# Tracking the big ones : novel dynamics of organelles and macromolecular complexes during cell division and aging

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# Chapter 1:

# General Introduction

# General introduction

To survive as a single cell or to function within a multi-cellular organism, cells need to continuously adapt their internal processes to changing internal and external signals and stressors. Cellular processes can be modulated by controlling the local abundance and activity of proteins. This is achieved by continuous and highly regulated synthesis, degradation, folding, modification and translocation of proteins. Protein dynamics therefore lies at the heart of virtually all cellular process and aberrations of it are implicated in several diseases. To understand cellular processes and possibly treat these diseases, knowledge of the underlying protein dynamics is essential. [1–4] In this Thesis we address two important aspects of protein dynamics: protein synthesis and distribution upon cell division and dynamics of the protein degradation machinery.

## Cell division: A matter of equal sharing?

When cells divide they give rise to two new cells. Cell division is as simple as that. However, as is often the case when things need to be divided, the exact distribution of components is a more complicated issue. The heritage of the original cell consists of both functional and damaged cellular components. Either type needs to be distributed adequately over the two new cells to support a successful life of the next generation. Adequate distribution of cellular components is an absolute prerequisite for life and therefore tightly regulated. The importance and tight regulation of this distribution is exemplified by the process of DNA replication and chromosome distribution during cell division.

'Adequate distribution of cellular components' does not necessarily mean 'equal distribution'. In fact, asymmetric sharing of cellular components appears to be an important aspect of cell divisions across the kingdoms of life. In single cell organisms asymmetric cell division is suggested to be important to generate phenotypic variation in a population, which allows this population to survive a variable environment. Moreover, asymmetric distribution of damaged cell components provides a way to restrict the consequences of aging in one cell at the expense of another. This prevents aging of the entire population, which may otherwise lead to mass extinction. In multicellular organisms asymmetrical distribution of cell components has been suggested to be essential for the creation of differentiated cells and the maintenance of germ lineages and stem cell populations. [9,10,21,22]

Asymmetric distribution of cellular components has both quantitative (one cells gets more than the other) and qualitative (one cell gets components with different characteristics) aspects. Quantitative aspects of asymmetrical inheritance are relatively easy to address and examples include asymmetric distribution of a nuclear transport factor [23] and of plasma membrane proton ATPase's [24]. Qualitative aspects of asymmetric distribution are usually caused by subtle changes in composition or small modifications of the protein (complex) leading to altered functionality. The functional significance of these subtle differences is often unknown, but may induce lineage differences. In budding yeast, for example, the asymmetric distribution of malfunctioning mitochondria defines an 'old' and a 'young' lineage [25,26]. Another example is formulated by the immortal DNA strand hypothesis, which suggests that stem cells retain a template copy of genomic DNA to avoid buildup of replication-induced mutations [27].

Asymmetric distribution of cell components and the potential lineage differences they induce are relatively easy to study in the budding yeast *Saccharomyces cerevisiae* [5]. This

unicellular eukaryotic organism produces two morphologically distinct cells upon cell division, a 'mother' and a 'daughter, that differ in age and define an 'old' and a 'young' cell lineage. Asymmetric distribution of cell components that consistently favor one of the two lineages may therefore be connected to the aging of the old lineage or the fitness of the young lineage [22]. The asymmetric distribution of cell wall components can be used to define both lineages. Upon cell division the mother cell keeps its own cell wall, whereas the cell wall of the emerging daughter cell (bud) is formed completely *de novo* [28]. When the daughter breaks away from the mother cell, chitinous scar tissue (the bud scar) is left on the mother cell. Every cell division leads to a new bud scar which allows tracking of the number of cell divisions a mother cell underwent [29]. This allows the study of the cumulative effects of multiple cell divisions with asymmetric distribution of cellular components.

Adequate distribution of cell components is of particular importance for organelles and macromolecular complexes since they are essential, often synthesized in a template-based manner and their size and complexity don't always allow rapid synthesis after cell division. Like chromosomes, the distribution of these large cell components is therefore actively controlled during cell division. This process is extensively studied in *S. cerevisiae* and involves three fundamental steps: cell polarization, transport and retention. Cell polarization is established by a complex signaling network that recruits formin proteins to the cell membrane at the future bud site. Formins act as a nucleation point for the assembly of unbranched actin cables. These cables function as the transport route for cell membrane and cell wall material which is deposited at the growing bud site. Later, these cables serve the transport of cellular organelles from the mother cell into the growing bud (Figure 1). The transport itself is mediated by class-V myosin motor proteins that recognize the different organelles by specific receptor molecules. Important exceptions are the nucleus and the nuclear ER, which depend on microtubules for their transport towards the daughter cell. Once at their destination, organelles need to be retained by tethering, e.g. to the cytoskeleton or the cell cortex, to prevent them from diffusion back into the mother cell. To make sure the mother cell keeps enough organelles for herself, some organelles are retained in the mother cell, as has been shown for peroxisomes [5]. Many of the key aspects of the mechanisms to distribute organelles upon cell division in yeast are conserved in mammalian cells and are reviewed by Jongsma *et al* [30].

To ensure that both new cells get sufficient amounts of organelles and macromolecular complexes, sharing the pre-existing components of the original cell (inheritance) is not enough. To support life in both new cells the pre-existing components need to be supplemented with synthesis of new components, which can either occur before or after cell division. Whether pre-existing (old) and newly synthesized components are equally shared between the two new cells, is unknown for most organelles and macromolecular complexes. One exception is the spindle pole body (SPB), the yeasts centrosome, for which it is reported that one cell inherits mainly old proteins and the other cell mainly new proteins [31]. This asymmetric distribution of old and new SPB proteins may imply functional differences between the two SPB's, which may induce lineage differences. Whether this qualitative asymmetric distribution is a curiosity of the SPB or a common topic for organelles and macromolecular complexes was unclear. A comprehensive analysis of the different organelles would greatly benefit from methods to simultaneously visualize old and new proteins.

Synthesis of new organelles and complexes can take place *de novo* or template-based. Template-based synthesis of an organelle or complex is a combination of growth by

**Fig. 1**

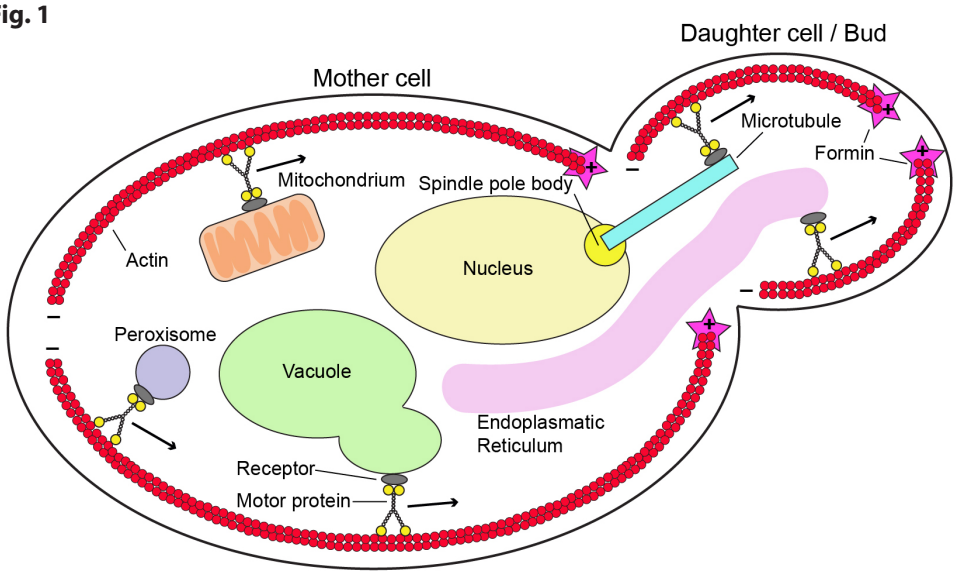


**Figure 1: Organelle transport in dividing budding yeast**
When a yeast cell divides, a daughter cell (bud) emerges from the mother cell. To equip the bud with all necessary organelles, a mother cell transports a portion of her organelles towards the emerging bud. For most organelles this transport is mediated by Myosin-V motor proteins that 'walk' along actin cables from the minus to the plus end. The directionality of this transport towards the bud is facilitated by the polar distribution of formin proteins, which position the formation of actin cables (and thus the plus end) at or near the emerging bud. To transport organelles, they are attached to the motor proteins by organelle-specific receptor molecules. In contrast to the actin-based transport of most organelles, the nucleus is transported along microtubules. Microtubules attach with their minus end to the nucleus at the spindle pole body and are oriented towards the bud by actin-based transport of their plus end. Pulling forces on the microtubules drag half of the nucleus towards the daughter cell and retain the other half in the mother cell.

addition of new components and division in two new organelles or protein complexes. A well-established example of this method is the formation of new mitochondria by growth and division [32]. Also the ER is thought to utilize this method as many of its constituents require functional ER for their synthesis. Macromolecular complexes like the proteasome or the ribosome on the other hand are formed completely *de novo* [33,34]. However, the contribution of *de novo* and template-based synthesis is much debated for other organelles and macromolecular complexes. Peroxisomes, for example, are proposed to form *de novo* using the ER as a platform for their synthesis [35,36], but also by growth and division [37]. As for their asymmetrical inheritance, the study of organelle and macromolecular complex synthesis would greatly benefit from methods to track old and new proteins simultaneously.

## How to distinguish and track old and new proteins?

Several methods have been developed to distinguish and simultaneously track old (synthesized long ago) and new (recently synthesized) proteins. These methods can be divided into four categories: differential chemical labeling, differential isotope labeling, fluorescent timers, and photo-transformable fluorescent proteins.
Differential chemical labeling involves the expression of a protein with a tag that reacts

with different chemical probes. Sequential administration of these probes allows tracking of old (labeled with the first probe) and new (labeled with the second probe) proteins. Several (differential) chemical labeling methods tag the protein of interest with an enzyme and use modified (eg. containing a fluorescent group) substrate molecules as chemical probe. Commonly used examples are SNAP-, CLIP-, and Halo tags [38–40]. The protein of interest can also be tagged with a small peptide tag, like the tetra-cysteine sequence used for FlAsH-ReAsH labeling [41].

Differential isotope labeling is usually achieved by stable-isotope labeling by amino acids in cell culture (SILAC) during a defined time (pulse), followed by a chase in the presence of the 'normal' amino acids [42]. New and old proteins can now be distinguished by mass spectrometry by the mass differences caused by the isotopic labeled amino acids. Commonly used isotope labeled amino acids include $^{2}$H leucine, $^{13}$C lysine and $^{15}$N arginine. Differential isotope labeling has been successfully used to track the inheritance of (very) old proteins in budding yeast and to reveal the long-lived proteome in rat brains [43,44].

A fluorescent timer is a protein tag that changes its fluorescent properties as a function of time. An example is dsRed, which undergoes a fluorescence shift during its slow maturation [45]. Newly synthesized dsRed-tagged proteins will first have green fluorescence, whereas the fluorescence of older proteins will have matured to red. This fluorescent timer was successfully used to address the distribution of old and new proteins of the yeast SPB upon cell division [31]. Another example is a series of mCherry derivatives developed by Subach *et al* that change their fluorescence over time from blue to red. As a result, newly synthesized proteins show blue fluorescence whereas older proteins show red fluorescence. These fluorescent timers were developed with a half-life for the shift in fluorescence of 0.25, 1.2 and 9.8h [46]. Unlike the other methods to distinguish and track old and new proteins, fluorescent timers entail a continuous flux from the new to the old population.

This limitation is largely overcome in the use of photo-transformable fluorescent proteins (PTFPs). PTFPs change their fluorescent properties upon exposure to light of a specific wavelength. A well-known example is photo-activatable GFP (PA-GFP), which only starts to fluoresce like a GFP molecule after exposure to intense 405 nm light [47]. PTFPs allow simultaneous tracking of new and old proteins by their fluorescent properties. The last ten years have shown a tremendous expansion of the collection of PFTPs, fuelling many new and exciting imaging techniques, like super resolution microscopy [48].

The different techniques to assess the (relative) age of proteins have led to valuable new insights in protein dynamics. However, with the exception of some differential chemical labeling methods, these techniques do not provide handles for selective purification or biochemical analysis of old and new proteins. Differential chemical labeling then has the drawback of the need for (expensive) chemicals. Also, presented techniques are not easily incorporated in (genetic) screenings. These limitations of the existing techniques are largely overcome by a novel technique that we present in this Thesis; Recombination-Induced Tag Exchange (RITE) [5]. RITE allows distinction and simultaneous tracking of old and new proteins and the used protein tags can be easily adjusted to the experimental needs.

**Protein Quality Control counteracts accumulation of damaged proteins during aging**

Another aspect of protein dynamics that is addressed in this Thesis is the dynamics of the protein degradation machinery in aging cells. In aging cells, damaged proteins tend to

accumulate, which is a hallmark of cellular aging and implicated in several age-related diseases. This suggests that the degradation of these proteins is insufficient and that protein degradation is a relevant factor in cellular aging. However, protein degradation is only one modality of a larger system that prevents the accumulation of damaged proteins: the Protein Quality Control (PQC) system. The cellular PQC system has two modalities, protein refolding and protein degradation, that often compete for the same damaged proteins [2].

Cellular proteins are continuously at risk for (partial) unfolding, e.g. as a result of post-translational modifications or altered concentrations of certain metabolites. Various cellular stresses, like osmotic stress or heat shock, can dramatically increase this risk. Not only are (partially) unfolded proteins less likely to function properly, they also tend to cluster with other (misfolded) proteins. This clustering is often caused by the exposure of hydrophobic patches as a result of protein unfolding and may lead to the formation of harmful aggregates. To maintain proper folding, cellular PQC employs a family of 'refolding' proteins; chaperones. Some chaperone proteins, like the small heat shock proteins, bind the (partially) misfolded proteins, thereby shielding their exposed hydrophobic patches and preventing aggregate formation. Other chaperones are also able to support the refolding of substrate proteins in an ATP-dependent process, like chaperones belonging to the HSP70 family [1,49]. How chaperones 'decide' when proteins are correctly folded, is unclear but probably relates to the absence of hydrophobic patches as detected by these various chaperones.

When a soluble protein cannot be refolded, the cellular PQC system can only destroy the protein to prevent the formation of aggregates. There are two 'degrading entities' to degrade these proteins; the proteasome and the lysosome. The proteasome is a multi-subunit protein complex containing protease activity, whereas the lysosome is a membrane enclosed compartment containing multiple proteases [50]. The proteasome and the lysosome have in common that they shield their catalytic activity from the rest of the cell to prevent unwanted degradation. Although safe, this necessitates the cell to 'present' the proteins to be degraded to the degrading entities. In case of the lysosome this is either mediated by direct import of the proteins to be degraded or by fusion with vesicles containing these proteins [51,52]. These vesicles can for example originate from autophagy or the endocytic machinery [52,53]. In case of the proteasome, substrate molecules are presented by the ubiquitin-proteasome system (UPS) [11].

The UPS is the primary degradation mechanism for the specific degradation of short-lived regulatory proteins and damaged soluble proteins. The UPS enables protein degradation in a time and place specific manner and is essential for virtually all cellular processes. The UPS is an extensive network of co-operating proteins (and protein complexes) and can be divided in a part that marks the proteins to be degraded (the ubiquitination machinery) and a protein complex that degrades the marked proteins, the 26S proteasome [11] (Figure 2).

The recognition signal for proteasomal degradation is a ubiquitin chain attached to the protein to be degraded. The covalent attachment of ubiquitin to the target protein is called ubiquitination. Ubiquitination is performed by an ATP-dependent enzymatic cascade involving three classes of enzymes; ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-ligating enzymes (E3). Together they form an isopeptide bond between the ε-amino group of substrate lysines and the carboxyl group at the C-terminal glycine of ubiquitin. The presence of internal lysines in ubiquitin allows

the formation of poly-ubiquitin chains. The signal conveyed by the poly-ubiquitin chain is highly dependent on the internal lysines used to make the chain. A lysine 63 linked ubiquitin chain for example is a signal to be degraded by autophagy, whereas a chain of at least four ubiquitins linked via lysine 48 is the typical recognition signal for the 26S proteasome [11,54].

The 26S proteasome is the macromolecular protease responsible for the degrading capacity of the UPS. The 26S proteasome consists of one 20S core particle (CP) flanked by one or two 19S regulatory particles (RP). Within the 26S proteasome the 20S CP is the degradation functionality, whereas the 19S RP's are needed for recognition of ubiquitinated substrates and their translocation in the 20S CP [33].

The 20S CP consists of four stacked heptameric rings, together forming a barrel-shaped structure with an inner catalytic chamber. The catalytic activity in this chamber comes from three catalytic active subunits in each of the two inner, or β, rings. The two outer, or α, rings close the catalytic chamber leaving only a small entrance. To prevent untargeted degradation of proteins, the access through this 'gate' is restricted by the N-terminal tails of the α-subunits. This renders the 20S CP on its own largely inactive towards folded peptides. To activate the 20S CP it needs to associate with one of several proteasome activators, the most common of which is the 19S RP [33].

The 19S RP recognizes ubiquitinated proteins, de-ubiquitinates them to recycle ubiquitin,

**Fig. 2**

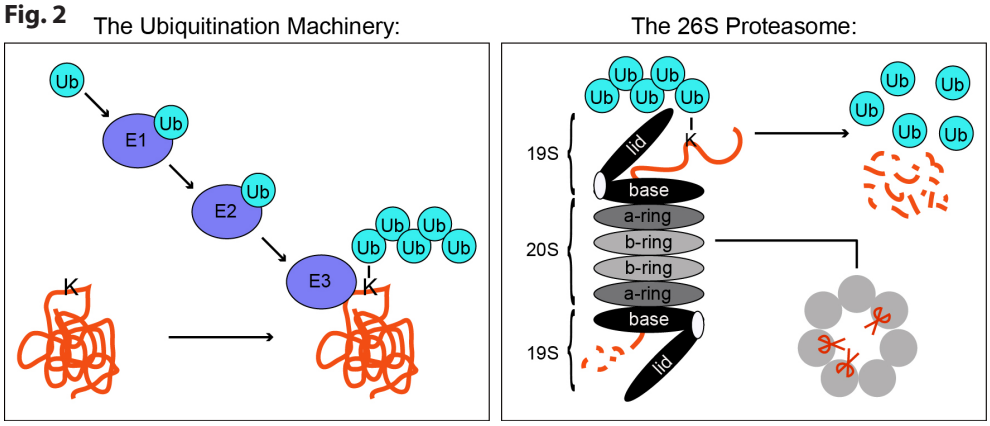


**Figure 2: The ubiquitin-proteasome system**

The ubiquitin-proteasome system (UPS) facilitates specific degradation of proteins and can be divided in two parts; the ubiquitination machinery and the 26S proteasome.

The ubiquitination machinery marks proteins for degradation by covalent attachment of ubiquitin (Ub). This is mediated by an extensive network of ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes. Together E1, E2 and E3 form an enzymatic cascade that attaches ubiquitin to an internal lysine residue (K) of the targeted protein. The presence of lysine residues in ubiquitin itself allows the formation of poly-ubiquitin chains. A chain of at least four ubiquitins attached via lysine 48 is the typical recognition signal for the 26S proteasome.

The 26S proteasome recognizes ubiquitinated substrates and degrades them into peptide fragments. The 26S is a proteins complex that can be subdivided in a central 20S particle and two flanking 19S particles. The 20S particle contains protein degrading activity inside a barrel-like structure formed by the stacking of four heptameric rings. The two inner (or β-) rings contain three catalytic active subunits each, which supply protein degrading activity to the 20S. The two outer (or α-) rings close the access to the degrading activity by blocking the entrance of the barrel-like structure with their N-terminal tails. This 'gate' can be opened by the 19S particle. The 19S particle consists of two sub-complexes; the base and the lid. The lid recognizes ubiquitinated substrates and removes the ubiquitin, whereas the base unfolds the substrates and transports them into the 20S barrel.

and then unfolds them before translocation into the 20S catalytic chamber where they are degraded. The 19S RP consists of a base and a lid complex. The lid complex is important for substrate recognition and de-ubiquitination and is loosely connected to the base by a 'hinge' subunit. Recognition of ubiquitinated proteins is mediated by two ubiquitin receptors; RPN10 and RPN13. De-ubiquitination is mediated by the RP resident RPN11, possibly aided by the proteasome associated dubs USP14 and UCH37. The base is attached to the 20S proteasome where it opens the 'gate' formed by the N-terminal tails of the α-subunits. Six AAA+ ATPase subunits in the base are required for unfolding and possibly translocation of the substrate protein [55]. In summary, the 26S proteasome is a multi-protein protease with a unit for substrate recognition and unfolding (the 19S) and a unit for degradation (the 20S).

The other degrading entity at the disposal of cellular PQC is the lysosome. Damaged cytosolic proteins, and even damaged organelles, are targeted to the lysosome by a process called autophagy. The autophagy lysosome system (ALS) was long considered a non-specific degradation mechanism for bulk degradation of cytoplasmic proteins and compartments [56]. However, over the years increasing specificity has been assigned to this process, like the identification of specific autophagy mechanisms for ribosomes and mitochondria [56,57].

Different ways of lysosomal targeting define three different forms of autophagy; macro-autophagy, micro-autophagy and chaperone-mediated autophagy (Figure 3). Macro-autophagy is most predominant and includes the formation of a double membrane, the phagophore, around cytosolic proteins or organelles that are destined for degradation. When the phagophore completely encloses its substrates it is called an autophagosome. The autophagosome then fuses with the lysosome, leading to the degradation of its contents. Substrates for macro-autophagy are recognized by autophagic adaptor proteins, like P62, which couple them to the growing phagophore [52]. Micro-autophagy is a more direct way of cargo delivery to the lysosome, as the lysosome acquires its substrates by their endocytosis. Central in the selection of cargo by the lysosome is the chaperone protein HSC70 [52,58]. The direct uptake of substrate proteins is also apparent in chaperone-mediated autophagy (CMA), though CMA uses a protein translocation complex instead of endocytosis to deliver substrates to the lysosome [51].

Apart from refolding and degradation, another important aspect of PQC mechanisms is the sequestration of damaged proteins in PQC compartments. Several different PQC compartments have been described with distinct composition, function and localization in the cell [59]. The JUxtaNuclear Quality control compartment (JUNQ), for example, is localized near the nuclear ER and contains soluble, ubiquitinated, damaged proteins as well as many chaperones and active proteasomes. Its proposed function is to enhance the efficiency of the PQC by sequestering (and thus concentrating) the important players. The Insoluble Protein Deposit compartment (IPOD) on the other hand resides near the lysosome and contains terminally aggregated proteins. Its function is thought to be the scavenging of potentially harmful misfolded proteins [60,61].

Apart from enhancing PQC efficiency and scavenging harmful proteins, sequestration of protein damage also facilitates asymmetric inheritance of damaged proteins. Association of these compartments with the cytoskeleton or organelles is proposed to restrict their presence to the older lineage upon cell division. In bacteria and fission yeast for example, aggregated proteins are sequestered at the old pole of a dividing cell [62,63]. In budding yeast on the other hand, protein aggregates are retained in the mother cell by association

**Fig. 3**

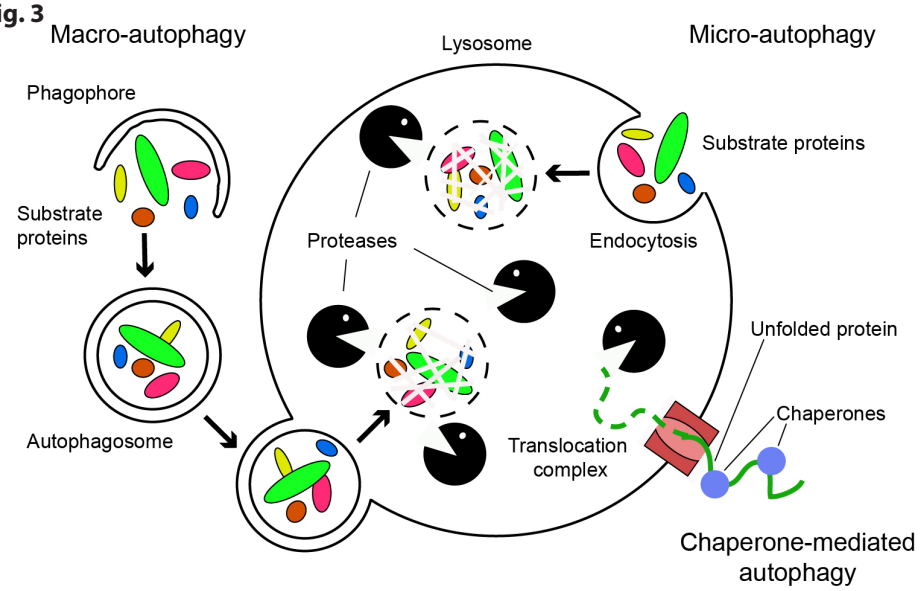


**Figure 3: The autophagy lysosome system**
The autophagy lysosome system (ALS) enables the degradation of proteins, complexes and even organelles in the lysosome. To this end, the lysosome is filled with proteases and other degrading enzymes. Substrates are targeted towards the lysosome by autophagy, which can be subdivided in three different classes; Macro-, Micro-, and Chaperone-mediated- autophagy.
Macro-autophagy entails the formation of a double membrane, the phagophore, around the cytosolic substrates. When the substrates are completely engulfed it is called an autophagosome. The autophagosome delivers its contents to the lysosome by fusing with the lysosomal membrane. Micro-autophagy is the direct endocytosis of substrates by the lysosome and their subsequent degradation. In chaperone-mediated autophagy, substrate proteins are recognized and unfolded by chaperones and transported across the lysosomal membrane by a translocation complex.
Together, the different modes of autophagy allow the ALS to (specifically) degrade a wide variety of substrates.

with the polarisome [64]. A recent study in mammalian cell lines, that are supposed not to have lineage differences, showed asymmetrical inheritance of JUNQ, possibly mediated by the intermediate filament vimentin [65].
In summary, cells prevent the accumulation of damaged proteins by the protein quality control (PQC) system. The PQC system initially tries to repair the damaged proteins, e.g. by employing chaperones to refold unfolded proteins. When repair fails, cells have two systems for the degradation of damaged proteins; the ubiquitin-proteasome system (UPS) and autophagy lysosome system (ALS). Together these systems prevent harmful accumulation of damaged proteins and thus support healthy cellular aging.

## Yeast as a model system for cellular aging

The budding yeast *Saccharomyces cerevisiae* is an important model organism in age-related research. Because it is easy to quantify longevity in budding yeast and because it is easy to manipulate its genome. This has allowed the identification of dozens of factors affecting longevity and the identification of several mechanisms underlying aging [22]. The search for homologues of these factors in higher eukaryotes has made major contributions

to mammalian aging research. Most notable is the identification of the sirtuins, a family of $NAD^+$ dependent protein deacetylases. Decreasing its activity in yeast and several other lower eukaryotes results in decreased longevity, whereas increased activity correlates with increased longevity. In mammalian cells, sirtuins have less drastic effects, although they have been implicated in several age-related processes and diseases [66].

When we define cellular aging as the accumulation of cellular damage resulting in a gradual loss of the cells functionality, we can distinguish two modes of aging in budding yeast: chronological aging and replicative aging (Figure 4). In chronological aging damage accumulates over time in a non-dividing cell, which as a result will eventually lose its capacity to divide and then dies. To measure chronological life span, one monitors the viability of a population of non-dividing cells over time. A population of non-dividing cells is obtained by starving cells in a liquid culture and viability is defined as the ability to resume cell growth once fresh medium is added [22]. Replicative aging is the accumulation of cell damage in the mother cell, due to the asymmetric distribution of cell damage upon cell division. Each time a yeast cell divides, the mother cell retains all cell damage in order to give rise to the fittest daughter cells possible. The cumulative effect of 25-30 of these asymmetric divisions will lead to a terminal replicative senescence [67]. Replicative life span

**Fig. 4**

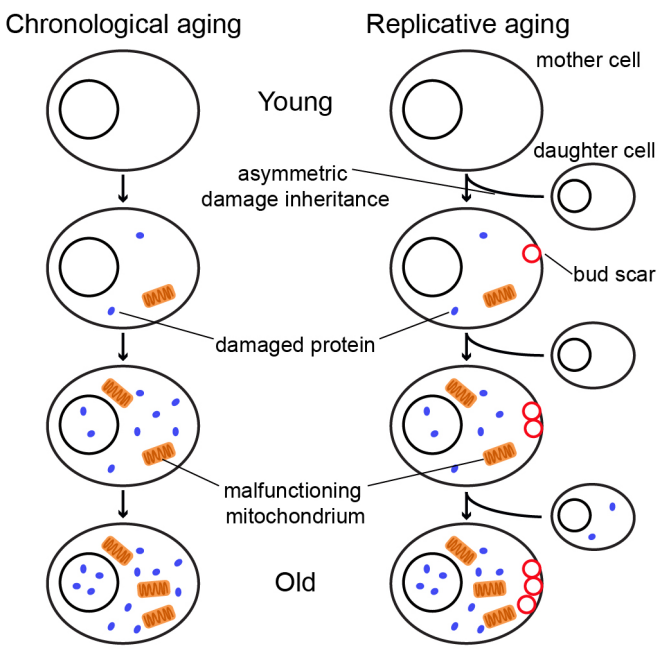


**Figure 4: Chronological and replicative aging in budding yeast**
Cellular aging is the gradual loss of the cells functionality and viability as a result of the accumulation of cellular damage. Many types of cell damage have been implicated in cellular aging, like protein damage and malfunctioning mitochondria. In budding yeast cell damage accumulates in two different ways; chronological aging and replicative aging. Chronological aging is the accumulation of cell damage as a function of time in non-dividing cells and can be assessed by measuring viability in time. Replicative aging is the accumulation of cell damage in mother cells as a result of asymmetric inheritance of cell damage. Upon cell division, the mother cell retains the cell damage to give rise to the fittest daughter cell possible. Replicative age can be assessed by counting the bud scars that are left on the cell wall of the mother cell, each time a daughter cell breaks away.

is defined as the amount of daughter cells one cell can produce and replicative age is the amount of cell divisions a cell underwent [22,68]. The replicative age of a yeast cell can be determined by counting the number of bud scars, which can be visualized by a staining with Calcofluor White [29].

Although replicative aging as a result of damage retention in the mother cell currently lacks a well-studied analogue in mammalian cells, the kind of damage (the aging factor) that is retained yields valuable information about the aging process. Several aging factors have been implicated in replicative aging in yeast [68]. Oxidized and aggregated proteins for example were found to accumulate with replicative age in mother cells and to show a mother-biased segregation upon cell division [69,70]. The retention of this protein damage in the mother cell is suggested to be the result of the association of protein aggregates with the actin cytoskeleton, which prevents their diffusion into the daughter cell [71]. These oxidized proteins may be the results of another aging-related factor; malfunctioning mitochondria. Malfunctioning mitochondria are found to accumulate in mother cells during replicative aging and suggested to be causative for this aging [25,72,73]. The mother-biased inheritance of malfunctioning mitochondria is suggested to be the result of an ingenious filtering mechanism [26]. A potential cause for the appearance of malfunctioning mitochondria is yet another aging factor; lost pH control of the vacuole (the yeast lysosome) in mother cells [74]. Although the role of the lysosomal pH in mammalian aging remains to be established, accumulation of damaged proteins and malfunctioning mitochondria are conserved aspects of cellular aging.

Chronological aging of budding yeast is a model system for the aging of post-mitotic cells and is studied in stationary phase yeast cultures [22]. A stationary culture is reached by growing rich liquid cultures to saturation, usually for 5-7 days at 30ºC. At the start of this period, yeast cells gain energy by fermentation of glucose to ethanol and are proliferating rapidly. When glucose gets limiting, proliferation is slowed down and the cells adjust their metabolism to utilize non-fermentable carbon sources, like ethanol. After this metabolic adjustment, the diauxic shift, cells make one or two very slow cell divisions before the non-fermentable carbon sources are depleted and the cells enter a starvation-induced quiescent state. The culture is now said to be in a stationary phase [75]. Upon the diauxic shift, cells start an extensive 'quiescence program' to prepare for a long survival in quiescence. An important part of this program is the adjustment of transcription and translation rates to the lower energy intake, reducing it to respectively ~20% and 0.3% of their original values. Another consequence is that this program makes the cell more resilient towards stress by up-regulation of genes involved in PQC and by thickening the cell wall. Furthermore, autophagy is induced under these conditions to scavenge damaged cell components and as a source of energy [75]. Also, several cellular proteins (like actin, proteasomes and several metabolic enzymes) get sequestered in so called storage compartments. These storage compartments are suggested to protect its constituents and provide a rapidly available pool of proteins once the growth conditions get more favorable [76–78]. Although the aging of mammalian post-mitotic cells is usually not starvation-induced, several factors and mechanisms are similar to yeast chronological aging, like the central role for TOR signaling and PQC mechanisms.

Replicative and chronological aging are intimately linked and many examples exist of chronological age affecting replicative life span and vice versa [79–81]. The tight link between both modes of aging is exemplified by the strong influence of replicative age on the chronological life span in a starving yeast culture. Based on their replicative age, two

populations of cells can be distinguished in stationary phase yeast cultures: Quiescent (Q) and Non-Quiescent (NQ) cells. Q cells are unbudded daughter cells formed after the diauxic shift that maintain their reproductive ability during long periods of starvation. NQ cells on the other hand, are replicative older cells with a reduced chronological life span [82]. NQ cells have a high load of reactive oxygen species and damaged proteins, which is consistent with the mother-biased inheritance of malfunctioning mitochondria and protein damage. Q cells on the other hand have low levels of both, possibly as a result of the observed up regulation of oxidative stress response genes [82–84].

These observations suggest that one can learn about age-related processes by observing phenotypic differences between cells of different replicative age in a starved yeast culture. In this Thesis we take this approach to study how a cell modulates its ubiquitin-proteasome system in response to aging.

**How does the cell modulate its UPS in response to aging?**

Selective degradation of damaged proteins by the UPS is an important PQC mechanism in the aging cell. Insufficient UPS activity leads to the accumulation of damaged proteins, which is a hallmark of cellular aging and implicated in several age-related diseases [3]. Insufficient UPS activity can be caused by limiting proteasome activity or limiting activity of the ubiquitination machinery. The accumulation of poly-ubiquitinated proteins that is often observed in aging cells, suggests that proteasome activity is limiting. Also, increased proteasome activity is found to be sufficient to reduce cytotoxicity upon oxidative stress and to increase longevity in budding yeast [85,86]. The concept of limiting proteasome activity as an important factor in aging and age-related diseases is getting increasing attention. This is exemplified by the growing interest for proteasome activators as potential therapeutics in the treatment of age-related diseases like Alzheimers disease [86].

Unfortunately for the cell, the chances of limiting proteasome activity increase during the aging process as a result of increased internal stress factors and an age-dependent decrease in said activity. The age-dependent decrease in proteasome activity is observed in several model organisms and is even suggested to be causative for this aging [14,85,87,88]. The age-dependent decrease of proteasome activity in human epidermal cells and the relatively high activity in fibroblast of healthy centenarians suggests that this phenomenon may also be relevant for mammalian cells [88,89]. Whether it is proteasome activity per se or whether there are other proteasome-related factors important during the aging process remains an open question.

The first indication of the involvement of another proteasome-related factor in the aging process came from a study in budding yeast. The proteasome in budding yeast is primarily localized in the nucleus when they grow in the presence of sufficient nutrients [90]. When yeast cells experience a limiting amount of glucose however, they export their proteasomes from the nucleus and sequester them in cytosolic foci termed Proteasome Storage Granules (PSG). These structures are stable when starvation is prolonged, but dissolve rapidly when nutrients are re-added, followed by the rapid import of proteasomes in the nucleus. PSGs are proposed to store proteasomes during starvation-induced quiescence, while allowing rapid release upon cell cycle re-entry [78]. Interestingly, proteasome localization is not the same for all yeast cells in a stationary phase culture. In this Thesis we show that the localization of the proteasome in starving budding yeast correlates with the replicative age of a cell. This may suggest that proteasome localization, like its activity, plays an important role in cellular aging.

**A PQC system for the proteasome?**

Although an age-dependent decline in proteasome activity is observed in several model systems for aging research, the cause of this decline remains obscure. Several factors are proposed to play a role, including decreased proteasome levels, altered proteasome conformation or an increasing number of damaged (less functional) proteasomes.

An age-dependent decrease in proteasome levels is observed in several model systems, often deduced from lower expression of proteasome subunits [12,87]. The reason for this counter-intuitive age-dependent decrease in proteasome levels is unclear. Conformational changes of proteasomes during aging were observed in a study in *Drosophila*, showing an age-dependent decline of the highly active 26S form of the proteasome in favor of the less active 20S form [14]. Since the 26S proteasome is stabilized by ATP, Vernace *et al* suggest that the decline in cellular ATP levels they observed in their aging cells is causative for this conformational change. This hypothesis may be supported by a similar shift from the 26S to the 20S form of the proteasome observed in yeast cells undergoing starvation [91]. Still, these data are merely a correlation at present.

A decreased proteasome activity due to an increased population of damaged proteasomes is consistent with several *in vitro* and *in vivo* studies showing decreased proteasome activity upon treatment with oxidizing agents like nitric oxide. However, none of these studies shows the actual damage to the proteasome [12,92]. Still, it is likely that proteasomes get (oxidatively) damaged during their life time given their extremely long reported half-life of 5-12 days [18–20]. Oxidative damage to the proteasome is even more likely in aging cells as many of these cells experience oxidative stress as a result of malfunctioning mitochondria. This (oxidative) damage may lead to reduced proteasome activity.

Given its central role in the protein quality control system, it is of vital importance to maintain a 'fit' population of proteasomes. To prevent the accumulation of damaged proteasomes during cellular aging, cells are therefore expected to employ quality control mechanisms on the proteasome. Degradation of the proteasome is reported in HeLa cells and rat livers and suggested to be mediated by the lysosome [18–20]. The way proteasomes are delivered to the lysosome and whether there is specificity towards damaged proteasomes, as PQC entails, is currently unknown.

The action of such a potential proteasome quality control mechanism would be of particular interest for long-lived non-dividing cells, like neurons. This type of cells cannot simply 'dilute' their malfunctioning proteasomes by cell division and since they are long-lived they will need exquisite UPS activity to maintain proteome fitness during their entire lifetime. The relevance of optimal UPS activity in these cells is highlighted by the appearance of protein aggregates, and thus insufficient PQC, in many neurodegenerative disorders. We believe that a deeper understanding of the proteasome quality control mechanisms may yield new therapeutical targets to increase UPS activity in the treatment of these and other age-related diseases.

**Conclusion**

In this Thesis we address two important aspects of protein dynamics: protein synthesis and distribution upon cell division and dynamics of the protein degradation machinery.

In Chapter 2, we present novel technology (Recombination-Induced Tag Exchange) to distinguish and simultaneously track old and new proteins. In Chapter 3 we used this technology to make a comprehensive analysis of the inheritance and synthesis of

organelles and macromolecular complexes in budding yeast. Thereby we resolved outstanding issues in organelle synthesis and uncovered symmetrical and asymmetrical patterns of inheritance. Asymmetrical inheritance of organelles and macromolecular complexes may induce lineage differences and could be involved in cell differentiation.
Next, we address two aspects of the dynamics of the protein degradation machinery that may be relevant for cellular aging: proteasome localization and degradation of the proteasome. In Chapter 4 we show that the localization of the proteasome, like its activity, may be a relevant factor in cellular aging and identify genetic factors affecting proteasome localization and longevity in budding yeast. In Chapter 5 we present data that is consistent with lysosomal degradation of damaged proteasomes, which may represent the first sketches of a quality control mechanism for the proteasome.

## References


1. Hartl, F. U., Bracher, A. & Hayer-Hartl, M. Molecular chaperones in protein folding and proteostasis. Nature 475, 324–32 (2011).
2. Balch, W. E., Morimoto, R. I., Dillin, A. & Kelly, J. W. Adapting proteostasis for disease intervention. Science 319, 916–9 (2008).
3. Vilchez, D., Saez, I. & Dillin, A. The role of protein clearance mechanisms in organismal ageing and age-related diseases. Nat. Commun. 5, 5659 (2014).
4. Morimoto, R. I. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. Genes Dev. 22, 1427–1438 (2008).
5. Fagarasanu, A., Mast, F. D., Knoblach, B. & Rachubinski, R. A. Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast. Nat. Rev. Mol. Cell Biol. 11, 644–54 (2010).
6. Verzijlbergen, K. F. *et al*. Recombination-induced tag exchange to track old and new proteins. PNAS 107, 64–68 (2010).
7. Terweij, M. *et al*. Recombination-induced tag exchange (RITE) cassette series to monitor protein dynamics in *Saccharomyces cerevisiae*. G3 3, 1261–72 (2013).
8. Menendez-Benito, V. *et al*. Spatiotemporal analysis of organelle and macromolecular complex inheritance. PNAS 110, 175–180 (2012).
9. Li, R. The art of choreographing asymmetric cell division. Dev. Cell 25, 439–50 (2013).
10. Macara, I. G. & Mili, S. Polarity and differential inheritance-universal attributes of life? Cell 135, 801–12 (2008).
11. Glickman, M. H. & Ciechanover, A. The Ubiquitin-Proteasome Proteolytic Pathway : Destruction for the Sake of Construction. Physiol. Rev. 82, 373–428 (2002).
12. Carrard, G., Bulteau, A.-L., Petropoulos, I. & Friguet, B. Impairment of proteasome structure and function in aging. Int. J. Biochem. Cell Biol. 34, 1461–1474 (2002).
13. Dasuri, K. *et al*. Aging and dietary restriction alter proteasome biogenesis and composition in the brain and liver. Mech. Ageing Dev. 130, 777–83 (2009).
14. Vernace, V. A., Arnaud, L., Schmidt-glenewinkel, T. & Figueiredo-, M. E. Aging perturbs 26S proteasome assembly in Drosophila melanogaster. Faseb J 21, 2672–2682 (2012).
15. Kruegel, U. *et al*. Elevated proteasome capacity extends replicative lifespan in *Saccharomyces cerevisiae*. PLoS Genet. 7, e1002253 (2011).
16. Chen, Q., Thorpe, J., Dohmen, J. R., Li, F. & Keller, J. N. Ump1 extends yeast lifespan and enhances viability during oxidative stress: central role for the proteasome? Free Radic. Biol. Med. 40, 120–6 (2006).
17. Van Deventer, S. J., Menendez-Benito, V., van Leeuwen, F. & Neefjes, J. N-Terminal Acetylation And Replicative Age Affect Proteasome Localization And Cell Fitness During Aging. J. Cell Sci. (2014).
18. Tanaka, K. Half-Life of Proteasomes ( Multiprotease Complexes ) in Rat Liver '. Biochem. Biophys. Res. Commun. 159, 1309–1315 (1989).
19. Hendil, K. B. The 19 S multicatalytic "prosome" proteinase is a constitutive enzyme in HeLa cells. Biochem. Int. 17, 471–478 (1988).
20. Cuervo, M., Palmer, A., Rivett, J. & Knecht, E. Degradation of proteasomes by lysosomes in rat liver. Eur. J. Biochem. 227, 792–800 (1995).
21. Kysela, D. T., Brown, P. J. B., Casey Huang, K. & Brun, Y. V. Biological Consequences and Advantages of Asymmetric Bacterial Growth. Annu. Rev. Microbiol. 67, 417–435 (2013).
22. Kaeberlein, M. Lessons on longevity from budding yeast. Nature 464, 513–9 (2010).
23. Van den Bogaart, G., Meinema, A. C., Krasnikov, V., Veenhoff, L. M. & Poolman, B. Nuclear transport factor directs localization of protein synthesis during mitosis. Nat. Cell Biol. 11, 350–6 (2009).
24. Henderson, K. A., Hughes, A. L. & Gottschling, D. E. Mother-daughter asymmetry of pH underlies

aging and rejuvenation in yeast. Elife e03504 (2014).
25. McFaline-Figueroa, J. R. *et al*. Mitochondrial quality control during inheritance is associated with lifespan and mother-daughter age asymmetry in budding yeast. Aging Cell 10, 885–95 (2011).
26. Higuchi, R. *et al*. Actin dynamics affect mitochondrial quality control and aging in budding yeast. Curr. Biol. 23, 2417–22 (2013).
27. Yadlapalli, S. & Yamashita, Y. M. DNA asymmetry in stem cells - immortal or mortal? J. Cell Sci. 126, 4069–76 (2013).
28. Park, B. P. U., Mcvey, M. & Guarente, L. Separation of Mother and Daughter Cells. Methods Enzymol. 351, 468–477 (2002).
29. Pringle, B. J. R. S t a i n i n g o f B u d S c a r s a n d O t h e r Cell Wall C h i t i n with Calcofluor. Methods Enzymol. 194, 732–735 (1991).
30. Jongsma, M. L. M., Berlin, I. & Neefjes, J. On the move : organelle dynamics during mitosis. Trends Cell Biol. 1–13 (2014).
31. Pereira, G., Tanaka, T. U., Nasmyth, K. & Schiebel, E. Modes of spindle pole body inheritance and segregation of the Bfa1p-Bub2p checkpoint protein complex. EMBO J. 20, 6359–6370 (2001).
32. Cerveny, K. L., Tamura, Y., Zhang, Z., Jensen, R. E. & Sesaki, H. Regulation of mitochondrial fusion and division. Trends Cell Biol. 17, 563–9 (2007).
33. Tomko, R. J. & Hochstrasser, M. Molecular architecture and assembly of the eukaryotic proteasome. Annu. Rev. Biochem. 82, (2013).
34. Woolford, J. L. & Baserga, S. J. Ribosome biogenesis in the yeast *Saccharomyces cerevisiae*. Genetics 195, 643–81 (2013).
35. Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P. & Tabak, H. F. Contribution of the endoplasmic reticulum to peroxisome formation. Cell 122, 85–95 (2005).
36. Van der Zand, A., Gent, J., Braakman, I. & Tabak, H. F. Biochemically distinct vesicles from the endoplasmic reticulum fuse to form peroxisomes. Cell 149, 397–409 (2012).
37. Motley, A. M. & Hettema, E. H. Yeast peroxisomes multiply by growth and division. J. Cell Biol. 178, 399–410 (2007).
38. Keppler, A., Pick, H., Arrivoli, C., Vogel, H. & Johnsson, K. Labeling of fusion proteins with synthetic fluorophores in live cells. PNAS 101, 9955–9 (2004).
39. Gautier, A. *et al*. An engineered protein tag for multiprotein labeling in living cells. Chem. Biol. 15, 128–36 (2008).
40. Los, G. V *et al*. HaloTag: A novel protein labeling technology for cell imaging and protein analysis. ACS Chem. Biol. 3, 373–382 (2008).
41. Gaietta, G. *et al*. Multicolor and electron microscopic imaging of connexin trafficking. Science 296, 503–7 (2002).
42. Mann, M. Functional and quantitative proteomics using SILAC. Nature 7, 952–958 (2006).
43. Thayer, N. H. *et al*. Identification of long-lived proteins retained in cells undergoing repeated asymmetric divisions. PNAS 111, 14019–26 (2014).
44. Toyama, B. H. *et al*. Identification of long-lived proteins reveals exceptional stability of essential cellular structures. Cell 154, 971–982 (2013).
45. Baird, G. S., Zacharias, D. a & Tsien, R. Y. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. PNAS 97, 11984–9 (2000).
46. Subach, F. V *et al*. report on cellular trafficking. Nat. Chem. Biol. 5, 118–126 (2009).
47. Patterson, G. H. & Lippincott-Schwartz, J. A Photoactivatable GFP for Selective Photolabeling of Proteins and Cells. Science (80-. ). 297, 1873–1878 (2002).
48. Zhou, X. X. & Lin, M. Z. Photoswitchable fluorescent proteins: ten years of colorful chemistry and exciting applications. Curr. Opin. Chem. Biol. 17, 682–90 (2013).
49. Lindquist, S. L. & Kelly, J. W. Chemical and biological approaches for adapting proteostasis to ameliorate protein misfolding and aggregation diseases: progress and prognosis. Cold Spring Harb. Perspect. Biol. 3, 1–34 (2011).
50. Ciechanover, A. Intracellular protein degradation: from a vague idea through the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. Bioorg. Med. Chem. 21, 3400–10 (2013).
51. Cuervo, A. M. & Wong, E. Chaperone-mediated autophagy: roles in disease and aging. Cell Res. 24, 92–104 (2014).
52. Feng, Y., He, D., Yao, Z. & Klionsky, D. J. The machinery of macroautophagy. Cell Res. 24, 24–41 (2014).
53. Luzio, J. P., Parkinson, M. D. J., Gray, S. R. & Bright, N. a. The delivery of endocytosed cargo to lysosomes. Biochem. Soc. Trans. 37, 1019–21 (2009).
54. Hershko, a & Ciechanover, a. The ubiquitin system for protein degradation. Annu. Rev. Biochem. 61, 761–807 (1992).
55. Liu, C.-W. & Jacobson, A. D. Functions of the 19S complex in proteasomal degradation. Trends Biochem. Sci. 38, 103–10 (2013).
56. Reggiori, F. & Klionsky, D. J. Autophagic processes in yeast: mechanism, machinery and regulation. Genetics 194, 341–61 (2013).
57. Kraft, C., Deplazes, A., Sohrmann, M. & Peter, M. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. Nat. Cell Biol. 10, 602–10 (2008).
58. Sahu, R. *et al*. Microautophagy of cytosolic proteins by late endosomes. 20, 131–139 (2012).

59. Sontag, E. M., Vonk, W. I. M. & Frydman, J. Sorting out the trash: the spatial nature of eukaryotic protein quality control. Curr. Opin. Cell Biol. 26, 139–46 (2014).
60. Kaganovich, D., Kopito, R. & Frydman, J. Misfolded proteins partition between two distinct quality control compartments. Nature 454, 1088–95 (2008).
61. Weisberg, S. J. *et al*. Compartmentalization of superoxide dismutase 1 (SOD1G93A) aggregates determines their toxicity. Proc. Natl. Acad. Sci. U. S. A. 109, 15811–6 (2012).
62. Lindner, A. B., Madden, R., Demarez, A., Stewart, E. J. & Taddei, F. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. Proc. Natl. Acad. Sci. U. S. A. 105, 3076–81 (2008).
63. Coelho, M. *et al*. Fission yeast does not age under favorable conditions, but does so after stress. Curr. Biol. 23, 1844–52 (2013).
64. Liu, B. *et al*. The polarisome is required for segregation and retrograde transport of protein aggregates. Cell 140, 257–67 (2010).
65. Ogrodnik, M. *et al*. Dynamic JUNQ inclusion bodies are asymmetrically inherited in mammalian cell lines through the asymmetric partitioning of vimentin. Proc. Natl. Acad. Sci. U. S. A. 111, 8049–54 (2014).
66. Finkel, T., Deng, C.-X. & Mostoslavsky, R. Recent progress in the biology and physiology of sirtuins. Nature 460, 587–591 (2013).
67. Mortimer & Johnston. Life span of individual yeast cells. Nature 183, 1751–1752 (1959).
68. Nyström, T. & Liu, B. The mystery of aging and rejuvenation - a budding topic. Curr. Opin. Microbiol. 18, 61–7 (2014).
69. Aguilaniu, H., Gustafsson, L., Rigoulet, M. & Nyström, T. Asymmetric inheritance of oxidatively damaged proteins during cytokinesis. Science 299, 1751–3 (2003).
70. Tessarz, P., Schwarz, M., Mogk, A. & Bukau, B. The yeast AAA+ chaperone Hsp104 is part of a network that links the actin cytoskeleton with the inheritance of damaged proteins. Mol. Cell. Biol. 29, 3738–45 (2009).
71. Liu, B. *et al*. Segregation of protein aggregates involves actin and the polarity machinery. Cell 147, 959–61 (2011).
72. Erjavec, N. *et al*. Deletion of the mitochondrial Pim1/Lon protease in yeast results in accelerated aging and impairment of the proteasome. Free Radic. Biol. Med. 56, 9–16 (2013).
73. Lai, C., Jaruga, E., Borghouts, C. & Jazwinski, S. M. A Mutation in the ATP2 Gene Abrogates the Age Asymmetry Between Mother and Daughter Cells of the Yeast *Saccharomyces cerevisiae*. Genetics 87, 73–87 (2002).
74. Hughes, A. L. & Gottschling, D. E. An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. Nature 492, 261–5 (2012).
75. Gray, J. V *et al*. Sleeping Beauty : Quiescence in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 68, 188–202 (2004).
76. Sagot, I., Pinson, B., Salin, B. & Daignan-Fornier, B. Actin Bodies in Yeast Quiescent Cells : An Immediately Available Actin Reserve ? Mol. Cell. Biol. 17, 4645–4655 (2006).
77. Narayanaswamy, R., Levy, M., Tsechansky, M. & Stovall, G. M. Widespread reorganization of metabolic enzymes into reversible assemblies upon nutrient starvation. PNAS 106, 10147–10152 (2009).
78. Laporte, D., Salin, B., Daignan-Fornier, B. & Sagot, I. Reversible cytoplasmic localization of the proteasome in quiescent yeast cells. J. Cell Biol. 181, 737–45 (2008).
79. Delaney, J. R. *et al*. Dietary restriction and mitochondrial function link replicative and chronological aging in *Saccharomyces cerevisiae*. Exp. Gerontol. 48, 1006–1013 (2014).
80. Kennedy, B. K. Daughter cells of *Saccharomyces cerevisiae* from old mothers display a reduced life span. J. Cell Biol. 127, 1985–1993 (1994).
81. Murakami, C. *et al*. pH neutralization protects against reduction in replicative lifespan following chronological aging in yeast. Cell Cycle 11, 3087–3096 (2012).
82. Allen, C. *et al*. Isolation of quiescent and nonquiescent cells from yeast stationary-phase cultures. J. Cell Biol. 174, 89–100 (2006).
83. Aragon, A. D. *et al*. Characterization of differentiated quiescent and nonquiescent cells in yeast stationary-phase cultures. Mol. Biol. Cell 19, 1271–80 (2008).
84. Davidson, G. S. *et al*. The proteomics of quiescent and nonquiescent cell differentiation in yeast stationary-phase cultures. Mol. Biol. Cell 22, 988–98 (2011).
85. Kruegel, U. *et al*. Elevated proteasome capacity extends replicative lifespan in *Saccharomyces cerevisiae*. PLoS Genet. 7, e1002253 (2011)
86. Schmidt, M. & Finley, D. Regulation of proteasome activity in health and disease. Biochim. Biophys. Acta 1843, 13–25 (2014).
87. Lee, C., Klopp, R. G., Weindruch, R. & Prolla, T. A. Gene Expression Profile of Aging and Its Retardation by Caloric Restriction. Science (80-. ). 285, 1390–1393 (1994).
88. Chondrogianni, N., Petropoulos, I., Franceschi, C., Friguet, B. & Gonos, E. . Fibroblast cultures from healthy centenarians have an active proteasome. Exp. Gerontol. 35, 721–728 (2000).
89. Bulteau, a.-L., Petropoulos, I. & Friguet, B. Age-related alterations of proteasome structure and function in aging epidermis. Exp. Gerontol. 35, 767–777 (2000).
90. Russell, S. J., Steger, K. A. & Johnston, S. A.

Subcellular Localization, Stoichiometry, and Protein Levels of 26 S Proteasome Subunits in Yeast. J. Biol. Chem. 274, 21943–21952 (1999).
91. Bajorek, M., Finley, D. & Glickman, M. H. Proteasome Disassembly and Downregulation Is Correlated with Viability during Stationary Phase. Curr. Biol. 13, 1140–1144 (2003).
92. Glockzin, S. Activation of the Cell Death Program by Nitric Oxide Involves Inhibition of the Proteasome. J. Biol. Chem. 274, 19581–19586 (1999).