sudden event resulting from an overwhelmed maintenance system or is it a programmed shutdown of functions? In multicellular organisms, a system of programmed cell death (PCD) has evolved that allows for old and damaged cells to be cleared from the tissue to ensure the survival and fitness of the whole organism. This process of PCD is essential for proper development and tissue maintenance, and is also implicated in the aging process (Tower, 2015). During aging, several tissues display a declined rate of PCD, so that the tissue contains an increasing number of old and less functional cells, which eventually will affect the function of the tissue and organism as a whole (Tower, 2015). This indicates that old and damaged cells are normally programmed to die, but that regulation of this cell death program deteriorates with progressive age. Age-related dysregulation of PCD can also be manifested as an *increasing* death rate in some tissues, leaving the tissue with fewer cells than is required for normal function (Tower, 2015).

The PCD signaling pathway represents a highly regulated system and cells going through this process display a number of distinct features. Interestingly, these characteristics of apoptotic cells could be seen also in stressed or senescent cells of unicellular organisms such as yeast, indicating that these cells might also die through the process of PCD (Madeo et al., 1997). While the relevance of apoptosis is discernable in multicellular organisms, the benefits of such a suicide program in unicellular organisms, such as yeast, is controversial (Hill and Nystrom, 2015). In this section I will give e brief overview of the process and purpose of apoptosis and provide a comparison between metazoan and yeast PCD.

5.1 Apoptosis

Apoptosis is a distinct mode of PCD, mediated by a family of cysteine proteases termed caspases (Kumar, 2007). A suicidal program such as apoptosis need to be under tight control, and therefore caspases are produced as inactive zymogens with a protective pro-domain and a C-terminal P10 domain that have to be removed for protein activation (Fig. 7). Upon apoptotic stimuli, initiator caspases are processed and induce a cascade, leading to the downstream activation of effector, or "executioner", caspases. Once activated, the effector caspases cleave a wide range of substrates, eliciting the typical cytological and morphological changes that are characteristic for apoptosis where cells self-degrade in a manner that is not damaging surrounding cells.

5.1.1 Apoptosis in yeast

The death of both replicatively and chronologically old yeast cells has been suggested to be an apoptotic event as these cells display characteristics similar to those observed in apoptotic metazoan cells. These characteristics include nuclear fragmentation, decreased membrane integrity and accumulation of intracellular ROS (Madeo et al., 1997, Laun et al., 2001, Ludovico et al., 2001, Ribeiro et al., 2006). The evolution on an apoptotic-like program in a unicellular organism has been explained as an altruistic event, where nutrients from the dead cells provide an advantage to the remaining population (Herker et al., 2004). This theory is conceivable when considering microbial growth in colonies and clusters, where the population can exhibit "multicellular behavior", and PCD might serve to constrain cell number to ensure development and survival of the clone (Vachova et al., 2012, Palkova et al., 2014). However, in a growing colony only a very small fraction of these cells will represent cells of advanced replicative age, thus it is difficult to see how

much the remaining young cells could actually benefit from the apoptotic death of such few replicatively old cells (Steinkraus et al., 2008).

5.1.2 Yeast metacaspase Mca1

Sequence analysis has revealed that unicellular species harbor caspase-related proteases that could be responsible for the proposed PCD in these organisms (Uren et al., 2000). The yeast genome encodes a single type I metacaspase Mca1 (metacaspase 1), also denoted Yca1 (yeast caspase 1), that has been proposed to function as an initiator caspase, activating an apoptotic-like program during oxidative stress and chronological aging (Madeo et al., 2002, Herker et al., 2004). Deletion of the *MCA1* gene increased the survival of cells upon H_2O_2 stress and aging, and the cell death observed in wild type cells coincided with proteolytic processing of Mca1, similar to that which occurs in mammalian initiator caspases upon their activation (Fig.7) (Madeo et al., 2002, Khan et al., 2005, Lefevre et al., 2012).

The reports of Mca1 involvement in initiation of apoptosis have been followed by studies suggesting that Mca1 might also possess beneficial functions in cell cycle control as well as in PQC (Lee et al., 2008, Lee et al., 2010). Mca1 was found to physically interact with protein aggregates and deletion of this gene significantly reduced the rate of aggregate clearance (Lee et al., 2010, Shrestha et al., 2013). These studies also showed that several PQC components, such as chaperones and proteasome subunits, were upregulated in the *MCA1* deletion mutant. Based on this, it was suggested that the increased survival and stress tolerance of this deletion strain could be an indirect effect caused by compensatory responses rather than the deletion of a pro-death gene (Lee et al., 2010, Hill and Nystrom, 2015).

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The yeast metacaspase exhibits structural similarities with canonical caspases, including the typical caspase-hemoglobinase fold and the conserved histidine-cysteine dyad known to mediate protease activity in metazoan caspases (Aravind and Koonin, 2002, Hill and Nystrom, 2015, Wong et al., 2012). There are however some significant differences between the yeast metacaspase and initiator caspases, as indicated by the low sequence similarity (10-11% when comparing Mca1 to caspase-3 and caspase-9, respectively). Metacaspases type I and initiator caspases differ in the structure of their pro-domain, where the yeast Mca1 does not contain any of the typical DEAD or CARD domains seen in initiator caspases (Chang and Yang, 2000). Instead, the Mca1 pro-domain contains a proline-rich stretch, as well as a QN rich domain, an aggregation-prone motif seen in prion-forming proteins (Fig. 7) (Alberti et al., 2009).

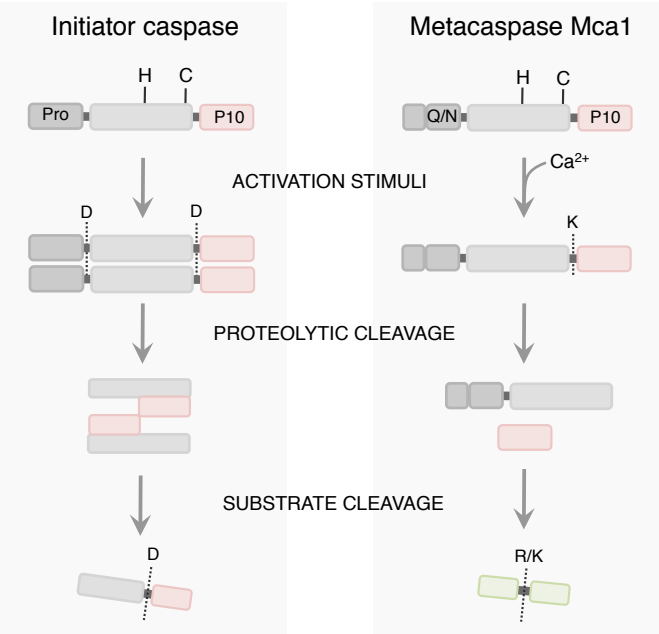

Figure 7. Comparison of initiator caspases and metacaspases

Both proteases share the same overall structure: an N-terminal prodomain, and a caspase domain with the conserved His-Cys dyad and a C-terminal P10 domain. Activational stimuli result in proteolytic cleavage. Induced proximity of initiator caspases results in two aspartate directed cleavage events, followed by dimerization, to form the fully activated caspase. Activation of metacaspases requires calcium and includes only one lysine-directed cleavage event to remove the P10 domain. Once cleaved, metacaspases do not form dimers, and are thought to act as monomers. Initiator caspases activate effector caspases by aspartate-directed cleavage, whereas metacaspases cleave substrates after arginine or lysine. Figure adapted from Hill and Nystrom, 2015.

Unlike initiator caspases, the pro-domains of metacaspases are not removed during activational processing and the structure of metacaspases does not enable dimerization (Wong et al., 2012, Hill and Nystrom, 2015). Additionally, metacaspases cleave proteins after arginine and lysine residues and lack the ability to cleave after aspartate residues, which is the typical recognition site of caspases (Fig. 7) (Vercammen et al., 2004, Watanabe and Lam, 2005). Another property of metacaspases that is not found among caspases is a dependence on calcium for activation and subsequent activity (McLuskey et al., 2012, Watanabe and Lam, 2005, Wong et al., 2012). In summary, comparative data suggest that metacaspases, like the yeast Mca1, are only distant relatives to caspases.

6. Results and discussion

The aim of this thesis was to decipher the mechanisms of damage segregation and elimination, as well as cellular rejuvenation in *Saccharomyces cerevisiae*. My work focused on the distribution of misfolded and aggregated proteins that accumulates during cellular aging, and that are asymmetrically segregated during cell division. In this section I will present my findings on this matter, based on the work in papers I-IV, and discuss their impact and scientific relevance in the context of contemporary research within the field.

6.1 Aggregate segregation is an active process

The asymmetric inheritance of proteins was reported by several studies to be an active and factor-dependent process reliant on actin cables, the polarisome, Sir2 and Hsp104 (Aguilaniu et al., 2003, Liu et al., 2010, Tessarz et al., 2009). However, a study from Rong Li's lab, using particle tracking and computer modeling, argued that aggregates move in a completely random manner, and that retention within the mother cell is due to the constraint by the small bud neck rather than by an active factor-dependent process (Zhou et al., 2011). Using time-lapse microscopy and tracking of Hsp104-GFP containing aggregates Zhou et al. found that, of the ~70 small-budded cells analyzed, only a minor fraction of the budding events showed aggregate movement across the bud neck. In these few cross-compartment movements that were observed, no significant direction could be detected, and aggregates seemed to move equally often from mother to bud compared to from bud to mother, thus arguing against a directional transport (Zhou et al., 2011). As this data directly contradicts the previously published observations of aggregate movements, we sought to clarify the abundance of aggregate

movements across the bud neck in dividing cells (***paper I***). By analyzing 393 budding events, we found that cross-compartment movements occurred in 15.5% and of these, and the portion of budding events showing movement from bud to mother was significantly more abundant (66.5%; p=0.03, ***paper I***: Fig.1a-b). Furthermore we used an ATPase negative version of Hsp104, Hsp104^{Y662A}-mCherry (Zhou et al., 2011) that can bind to but not dissolve aggregates, and found that aggregates bound by this chaperone forms fibrillar structures along the mother-bud axis that colocalize with the actin binding protein Abp140 (***paper I***; Fig. 1c-d). This colocalization was further confirmed using high-resolution 3D structure illumination microscopy (SIM), revealing that heat induced aggregates as well as Huntingtin aggregates (Htt103Q) form long structures that are wrapped closely around actin cables (***paper II***; Fig. 6a-f). As the actin cytoskeleton moves in a directional fashion, it is unlikely that aggregates associated with these structures are moving simply by random diffusion.

In ***paper I*** we also substantiated the importance of the polarisome component Bni1, demonstrating that this formin is needed for the segregation of disease-related Htt103Q aggregates (***paper I***; Fig. 1e-g) in addition to its established role in segregation of heat-induced aggregates (Liu et al., 2010). The identification of factors that are required for damage asymmetry during cell division further supports the notion that this is an active process, active in the sense that aggregate movement is directed and factor-mediated rather than the result of passive diffusion.

In addition to our experimental data supporting an active segregation model, one can also point out several shortcomings in the methods used by Zhou et al in favor of the passive diffusion model. For instance the particle tracking that was performed on aggregates upon heat shock was based on images from one hour time-lapse experiments, yet the figure illustrating the

average mean square displacement (MSD) only covers the initial 5-6 minutes (Zhou et al., 2011). As this corresponds to the very first minutes directly following the heat shock it might be true that aggregates during this time-frame move randomly, since heat causes a transient collapse in the actin cytoskeleton making it impossible for aggregates to become tethered to actin cables for retention or retrograde transport. Moreover, the seemingly random tracking pattern of aggregates recorded by Zhou et al, was explained by another study demonstrating that aggregates move together with organelles to which they are tethered (Spokoini et al., 2012).

The passive diffusion model for aggregate segregation postulates that asymmetric distribution of aggregates is the result of a small bud neck size and the limited window of time that the bud neck is open (i.e. generation time) (Zhou et al., 2011). In ***paper II*** we tested this correlation using Shs1-GFP as a marker for the yeast bud neck and show that for *sir2Δ* cells, the increased inheritance of aggregates into the bud cannot be explained neither by an increase in bud neck size, nor by an increased generation time (***paper II***: Fig. 2a-c). Furthermore, increasing the generation time through treatment with low concentrations of cycloheximide did not have any impact on the asymmetric distribution of protein aggregates in wild type cells (***paper II***: Fig. 2g).

The correlation between aggregate inheritance and bud neck width that was suggested in the study by Zhou et al, was based on computer simulations in which two perfectly round spheres were used to represent mother and daughter cell, and where the size of the daughter cell was set to 85% of the size of the mother cell (Zhou et al., 2011). Such a large daughter cell would only be present at the end of a budding event, past the S/G2 cell cycle phase and after actin repolarization has occurred (Pruyne and Bretscher, 2000). In budding events with such a large daughter cell, the physiological features of

the actin cytoskeleton no longer allows for a directed transport along the mother-bud axis. Thus, the computer model used by Zhou et al to demonstrate that aggregates move in a passive manner is biased and based on a setup that does not allow the option of active transport.

In conclusion, the data presented in ***paper I and paper II,*** along with the identification of several asymmetry generating genes (AGGs) in ***papers III-IV*** (described in following sections), support the active segregation model, where aggregates are tethered to the cytoskeleton and sequestrated to certain quality control sites, to retain the damaged proteins within the mother cell and ensure proper progeny rejuvenation.

6.2 Calmodulin and the myosin motor Myo2 are asymmetry generating factors

While we have established that retrograde transport of aggregates from bud to mother does occur, and is an active and factor-dependent process, this is not the most prevailing way in which a damage-free daughter cell is produced. Instead damage asymmetry is mainly achieved by inhibiting the aggregates from entering the bud in the first place (Liu et al., 2010). This retention process is linked to spatial quality control and the deposition of protein damage at certain quality control sites (Kaganovich et al., 2008, Spokoini et al., 2012). We developed a method to distinguish between the process of aggregate retention in the mother cell and aggregate removal of aggregates inherited by the daughter (by degradation and/or retrograde transport; ***paper I and paper IV)*** and found that the actions of Sir2 are mostly affecting the retention process (***paper II***; Fig. 2a-e). The genetic interactome of *SIR2* has been used previously to find players involved in aggregate retention, upon which the actin cytoskeleton and the polarisome were identified (Liu et al., 2010). We now expanded on this study

and identified negative genetic interactions between *SIR2* and temperature sensitive (ts) alleles of essential genes (***paper II***). In addition to confirming previous data of *SIR2* connections to functions in actin cytoskeleton and cell polarity, this screen revealed previously unknown connections to functions in ER-Golgi trafficking, microtubule nucleation, sister chromatid segregation and proteasome regulatory subunits (***paper II***; Fig.2a-b). Further testing of these essential interactors revealed that many of these genes are, similar to *SIR2*, involved in aggregate retention (40% of all interactors; ***paper II***), thus extending the list of identified asymmetry generating genes (AGGs). Among these newly identified AGGs were the actin myosin V motor protein Myo2, and the actin organization protein calmodulin (Cmd1), both being involved in aggregate retention as well as aggregate removal (***paper II***; Fig.4a-b). The aggregate segregation phenotype of the *cmd1-1* mutant was accompanied by an increase in the number of aggregates per cell (***paper II***; Fig 4c-d). Additionally, we report that both Myo2 and Cmd1 colocalize with heat-induced aggregates bound by $Hsp104^{Y662A}$, as well as to aggregates of Htt103Q (***Paper II***; Fig.5e). These data suggest that aggregates could be tethered to actin cables through interaction with Myo2 and Cmd1 and that the deposition of aggregates into cytoprotective inclusions such as JUNQ and IPOD may be dependent on actin re-organization mediated by Cmd1.

6.3 Aggregate segregation requires vacuolar functions and vesicle trafficking

The importance of actin motors and genes involved in ER-Golgi trafficking identified in ***paper II*** opens up the possibility that aggregate tethering and deposition could be linked to the vesicle trafficking system of the cell. This notion was corroborated by the findings in ***paper III***, where we performed a genome-wide imaging screen to identify new AGGs and found that genes involved in vacuolar functions and vesicle trafficking were highly enriched among genes that are important for generating damage asymmetry (***paper III***; Fig 1c-d). Among these newly identified AGGs were many factors required for endosome maturation and membrane fusion; including SNARE proteins, components of the HOPS and CORVET membrane tethering complexes and regulators of phosphatidyl inositols (PtdIns) involved in vesicle sorting (***paper III***; Fig 1c-e). Moreover, we identified several components important for proper function of the vacuolar proton pump (V-ATPase), indicating that pH control and vacuolar acidification are necessary in the establishment of damage asymmetry. Interestingly, this provides a possible link between the reported age-related loss of vacuolar pH control (Hughes and Gottschling, 2012) and proteostasis decline, as membrane fusion and endocytosis have been demonstrated to be reliant on a functional V-ATPase (Baars et al., 2007, Coonrod et al., 2013, Tang et al., 2008).

The loss of vacuolar acidification, that has been reported to be an event occurring early during replicative aging, would have a negative impact on membrane fusion and vesicle tethering to the vacuole (Fig. 8). If aggregate segregation is connected to actin-dependent vesicle trafficking, then this would in turn decrease the efficiency of the spatial quality control. Intriguingly, one of the cellular quality control sites, the IPOD inclusion, was shown to be localized

to the vacuolar membrane (Spokoini et al., 2012). It is therefore possible that a vesicle transport-dependent mode of aggregate sequestration is linked to this quality control site. Thus, a diminished control of vacuolar pH, similar to that occurring during aging, would result in less damage being sequestered into cytoprotective IPOD inclusions (Fig. 8). This in turn would increase the risk of damage being inherited by the daughter cell during cell division.

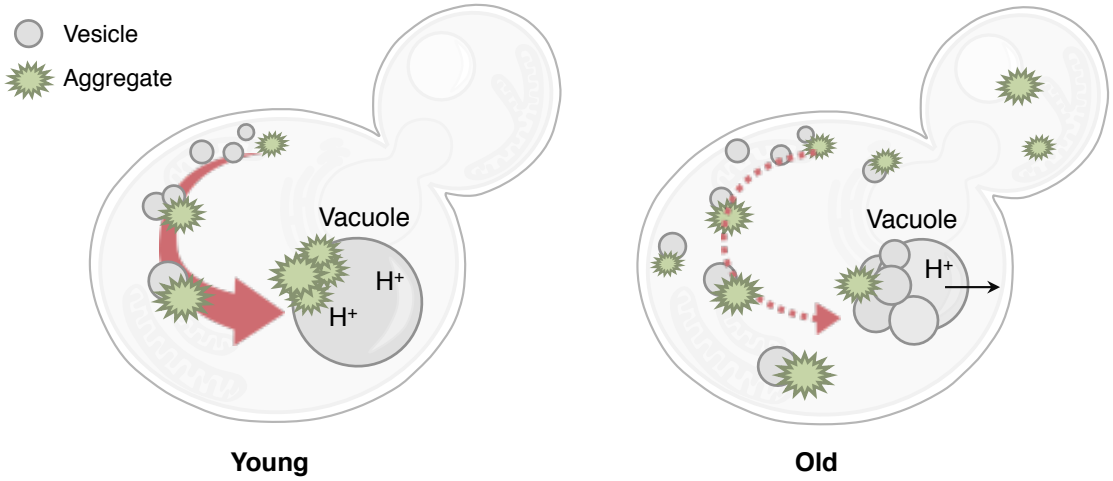

Figure 8. Model for a connection between vacuolar pH control and aggregate segregation

Aggregate sequestration to vacuolar inclusion sites (IPOD) is dependent on vesicle trafficking and fusion of vesicles to the vacuolar membrane (left). Membrane fusion is reliant on the presence and function of the vacuolar proton pump, maintaining a low pH in the vacuolar lumen. In aged cells (right) the pH control is lost and the vacuolar pH rises. This causes a decline in vesicle trafficking, which in turn decreases the fusion of aggregates to the IPOD compartment. As the efficiency of the spatial quality control declines, the risk of damage being inherited to the daughter cell increases.

6.4 The vacuole adaptor protein Vac17 regulates damage asymmetry and controls lifespan

Among the vacuole trafficking related genes identified as AGGs in ***paper III***, we identified several known components of the machinery for vacuole inheritance. In addition to the demonstrated requirement of actin and Myo2 in damage asymmetry (Erjavec et al., 2007, Liu et al., 2010, Specht et al., 2011) (**papers I & II**), we found a similar requirement of Vac17 and Vac8 (***paper III***; Fig 1a-c). Vac17 is the vacuolar adaptor protein, which binds vacuoles through interaction with the vacuolar membrane protein Vac8, and couples vacuoles to actin cables by binding the actin motor protein Myo2 (Fig. 9). During cell division, vacuolar fission enables small vacuolar vesicles to be released from the mother cell. These small vesicles are subsequently transported along actin cables towards the protruding daughter cell, into which they are released upon daughter-cell specific degradation of the Vac17 adaptor protein (Weisman, 2006). Interestingly all the components of this inheritance machinery, as well as Pfa3, the palmitoyltransferase regulating Vac8 localization to the vacuole, was identified as AGGs.

Vac17 is a very low abundant protein in the cell, and appears to be limiting for the aggregate segregation process since overproduction of this adaptor protein rendered the cells even more efficient in establishing damage asymmetry (***paper III;*** Fig. 2e-f). This increase in asymmetry by Vac17 overproduction was associated with an enhanced fusion of aggregates into inclusions. This accelerated formation of cytoprotective inclusions by Vac17 overproduction was observed in both heat-stressed and replicatively old cells (***paper III***; Fig 3a-d).

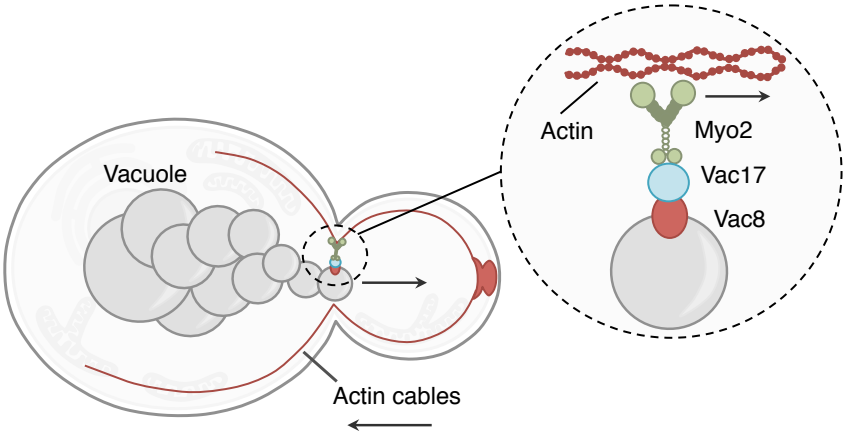

Figure 9. Vacuole inheritance during cell division

The vacuole in the mother cell becomes fragmented, so that smaller structures can detach and become transported into the daughter cell. Vac17 anchors vacuoles to actin cables through interactions with the actin motor protein Myo2 and Vac8 on the vacuolar membrane.

Intriguingly, we find that the function of Vac17 in aggregate asymmetry is uncoupled from its function in vacuole inheritance (***paper III***; Fig 2g). Instead, the role of Vac17 seems to be related to vesicle trafficking processes. The *vac17Δ* cells displayed a fragmented vacuole and decreased endocytosis and both vacuole and endosome fusion, similar to aggregate fusion, was accelerated upon elevated levels of Vac17 (***paper III***; Fig. 3a-f & 4a-d). Concomitantly, we demonstrated that these beneficial effects of Vac17 overproduction were completely lost upon deletion of *VPS16*, encoding a subunit of the HOPS and CORVET tethering complexes (***paper III***; Fig 2h & 3g). Thus, the Vac17-mediated fusion and sequestration of aggregates into IPOD inclusions is dependent on vesicle fusion to the vacuolar membrane.

Improved spatial quality control upon Vac17 overproduction was further reflected in an improved fitness and longevity of this strain. Overexpression of *VAC17* extended lifespan in a manner dependent on the cytosolic Hsp70 system, representing further evidence for damaged and aggregated proteins acting as true aging factors (***paper III***; Fig. 4e; also see section 3.2 on aging factors).

Furthermore, the lifespan extension mediated by Vac17, similar to the effect on aggregate segregation, was dependent on physical interactions with Myo2 and functional vesicle fusion (***Paper III***; Fig. 4f-i)

The data on *VAC17* further supports a model in which aggregates interact with vesicles, and are sequestered to IPOD inclusion sites through actin-dependent vesicle transport and/or subsequent fusion of vesicles to the vacuolar membrane (Fig. 8). This model further explains the involvement of Cmd1 and Myo2, which were previously identified as AGGs (***paper II***). Myo2 is the actin motor protein required for Vac17 function in aggregate fusion and lifespan regulation and Cmd1 is required both for actin reorganization as well as docking and fusion of vacuolar membranes (Eitzen et al., 2002).

The model presented where aggregates rely on vesicle trafficking for sequestration into quality control compartments involves only the perivacuolar IPOD compartment, as the trafficking genes identified as AGGs were mainly involved in endosomal transport. It is however possible that the sequestration of aggregates towards the nuclear JUNQ/INQ might also involve vesicle trafficking. In the screen presented in ***paper III***, components of other vesicle transport routes, including transport in both directions between ER-Golgi and endosomes-Golgi, were also found to be important for spatial quality control and establishing damage asymmetry. Additionally, a large group of *SEC*-mutants was identified in ***paper II*** as genetic interactors of *SIR2*, and shown to also exhibit an asymmetry defect, further implicating a role for ER-Golgi transport in the management and deposition of protein aggregates. This notion is supported by studies in *C. elegans*, reporting on a role for ER-Golgi trafficking in the regulation of inclusion formation and aggregate toxicity (van Ham et al., 2008).

Moreover, vesicle mediated incorporation of membrane material into the nuclear envelope seem to occur at a distinct site, close to the nucleolus (Webster et al., 2010). Interestingly, the juxtanuclear inclusion (JUNQ/INQ) harboring

protein aggregates have also been reported to localize in close proximity to the nucleolus (Miller et al., 2015, Oling et al., 2014) (***paper IV***). If vesicle transport is important for the spatial deposition of aggregates into the two different compartments, then the interaction of aggregates with lipid membranes might be important for their sorting to a specific inclusion. Diverse types of aggregates could have different propensity to bind vesicles and organellar membranes, thus membrane interaction might function in spatial sorting, guiding aggregates to their final deposit site. This idea fits with the data on aggregates interacting with the membranes of both ER and mitochondria (Escusa-Toret et al., 2013, Zhou et al., 2014). However, experimental data is still lacking on this topic, and future research is needed to elucidate the potential role of membrane interaction and different routes of vesicle transport in spatial quality control.

6.5 The metacaspase Mca1 regulates damage asymmetry through its role in aggregate removal

As described previously, the asymmetric distribution of damaged and aggregated proteins is accomplished through the interconnected processes of limiting aggregate inheritance by retention within the mother cell, and removing aggregates that leak into the daughter. In ***paper II*** and ***paper IV*** we found that most AGGs such as actin remodeling proteins and chaperones are affecting both of these processes. However, we did notice some exceptions to this rule: lack of the Hsp70 chaperone Ssa3 or the metacaspase Mca1 only affected aggregate removal (***paper IV***; Fig. 1a-c). As the aggregate removal process is not the major contributor to asymmetric segregation during cell divisions, these genes were not picked up in the genome-wide screen performed in ***paper III***.

The yeast metacaspase Mca1 had previously been demonstrated to colocalize with the Hsp104 disaggregase, and it was reported that the clearance of heat-induced aggregates was reduced in a *mca1Δ* strain (Lee et al., 2010). We elaborated upon these results, and found that Mca1 is relocalized to aggregates formed upon both heat-shock and aging, and is present in both the IPOD as well as in the JUNQ/INQ inclusion site (***paper IV***; Fig. 3a-c). This relocalization was independent of cytosolic chaperones, indicating that Mca1 could be one of the first proteins recognizing and binding to misfolded and aggregated proteins upon stress (***paper IV***; Fig. 3d). Furthermore, overproduction of Mca1 buffered for the absence of the Hsp40 co-chaperone Ydj1 and rendered cells better capable of aggregate removal through the degradation of terminally misfolded proteins (***paper IV***; Fig. 1d & 3e-h). The beneficial effects of Mca1 were reflected also in replicative longevity, as overproduction of the metacaspase could extend lifespan both in WT and *ydj1Δ* genetic backgrounds. The effects of *MCA1* overexpression were partly dependent on its caspase activity, as a catalytic inactive version of Mca1 ($Mca1_{C276A}$) was not as efficient in improving aggregate removal or extending lifespan (***paper IV***, Fig. 4g-h). In Ydj1-deficient cells, overexpression of the catalytically inactive Mca1 reduced rather than extended life span (***paper IV***, Fig. 4i), indicating that Mca1 harbors both protease-dependent and independent functions in lifespan control and PQC, of which the protease-independent ones can only be accomplished in the presence of a fully functional Hsp40 system (Ydj1).

The protease activity of Mca1 may aid in aggregate removal by assisting the proteasome through pre-cleavage of substrates that are destined for degradation (Hill and Nystrom, 2015, Kampinga, 2014). In agreement with this statement, we found that the removal of ΔssCPY*, a known substrate of the 26S proteasome, was impeded upon *MCA1* deletion and accelerated by Mca1 overproduction (***paper IV***, Fig 3g-h). Additionally, the lifespan extending effect

of Mca1 was dependent on a functional proteasome, providing further evidence for a connection between Mca1 and proteasome activity (***paper IV***; Fig 4e).

Another possible function of Mca1, also linked to proteasomal degradation but independent on Mca1 protease activity, is the recruitment of PQC factors to sites of protein aggregation. One such factor could be the AAA ATPase Cdc48, a segregase that acts in extracting proteins from complexes and aid in their subsequent degradation by the proteasome (Baek et al., 2013). We demonstrated that Mca1 interacts genetically with Cdc48 (***paper IV***; Fig. 2a), and other studies have reported that Cdc48 localization to the insoluble fraction is dependent on Mca1 (Shrestha et al., 2013). Furthermore, in ***paper II*** we identified a negative genetic interaction between *CDC48* and *SIR2*, and show that Cdc48 is required for proper damage segregation, mostly by affecting aggregate removal (***Paper II;*** Fig. 3a-b & 4b).

Another protease-independent function of Mca1 could be by acting as a holdase; i.e. binding to misfolded proteins, stabilizing the structure and thereby limiting further aggregation and toxicity (Hill and Nystrom, 2015, Kampinga, 2014). The pro-domain of Mca1 contains a Q/N rich stretch with a similar sequence to that found in aggregation-prone prions, and it has been shown that this pro-domain is important for Mca1 localization to aggregates (Lee et al., 2010). Nevertheless, further experimental data is required to elucidate the importance of this pro-domain in the beneficial functions reported for Mca1 in PQC and lifespan control (***paper IV***).

The proposed functions of Mca1 are not mutually exclusive, as binding to aggregates could be followed by activational cleavage and induction of proteolytic functions. Regardless of the exact mechanism however, the beneficial roles of Mca1 demonstrated in ***paper IV*** contradicts the previous reports of Mca1 acting, similar to an initiator caspase, in inducing PCD. The involvement of Mca1 in an apoptotic-like event has been questioned, and studies

have suggested that the increased survival seen in *mca1Δ* cells is due to compensatory mechanisms such as upregulation of chaperones and proteasome subunits as a response to the stress inflicted by *MCA1* deletion (Hill and Nystrom, 2015, Lee et al., 2010). Another explanation could be that Mca1 exhibit dual functions, with proteolytic activation acting as a switch between pro-survival and pro-death functions. As Mca1 activation as well as its proteolytic activity requires the presence of calcium, regulation of Mca1 activity could be achieved through spatial and temporal control of cellular calcium concentrations (Hill and Nystrom, 2015, Wong et al., 2012). However, this does not explain the fact that the proteolytic activity of Mca1 was shown to be part of its beneficial role during cellular aging (***paper IV***). Instead, it could be that the downstream actions of Mca1 are either cytotoxic or cytoprotective depending on the cellular context. The activity of Mca1 may aid in PQC and in the degradation of proteins in a healthy cell, but as the cell ages or is subjected to stress; the activity of Mca1 might eventually causes more harm than good. Such a dichotomy has been described for the 26S proteasome activity, where an elevated proteasome activity is beneficial in a normal cell, but detrimental in a cell with reduced chaperone activity (Baxter and Craig, 1998, Oling et al., 2014). In a cell with reduced chaperone activity, many proteins will be non-native and partially misfolded, but could potentially still retain a basal function. If the proteasome activity is elevated under such circumstances, this will cause the degradation of such proteins and the subsequent loss of essential functions, eventually leading to homeostatic collapse and cell death.

In this scenario, the reported Mca1-mediated cell death could be a result of overactivation of Mca1 upon prolonged or severe stress, and such overactivation might be linked to a breakdown in cellular calcium regulation.

6.6 Main findings paper I

- Retrograde movement of aggregates from daughter cell to mother cell is the most prevalent cross-compartment movement of aggregates.
- Aggregates form fiber-like structures stretching along the mother-bud axis that colocalize with the actin binding protein Abp140.
- The formin Bni1 is essential for segregation of both heat induced aggregates as well as aggregation-prone disease proteins.

6.7 Main findings paper II

- Asymmetric distribution of aggregates cannot be accomplished solely by the restriction of bud neck size and generation time.
- Sir2 plays a role in the retention of aggregates in the mother cell during cell division, rather than the removal of aggregates in the daughter cell.
- Genetic interactions of *SIR2* among essential genes identify functional groups involved in aggregate retention: ER-Golgi trafficking, microtubule nucleation, sister chromatid segregation and proteasome regulatory subunit.
- Aggregates colocalize with the actin motor protein Myo2 and the actin regulating protein Cmd1, and both of these proteins are required for proper damage segregation.

6.8 Main findings paper III

- A genome wide screen for AGGs reveals an important role for the vacuole and vesicle trafficking in generating damage asymmetry.
- The vacuole adaptor protein Vac17 affects aggregate asymmetry in a manner unrelated to its role in vacuole inheritance.
- Elevated levels of Vac17 increase damage asymmetry during heat shock and aging and accelerate fusion of aggregates with the IPOD compartment at the vacuolar surface.
- Increased levels of Vac17 extend replicative lifespan in a chaperone dependent manner.
- The effects of Vac17 on lifespan regulation and damage asymmetry are dependent on Myo2-binding and functional machinery for membrane tethering and fusion.

6.9 Main findings paper IV

- The metacaspase Mca1 is affecting damage asymmetry by acting on the removal of aggregates.
- Mca1 can to some extent buffer for the lack of Ydj1, the major cytosolic Hsp40 co-chaperone.
- Increased levels of Mca1 extend replicative lifespan and increase the degradation of a misfolded substrate.

- Lifespan extension by Mca1 is dependent on proteasome and chaperone activity.
- Mca1 auto-processing and activation occur during aging and H_2O_2 treatment, but the beneficial effects of Mca1 overproduction is only partly dependent on its caspase activity.

7. Concluding remarks

As a cell ages, different aging factors accumulate and eventually overwhelm the system, leading to a decline in functionality. Studies have provided evidence that aging factors act in parallel and that they also influence the occurrence and accumulation of one another. Taken together, the accumulated data have created a complex network of functions and genes that regulate cellular aging and longevity. Since many of these aging factors and longevity pathways have been conserved throughout evolution, studies in simpler organisms such as yeast can provide useful clues to how the system works and declines. Increasing our knowledge on how cellular systems normally deal with the increased burden of aging might also help us understand the mechanisms of age-related diseases as well as other disorders where the cell's normal maintenance system is incapacitated.

My work has been focused on the role of misfolded and aggregated proteins as potential aging factors, with the aim of elucidating how such damage is managed within the cell, and how this damage is prevented from entering the daughter cell during cell division. Revealing the mechanisms of protein quality control will aid our understanding of proteopathies such as Alzheimer's, Parkinson's and Huntington's disease where improperly folded proteins accumulate and interfere with the cellular function in neurons (Hipp et al., 2014). The relevance of my work can also be extended to research in the stem cell field, as stem cells have been shown to exploit a mechanism of asymmetric damage segregation, similar to that found in yeast, involved in ensuring self-renewal and "immortality" of the stem cell line (Bufalino et al., 2013, Fuentealba et al., 2008).

In ***papers I-IV*** we establish that the segregation of misfolded and aggregates during cell division is an active and factor-dependent process, and we further identify several of the factors that are involved in this process. We validate the previously reported importance of the actin cytoskeleton and establishment of cell polarity, and further report that many genes involved in vacuolar functions and vesicle trafficking are of importance for the establishment of damage asymmetry. Our findings have led us to hypothesize that aggregates might associate with membrane vesicles, and that the spatial deposition of aggregates to inclusions are dependent on vesicle trafficking. We specifically show that the sequestration of aggregates to perivacuolar inclusion sites (IPODs) involves endosomal transport, and that factors for fusion of vesicles to the vacuolar membrane are of importance. These factors include calmodulin, SNARE proteins, HOPS and CORVET tethering complexes, and our data also indicate a role for the vacuole adaptor protein Vac17 in this process. Further experimental data is needed to assess if there is an actual physical interaction of aggregates and vesicles, or whether the dependence of vesicle trafficking is due to some other regulatory factors exploiting this transport route.

While our data points to a role for the vacuole in the sequestration of aggregates, one wonders if this organelle has a subsequent function also in determining the fate of aggregated proteins deposited in the IPOD. Upon stress relief, misfolded proteins trapped in inclusions gradually disappear, but it is not known whether this disappearance is due to refolding or degradation, and whether aggregates in different inclusions share the same fate. Studies have shown that misfolded proteins in the JUNQ/INQ compartment are imported into the nucleus (Park et al., 2013, Miller et al., 2015), where protein degradation can occur. Thus, the nuclear deposition of aggregates has both an immediate spatial protective function as well as a consecutive function in degradation. The same could be true for misfolded proteins deposited at the vacuolar surface: upon cell recovery,

a proportion of the proteins could be translocated into the vacuolar lumen to be degraded by vacuolar proteases. In mammalian cells, misfolded proteins are degraded in the lysosome through the process of autophagy (Cuervo and Wong, 2014). Although reports have shown that the yeast IPOD colocalizes with autophagic markers, it remains uncertain whether proteins in this inclusion are actually substrates for autophagic degradation (Kaganovich et al., 2008, Spokoini et al., 2012).

The vacuole might also be of importance for the progeny rejuvenation occuring during meiosis. When diploid yeast cells are deprived of their carbon or nitrogen source, they undergo meiosis to produce four haploid spores that are extremely stress-resistant. Any damage that has accumulated during the lifetime of the diploid cell disappears during such meiosis, and all four spores are rejuvenated (Unal et al., 2011). Most aggregate inclusions found in old cells represent perivacuolar IPODs (***paper IV***), probably due to a higher turnover rate of proteins in JUNQ/INQ, and thus these are the aggregates in need of elimination during sporulation. As vacuoles are excluded from cells during sporulation (Roeder and Shaw, 1996), this process would also ensure the elimination of vacuole-localized IPOD inclusions.

In ***paper IV*** we looked deeper into the process of aggregate removal, and presented evidence for the metacaspase Mca1 being involved in proteasome-dependent degradation of misfolded proteins. It would be interesting to further test this beneficial function of Mca1 and investigate whether there is a connection to vacuolar function and vesicular trafficking. Such a connection has been implicated, as a *MCA1* deletion was shown to cause upregulation of many genes in vacuolar catabolism (Lee et al., 2010) and we found that several vesicle trafficking genes were among the genetic interactors of *MCA1*. A connection between Mca1 and the vacuole is also indicated by the dependence of Mca1

activation on high concentrations of Ca^{2+}, and the vacuole being the main storage of this divalent anion.

Our data also points to a role for vesicular trafficking between ER and Golgi in damage segregation. Among the genetic interactions identified for *MCA1* and *SIR2* (***papers II and IV)***, numerous shared interactors are found, including several *SEC*-genes involved in ER-Golgi trafficking. Mutations in these genes were shown to cause a collapsed damage asymmetry, but the exact role for these genes remains to be elucidated. It would be interesting to test whether ER-Golgi trafficking are involved in sequestration of aggregates to the JUNQ/INQ compartment, similar to how endosome trafficking seems to be driving aggregate sequestration into IPOD.

Further research will also have to clarify the factors involved in spatial sorting, and whether genes of the cellular trafficking system is involved also in the determination of aggregate destination. Furthermore, it will be interesting to learn to what extent these pathways for aggregate segregation are conserved in higher eukaryotes and whether any of the identified AGGs could provide potential therapeutic targets for treating proteopathies or induce tissue rejuvenation.

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