MINIREVIEW

Linking cellular proteostasis to yeast longevity

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One sentence summary: This mini-review overviews the main mechanisms contributing for the maintenance of *Saccharomyces cerevisiae* proteostasis network. Particularly, the age-dependent changes and the multidimensional nature of the proteostasis network are highlighted.

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ABSTRACT

Proteostasis is a cellular housekeeping process that refers to the healthy maintenance of the cellular proteome that governs the fate of proteins from synthesis to degradation. Perturbations of proteostasis might result in protein dysfunction with consequent deleterious effects that can culminate in cell death. To deal with the loss of proteostasis, cells are supplied with a highly sophisticated and interconnected network that integrates as major players the molecular chaperones and the protein degradation pathways. It is well recognized that the ability of cells to maintain proteostasis declines during ageing, although the precise mechanisms are still elusive. Indeed, genetic or pharmacological enhancement of the proteostasis network has been shown to extend lifespan in a variety of ageing models. Therefore, an improved understanding of the interventions/mechanisms that contribute to cellular protein quality control will have a huge impact on the ageing field. This mini-review centers on the current knowledge about the major pathways that contribute for the maintenance of *Saccharomyces cerevisiae* proteostasis, with particular emphasis on the developments that highlight the multidimensional nature of the proteostasis network in the maintenance of proteostasis, as well as the age-dependent changes on this network.

Keywords: proteostasis; ageing; *Saccharomyces cerevisiae*; molecular chaperones; ubiquitin-proteasome system; autophagy

INTRODUCTION

Protein homeostasis or 'Proteostasis' refers to the healthy maintenance of the cellular proteome and involves highly complex and interconnected pathways that govern the fate of proteins from synthesis to degradation. Proteome fidelity is preserved through a multi-compartmental system that has the ability to coordinate protein synthesis, processing, trafficking, folding, localization, assembly/disassembly and degradation.

Proteins are permanently challenged by intrinsic (such as mutations and translational errors) and extrinsic/environmental stressors that can impair proteostasis over time. The first line of defence against loss of proteostasis relies mainly on molecular chaperones and degradation pathways that attempt to solubilize, refold or degrade accumulated misfolded proteins. Nevertheless, age compromises the protein quality control components and promotes a general deterioration of proteostasis (Morimoto 2008; Douglas and Dillin 2010). Thus, aged cells show an enhanced incapability to maintain metastable proteins in properly folded states in the face of deteriorating proteostasis networks, initiating a cascade of protein destabilization and accumulation of misfolded protein, prone to form potentially toxic aggregates (Gidalevitz *et al.* 2006; Ben-Zvi, Miller and Morimoto 2009). The yeast *Saccharomyces cerevisiae* is a well-recognized ageing model that provides two distinct paradigms for ageing studies, the replicative lifespan (RLS), which represents the number of divisions that each individual cell can undergo, and the chronological lifespan
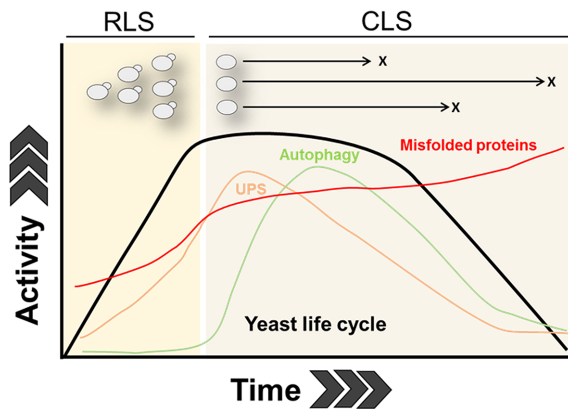
Figure 1. Temporal activity of the degradation pathways during yeast life cycle. The yeast *Saccharomyces cerevisiae* provides two distinct paradigms for ageing, the RLS and the CLS. These two distinct ageing stages are related with pronounced changes in the degradation pathways, particularly the UPS (orange line) and autophagy (green line), and the onset of protein aggregation (red line). See text for more details.

(CLS), which determines the time that cells can remain viable in a non-dividing state (Fig. 1), reviewed in (Longo *et al.* 2012; Sampaio-Marques, Burhans and Ludovico 2014). Herein, we present a mini-review on the molecular mechanisms contributing to maintenance of *S. cerevisiae* proteostasis network and how these processes are affected by age and contribute to the global breakdown of proteostasis during ageing.

THE YEAST PROTEOSTASIS NETWORK

Cells take advantage of three main processes to face proteotoxic stress: refolding, degradation or sequestration into spatially organized protective inclusions (Tyedmers, Mogk and Bukau 2010; Sontag, Samant and Frydman 2017). A brief overview of these three main processes operating in yeast as well as of the specific protein quality control of different organelles is presented.

Molecular chaperones

Molecular chaperones are crucial components of proteostasis network having multiple functions in different branches of the system. Molecular chaperones promote folding of newly synthesized proteins, refold of misfolded proteins and help in the degradation of aberrant proteins as well as on the formation of protective inclusions of misfolded and aggregated proteins (Fig. 2). Together with degradation pathways, molecular chaperones are the first line of defence to solubilize, refold or degrade misfolded proteins.

Many molecular chaperones are constitutively expressed, whereas others can be predominantly induced under different conditions of stress. Inducible molecular chaperones have originally been described in response to heat shock stress and are thus called heat shock proteins (HSPs), the name still given to different classes of molecular chaperones. Yet, it is currently known that molecular chaperones can also be induced as a consequence of other stressors (pH, osmotic conditions, energy depletion, ion concentration, etc.). Transcriptional regulation of molecular chaperones in yeast is accomplished by three transcription factors, Hsf1 (heat shock factor 1), Msn2 and the partially redundant Msn4 (Trott and Morano 2003).

Molecular chaperones generally recognize exposed hydrophobic regions, normally hidden in a properly folded protein, and prevent aggregation with other denatured proteins, but the mode of action varies between the different classes. Molecular chaperones also control denatured proteins by assisting on the delivering of unrepairable unfolded proteins to the proteosome for degradation (Fig. 2). The chaperone repertoire in the yeast *S. cerevisiae* proteome consists of 69 molecular chaperones and their co-chaperones that commonly assist chaperones on folding or unfolding of proteins (Voos and Rottgers 2002; Young *et al.* 2004; Kampinga and Craig 2010). They are classified into different families based on the molecular weight of monomers (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and small Hsp) (Diaz-Villanueva, Diaz-Molina and Garcia-Gonzalez 2015).

Based on the sort of interaction with client proteins, chaperones are also classified as holdases, foldases and disaggregases (Diaz-Villanueva, Diaz-Molina and Garcia-Gonzalez 2015). Some chaperones, as Hsp90 and Hsp104, play relevant functions during ageing (Leak 2014). *S. cerevisiae* genome encodes for two Hsp90 homologs, Hsp82 and Hsc82, which shares almost 90% sequence identity (Borkovich *et al.* 1989). While *HSC82* is constitutively expressed, expression of *HSP82* is usually induced under stress conditions (Borkovich *et al.* 1989). The two Hsp90 isoforms have also been shown to have distinct and opposite roles on the control of selective translation during stress and cell death (Silva *et al.* 2013). The Hsp90 chaperone activity is particularly relevant, since it acts on nascent, partially folded proteins (Li, Soroka and Buchner 2012). Nevertheless, it is not able to refold denatured proteins, instead it needs the participation of other chaperones to complete the task, particularly Hsp40, which binds to unfolded proteins, and Hsp70 that under stress conditions solubilizes protein aggregates and refolds aggregated proteins (Ran, Bali and Michels 2008; Kampinga and Craig 2010; Sakurai and Ota 2011). Importantly, *HSP90* genes are under control of Hsf1, and the essential roles of this transcriptional factor in response to various conditions including proteotoxic stress and ageing are partially mediated by Hsp90 together with five additional mitochondrial HSP genes (Sakurai and Ota 2011). Several observations have implicated Hsf1 in the regulation of longevity in yeast (Harris *et al.* 2001; Cheng *et al.* 2007) as well as in other ageing models as *Caenorhabditis elegans* (Morley and Morimoto 2004). Besides Hsp90, the yeast molecular chaperone Hsp104, a member of the Hsp100 family, is also considered a chaperone with a role in the modulation of chronological ageing (Speldewinde and Grant 2017). *HSP104* expression is induced by a variety of environmental stresses and when cells enter stationary phase or sporulate (Sanchez and Lindquist 1990; Sanchez *et al.* 1992). In contrast to Hsp90, Hsp104 cannot prevent aggregation of denatured proteins but acts by re-solubilizing aggregated proteins in cooperation with the Hsp70 family of chaperones and co-chaperones (Parsell *et al.* 1994; Glover and Lindquist 1998). Hsp104 can also interact with Hsp26, along with other small HSPs, such as Hsp42, which assist in the refolding of the aggregation-prone proteins (Cashikar, Duennwald and Lindquist 2005). The complex constituted by Hsp104 and Hsp70 was recently found to be recruited by the peroxiredoxin Tsa1 and to mediate the disaggregation of the H_2O_2-generated aggregates that are formed during ageing, leading to ageing delay (Hanzen *et al.* 2016). Furthermore, Hsp104 seems to have a role on the asymmetrically inheritance of aggregates by mother cells during division and thus, its deletion results in a shortened RLS (Erjavec *et al.* 2007). In the yeast models of Huntington's disease, in contrast to the transgenic mice model, Hsp104 has a paradoxal function, promoting the aggregation
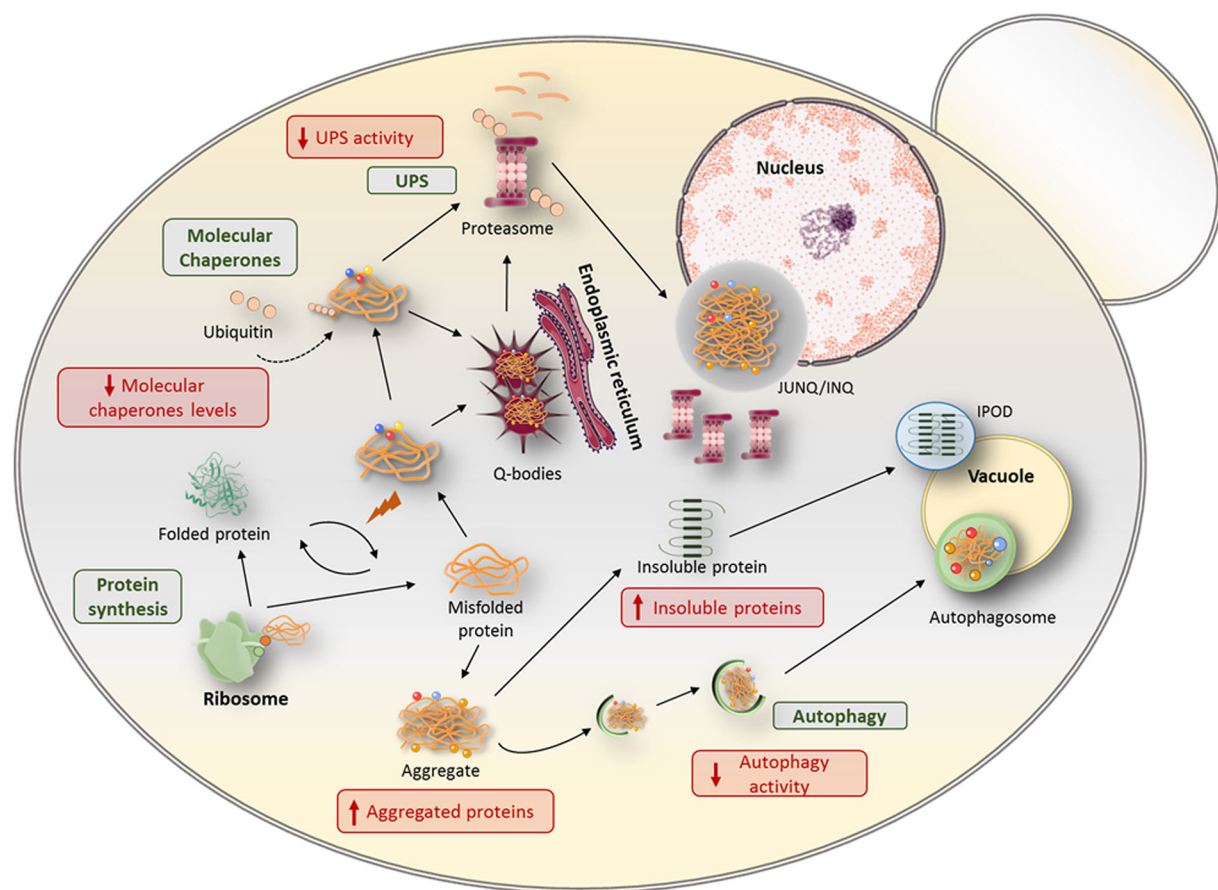
Figure 2. Impact of ageing in the yeast proteostasis network. Proteostasis is maintained essentially by the action of molecular chaperones and the two degradation pathways, the UPS and autophagy. Molecular chaperones assist de novo protein synthesis and promote protein refolding or prevent aggregation of misfolded or damaged proteins. In the presence of misfolded proteins, molecular chaperones associate with them to promote their refolding. When protein refolding is not possible, molecular chaperones target the unfolded protein for degradation by the proteasome, after ubiquitination, or by vacuoles. Once misfolded proteins organize into insoluble aggregates, they are target to be degraded by autophagy. Misfolded and aggregated proteins can also be sequestered into larger spatially organized protective inclusions: the JUNQ/the INQ and the peripheral vacuole-associated, IPOD. Red boxes indicate the changes in the proteostasis network machinery promoted by ageing. See text for more details.

and toxicity of polyglutamine (polyQ) (Krobitsch and Lindquist 2000).

In summary, the observations described above support the important functions of molecular chaperones on the maintenance of protein folding and cellular homeostasis required for longevity.

Degradation pathways

Molecular chaperones are crucial players to refold and/or to redirect non-native proteins toward degradation pathways, the ubiquitin-proteasome system (UPS) and autophagy. Protein degradation is a ubiquitous process critical for cell repair and survival, regulating several processes such as cell cycle, signaling, DNA transcription, repair and translation, among others. Protein degradation is particularly relevant during ageing, a scenario of cellular waste accumulation, where degradation pathways have to assist on the cleaning of ageing-accelerating factors.

The UPS mainly serves to target individual proteins to the proteasome, while autophagy clears larger aggregates or membrane-associated proteins (Menzies, Fleming and Rubinsztein 2015; Kwon and Ciechanover 2017). Both degra-

dation pathways allow the recycling of macromolecules and energy, being central in the regulation and maintenance of cellular homeostasis.

The ubiquitin-proteasome system

The primary route for the degradation of numerous short-lived proteins is the ubiquitination-dependent degradation by the proteasomal machinery, UPS, which consists of both substrate-recruiting and substrate-degrading machinery (Low 2011). Proteasome is organized into the 19S regulatory subunit, which recognizes substrates to be degraded, and the 20S core subunit that contains the proteolytic active sites (Groll *et al.* 1997). The mechanism of degradation assured by the proteasome is linked to the ubiquitin system, which consists of a cascade of enzymes that targets the proteins to be degraded with ubiquitin (Finley *et al.* 2012) (Fig. 2). Covalent attachment of ubiquitin to a substrate is catalyzed by the E1-E2-E3 enzyme cascade, reviewed in Finley *et al.* (2012). The reaction is initiated by the ubiquitin-activating enzyme E1 (Uba1) and activated ubiquitin is transferred to one of the ubiquitin-conjugating enzymes (E2 or Ubc enzymes). Finally, E3 enzymes (ubiquitin ligases) catalyze the formation of isopeptide bonds between lysine residues in substrate proteins and the activated carboxyl group of ubiquitin. The conjugation

machinery shows hierarchical organization and in yeast is composed by one E1, 11 E2s and 60–100 E3s enzymes reviewed in Finley *et al.* (2012).

Multiple repetitions of the ubiquitin-conjugation process results in the polyubiquitination of the targeted substrate (Finley *et al.* 2012). The polyubiquitin-tagged substrates are recognized by the 19S proteasome subunit that removes the molecules of ubiquitin (Ub) and partially unfolds the substrates, allowing the substrates entrance and proteolysis within the 20S core (Tanaka 2009) (Fig. 2). Alongside this process, Ub can also be removed by deubiquitinating enzymes (DUBs), which do not integrate the 19S proteasome. Besides recycling Ub, these DUBs also function as a regulatory mechanism where proteins can be rescued from degradation and reactivated (Finkel, Deng and Mostoslavsky 2009; Oling *et al.* 2014). Several reports on different human tissues, senescent primary cultures and whole organisms suggest that the maintenance of proteasome activity is directly correlated with longevity, reviewed in Saez and Vilchez (2014). In *S. cerevisae*, an enhanced preservation of proteasome-mediated protein degradation promoted by the overexpression of Ump1, a protein needed for proteasome assembly, increased viability during chronological ageing (Chen *et al.* 2006). In another study, proteasome activity was enhanced by increasing the levels of the transcription factor Rpn4, which regulates the levels of proteasome subunits (Dohmen, Willers and Marques 2007). This modulation resulted in increased RLS and resistance to proteotoxic stress (Kruegel *et al.* 2011). Calorie restriction, an intervention known to extend the maximum lifespan of a variety of species including *S. cerevisiae* (Lee and Longo 2016), was found associated with increased proteasome activity and maintenance of UPS activity (da Cunha, Demasi and Kowaltowski 2011). Accumulating evidence indicates that the UPS, particularly the proteasome, is a key component of the ageing process.

Autophagy

Autophagy refers to the vacuolar/lysosomal degradation of intracellular macromolecular components. Autophagy was initially viewed as a non-selective bulk process induced by starvation. However, this process is now recognized as a central regulator of cellular homeostasis with intricate links to cell metabolism and growth control. Autophagy is a determinant factor on cell survival and death decisions with a major impact on ageing (Reggiori and Klionsky 2013).

In yeast, autophagy can be divided into two main types, microautophagy and macroautophagy, hereafter named autophagy, and both of them include nonselective and selective processes (Shintani and Klionsky 2004). In microautophagy, the cargo is sequestered directly by the vacuole membrane, and the following invagination leads to its delivery into the vacuole lumen. In contrast, during autophagy the cargo is sequestered within cytosolic double-membrane vesicles that subsequently fuse with the vacuole, allowing breakdown of the cargo and recycling of the resulting macromolecules (Eskelinen *et al.* 2011). The budding yeast *S. cerevisiae* has also another type of selective autophagy—the cytoplasm to vacuole targeting (Cvt) pathway—that operates in nutrient-rich conditions. Cvt pathway is usually responsible for constitutive and selective transport of vacuolar hydrolases such as α-mannosidase (Ams1) and aminopeptidase I (Ape1) to the vacuoles using many of the same molecular components as autophagy. Therefore, this pathway is considered a biosynthetic rather than a degradative pathway (Scott *et al.* 1997; Lynch-Day and Klionsky 2010).

A hallmark of autophagy is the formation of an autophagosome, a double-membrane vesicle that sequesters cytosol and organelles, that fuses with the vacuole releasing the content to be degraded. This process is supported by two main groups of genes, autophagy-related genes (ATG) and vacuolar protein sorting genes. Initiation of autophagy is tightly regulated by Atg1 complex that responds to nutrient starvation by recruiting multiple Atg proteins to the pre-autophagosomal structure (Reggiori and Klionsky 2013; Tyler and Johnson 2018). After initiation, autophagy proceeds with vesicle nucleation, expansion and completion ending in its fusion with the vacuole (Fig. 2).

Selective autophagy encompasses the degradation of specific cargos such as peroxisomes by pexophagy, mitochondria by mitophagy or endoplasmic reticulum (ER) by reticulophagy, reviewed in (Galluzzi *et al.* 2017). The selective degradation of yeast peroxisomes (micropexophagy) or even nuclear regions (piecemeal microautophagy) can be also performed by microautophagy (Klionsky *et al.* 2007). Overall, selective autophagy, including the Cvt pathway, is largely similar to non-selective autophagy but relies on cargo-specific receptors that interact with Atg8.

Mounting evidence indicates that functional autophagy is essential for longevity in several scenarios, reviewed in Sampaio-Marques, Burhans and Ludovico (2014), Madeo *et al.* (2015), and Tyler and Johnson (2018). Importantly, lifespan and autophagy share as common regulators the major nutrient-sensing pathways. In fact, many of the interventions that extend lifespan of diverse organisms decrease activity of nutrient-signaling pathways. These pathways, thus, well-recognized as 'longevity pathways', are important regulators of autophagy that in turn are essential for the maintenance of the proteome and a healthy cellular ageing (Sampaio-Marques, Burhans and Ludovico 2014). This has recently been nicely reviewed by Tyler and Johnson (2018).

Besides the genetic abrogation (Powers *et al.* 2006) and the inhibition by rapamycin (Alvers *et al.* 2009) of the TOR signaling, other interventions known to extend yeast lifespan, such as caloric restriction or treatment with spermidine or resveratrol, are dependent on autophagy induction (Eisenberg *et al.* 2009; Morselli *et al.* 2011). Autophagy also plays a role in the extension of CLS by methionine restriction, a dietary intervention that has been demonstrated to extend the lifespan of a variety of multicellular model organisms, including yeast (Johnson and Johnson 2014; Ruckenstuhl *et al.* 2014). Consistently, a large-scale screen for genes that regulate longevity in *S. cerevisiae* identified several autophagy gene mutants with reduced lifespan (Matecic *et al.* 2010). Epigenetic regulation of yeast lifespan has also been described as dependent on autophagy. In this context, we have shown that Sir2 is an important regulator of autophagy during ageing of alpha-synuclein expressing cells (Sampaio-Marques *et al.* 2012; Guedes, Ludovico and Sampaio-Marques 2017). Studies by Eisenberg *et al.* (2014) showed that accumulation of acetyl-coenzyme A downregulates the transcription of different *ATG* genes during ageing due to histone hyperacetylation.

The contribution of autophagy to lifespan extension is associated with maintenance and/or improvement of several cellular processes. Autophagy is involved in the turnover and clearance of organelles and aberrant damaged or toxic macromolecules; enhances the resistance to oxidative stress, through regulation of ROS production/accumulation; modulates the DNA damage response by the regulation of several processes including cell cycle checkpoints and DNA repair, among others, reviewed in Tyler and Johnson (2018). This brief review demonstrates that autophagy has a deep impact on ageing modulation and longevity extension.

Spatial protein quality control

As described above, cells have evolved an elaborated proteostasis network to preserve the functionality of their proteomes. Nevertheless, while the chaperones and degradation systems are the first line of defence for repair or elimination of misfolded proteins providing a temporal quality control, cells also possess a spatial protein quality control system. Accumulating evidence suggests that damaged proteins are not randomly distributed inside the cells—instead, misfolded and aggregated proteins are sequestered into larger protective inclusions that are spatially organized (Alberti 2012). This spatial protein quality control limits the toxicity of damaged and misfolded/aggregated proteins and guarantees that the damage is not inherited by succeeding cell generations (Hill, Hanzen and Nystrom 2017).

An early cellular event of spatial protein quality control, occurring immediately upon misfolding, seems to be the active and chaperone dependent (such as sHsps, Hsp40, Hsp70 and Hsp104; Kaganovich et al. 2008; Specht et al. 2011; Escusa-Toret, Vonk and Frydman 2013) transport of damaged proteins to dynamic compartments called Q-bodies, which are attached to the ER en route to clearance by the UPS (Escusa-Toret, Vonk and Frydman 2013). The damaged proteins inside of these Q-bodies are rapidly cleared through the UPS, but if clearance is impaired, these misfolded proteins concentrate in a protein quality control compartment (Kaganovich et al. 2008; Escusa-Toret, Vonk and Frydman 2013) (Fig. 2). Two major protein quality control compartments sequester misfolded cytosolic proteins, the juxta nuclear quality control site (JUNQ) and the peripheral vacuole-associated, insoluble protein deposit (IPOD) (Kaganovich et al. 2008; Tyedmers et al. 2010; Chen et al. 2011). A ubiquitin-based sorting mechanism targets misfolded proteins to JUNQ, while non-ubiquitinated proteins are sent to IPOD (Kaganovich et al. 2008). However, the disaggregase Hsp104-Hsp70 complex is also associated with this JUNQ (Specht et al, 2011), raising the hypothesis that sequestration of misfolded protein precedes the determination of their proteasomal degradation or chaperone-assisted refolding. Damaged proteins can travel in and out of the JUNQ; thus, the JUNQ is considered a highly dynamic deposit, with rapid protein turnover due to the high concentration of proteasomes (Kaganovich et al. 2008). The IPOD is in contrast less dynamic, and is typically formed upon prolonged stress, and remains longer after stress relief. More recently, JUNQ was found inside the nucleus defining a new intranuclear quality control compartment (INQ) for the deposition of both nuclear and cytosolic misfolded proteins, irrespective of ubiquitination (Miller et al. 2015) (Fig. 2). It is now well established that chaperones and co-chaperones assist the sorting of proteins to quality control compartments. Hsp42 is required for sorting proteins into peripheral inclusions and IPOD, while the co-chaperone Sis1 delivers misfolded proteins to the nucleus for degradation (Specht et al. 2011; Park et al. 2013). The v-SNARE binding protein Btn2 and its paralog Cur1 are crucial to regulate protein sorting to inclusions (Malinovska et al. 2012; Miller et al. 2015). Btn2 can either directly interact with Hsp42 directing the substrates to IPOD or bind to Sis1 promoting the sorting to JUNQ (Malinovska et al. 2012). In these events, Btn2 and Hsp42 work as aggregases, assisting inclusion formation of misfolded substrates in the nucleus and cytoplasm, respectively. Cur1 in turn regulates Sis1 availability in the cytosol. It binds and recruits Sis1 to the nucleus without substrates, allowing the interaction between the free substrates and Btn2/Hsp42 in cytosol (Alberti 2012; Nystrom and Liu 2014).

The molecular principles governing the sorting and distribution of misfolded, damaged and aggregation prone-proteins are not fully resolved. Yet, it is evident that the cell uses specific factors in a dynamic fashion to be as efficient as possible on the management of misfolded proteins and in maintaining cellular homeostasis.

Organelle protein quality control

A communication between the cytoplasm proteostasis network and the specific protein quality control of different organelles seems to be relevant for the maintenance of proteostasis. Nevertheless, little is known about the factors involved in this communication as well as about the hierarchical order whereby preservation of proteostasis in some compartments is prioritized.

The specific protein quality control system of ER and mitochondria are among the most well studied and explored. The ER is an organelle hosting protein synthesis and thus needs a tightly protein-quality surveillance (Walter and Ron 2011). Newly synthesized secretory and membrane proteins that do not pass ER quality control are usually retained by ER resident chaperones and routed to ER-associated degradation (ERAD) pathways (Hegde and Ploegh 2010). However, when nascent unfolded and misfolded proteins increase and overcome ER processing capacity, a process known as the unfolding protein response (UPR), a signaling pathway that transcriptionally up-regulates genes involved in expanding the ER protein folding capacity, is activated. In yeast, UPR signaling pathway is triggered by the ER-located transmembrane protein Ire1, which promotes splicing of the HAC1 premRNA (HAC1u) to produce the translatable transcription factor mRNA (HAC1i) (Kimata et al. 2006). This transcriptional factor induces the up-regulation of the expression of several genes involved in protein processing and maturation in an attempt to maintain homeostasis. Yet, if the amount of ER unfolded proteins exceeds the ability of the UPR to restore homeostasis, unfolded proteins will be exported to the cytosol (Brodsky 2012). A novel mechanism to eliminate misfolded glycosylphosphatidylinositol (GPI)-anchored proteins via the secretory pathway, termed 'rapid ER stress-induced export' (RESET) has been described (Satpute-Krishnan et al. 2014). RESET involves the export of misfolded GPI proteins to the plasma membrane for subsequent degradation by the lysosome. In yeast, this secretory pathway is dependent on two GPI-linked aspartyl proteases (Yps1/2) and components of the UPR (Ire1 and Hac1) but is independent of ERAD elements (Miller, DiDone and Krysan 2010).

The maintenance of mitochondrial proteostasis is particularly challenging due to the oxidative environment of this organelle. The first line defence against loss of mitochondria proteostasis involves molecular chaperones, which have been conserved from bacteria to higher eukaryotic cells, as well as various proteolytic enzymes, reviewed in Baker, Tatsuta and Langer (2011). The vast majority of the mitochondrial proteome is synthesized in the cytosol and imported, in a relatively unfolded state, with guidance by members of the Hsp70 and Hsp90 family, into the organelle. Once inside mitochondria, additional chaperones aid the correct folding of imported proteins. There are two major matrix mitochondrial chaperone systems—mtHsp70, part of pre-sequence translocase-associated import-motor (PAM) complex, which directly folds the incoming proteins, and multimeric HSP60–HSP10 chaperonins that also assist on folding of proteins to their native state (reviewed in Chacinska et al. 2009).

Complementary to the chaperones' action to maintain mitochondria proteostasis, specific mitochondrial proteases recognize and degrade proteins that fail to fold or assemble properly (Tatsuta and Langer 2008). The Lon protease family resides in the mitochondrial matrix and is required for the removal of oxidatively damaged proteins (Suzuki *et al.* 1994; Bota and Davies 2002). Importantly, Lon seems to impact fungal ageing given that its overexpression in *Podospora anserina* results in an increased lifespan (Luce and Osiewacz 2009). The mitochondrial inner membrane has different classes of proteases being the two AAA + metalloprotease complexes highly relevant to ensure protein quality control (Koppen and Langer 2007).

The mitochondrial protein quality control is also influenced by the UPS and includes a mitochondria-specific unfolded protein response (UPRmt) (Heo *et al.* 2010; Braun *et al.* 2015). The UPRmt senses the perturbations that overload mitochondria quality control network capacities, and activates the transcription of nuclear-encoded protective genes in order to re-establish mitochondrial homeostasis (Haynes and Ron 2010).

Another level of mitochondrial protein quality control is provided by the dynamic nature of this organelle and the fusion and fission phenomenon. Mitochondrial fusion allows dysfunctional damaged mitochondria to fuse with healthy neighbor mitochondria facilitating quality control and diluting the toxic effects of the accumulation of misfolded/aggregated proteins. In contrast, fission allows for the separation of damaged mitochondria and to promote their turnover through mitophagy (Wang and Klionsky 2011). Mitophagy, which is the selective degradation of mitochondria by autophagy, is indeed the most common route for degradation of dysfunctional mitochondria (Lemasters 2014). Several studies reported that the impairment of mitophagy and consequently mitochondrial clearance, in aged cells, is the basis of several neurodegenerative disorders. Finally, a last level of quality control occurs when extensive mitochondrial damage promotes the release of death factors that result in turnover of the entire cell (Baker, Tatsuta and Langer 2011)

AGE-RELATED EFFECTS ON PROTEOME INTEGRITY AND PROTEOSTASIS NETWORK

Ageing is a complex and multifactorial physiological process that results in the progressive accumulation of molecular alterations associated with the decline of different cellular functions. Loss of proteostasis, promoted among other factors by the accumulation of misfolded and damaged proteins, is proposed to be one of the primary hallmarks of ageing (Lopez-Otin *et al.* 2013) observed in different ageing models (David *et al.* 2010; Reis-Rodrigues *et al.* 2012; Sampaio-Marques, Burhans and Ludovico 2014). Accumulation of these aberrant proteins predisposes to the formation of aggregates that are potentially toxic to the cell. Protein aggregates are associated with several neurodegenerative diseases but their role in physiological ageing remains largely unexplored. Nevertheless, studies in *C. elegans* suggest that the decline in proteome integrity may not be caused by a random and gradual accumulation of molecular damage—instead, it can be the result of early programmed events (Ben-Zvi, Miller and Morimoto 2009). Further contributing to loss of proteostasis during ageing is the decline of the protein quality control machinery (Fig. 1). The functioning of molecular chaperones, the first line of defence of the proteostasis network, is impaired by ageing. Particularly the chaperones with ATP-dependent activity are repressed due to the limited amounts of ATP available in aged cells (Stroo *et al.* 2017). The activity of

molecular chaperones could also be affected by their availability, which does not meet the needs of aged cells, and by the lack of substrate recognition due to undesired ageing protein alterations (Csermely 2001; Nardi *et al.* 2006; Kumar, Kumar and Reddy 2007). Consistently, recent studies in *C. elegans*, demonstrated that during ageing a more prominent function for sHSPs, ATP-independent, is observed in the preservation of proteostasis, instead of the HSP70 and HSP90 families (Walther *et al.* 2015).

The age-associated alterations on proteostasis are not restricted to molecular chaperones—the degradation pathways, UPS and autophagy, are also affected by age. Aged cells display decreased proteasome subunit expression associated with alterations and/or replacement of proteasome subunits culminating in decreased UPS activity throughout lifespan (Carrard *et al.* 2002; Vernace, Schmidt-Glenewinkel and Figueiredo-Pereira 2007). It is also suggested that the factors required for UPS activity, such as Ub ligases/proteases or proteasome activators, might be sequestered in aggregates during ageing (Andersson *et al.* 2013). Consistently, our experimental results showed an accumulation of ubiquitinated proteins associated with an increase in levels of *RPN4* along chronological ageing, reflecting an impairment of proteasome activity (Figs 1 and 3). The relationship between UPS and ageing is nicely shown by the genetic studies performed in *S. cerevisiae* demonstrating that abrogation of *RPN4* proteasome assembly factor promotes a shortening of RLS (Kruegel *et al.* 2011), while overexpression of proteasome-related genes, like the *UMP1,* increases longevity (Chen *et al.* 2006). Caloric restriction has been described as an inducer of UPS activity and enhanced UPS activity is linked to increased lifespan in yeast, worms, rodents and humans (Carrano *et al.* 2009; da Cunha, Demasi and Kowaltowski 2011; Kruegel *et al.* 2011; Vilchez *et al.* 2012). In aged yeast cells, the proteasome activity is decreased and the ubiquitination process is impaired but caloric restriction is able to prevent/delay these effects that occur during ageing (da Cunha, Demasi and Kowaltowski 2011).

As mentioned above, the other main degradation pathway, autophagy, is also affected during ageing. Similar to UPS, decreased autophagy during ageing has been reported in different model systems, reviewed in Rubinsztein, Marino and Kroemer (2011). However, due to the autophagy process complexity and the stochastic nature of ageing, the mechanisms underlying the decreased autophagy remain largely unclear. Nevertheless, down-regulation of the autophagy genes *ATG5* and *ATG7* was reported in human brain during normal ageing (Lipinski *et al.* 2010; Martinez-Lopez, Athonvarangkul and Singh 2015) and decreased autophagic activity have been detected in rodent models (Bergamini *et al.* 1993; Donati *et al.* 2001). The mechanisms responsible for the decrease in *ATG* levels remain to be elucidated. Besides the molecular alterations observed in autophagy, an expansion of lysosomal compartments, accumulation of autophagic vacuoles, as well as the deposition of autofluorescent pigment lipofuscin was observed in aged cells of different tissues (brain, heart, muscle and kidney) (Donati *et al.* 2001; Del Roso *et al.* 2003). These alterations in the lysosome led to a decreased lysosomal activity due to a dysfunctional proteolytic activity, which resulted in the decline of autophagic activity (Donati *et al.* 2001; Del Roso *et al.* 2003). In *S. cerevisiae,* vacuolar homeostasis is also affected during ageing due to increased vacuolar pH that limits the activities of vacuolar proteases, resulting in impaired autophagy (Nakamura *et al.* 1997) (Fig. 1). Since pharmacologic or genetic inhibition of autophagy induces changes that resemble the phenotype associated with ageing (Simonsen *et al.* 2008; Toth *et al.* 2008; Matecic *et al.* 2010), it is assumed that decline of autophagy promoted by ageing will impact on
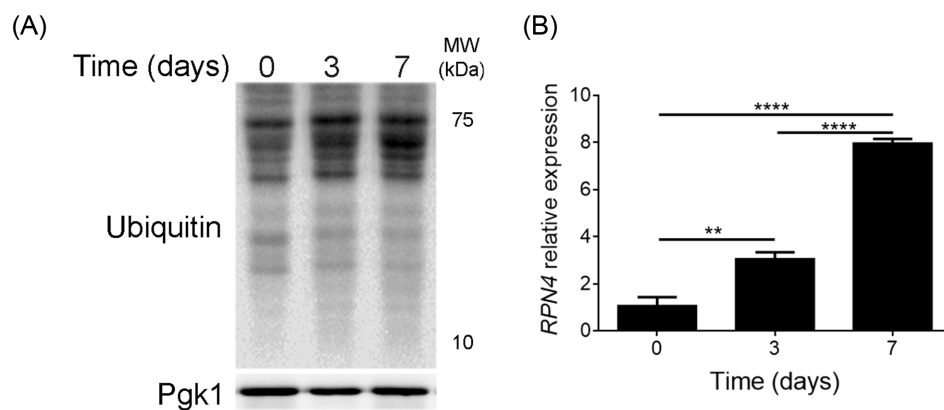
Figure 3. Proteasome activity is decreased during yeast ageing. (**A**) Ubiquitination profile and (**B**) *RPN4* mRNA relative expression levels of wild type BY4742 yeast cells during CLS. Three reference genes (*ACT1*-actin, *PDA1*-alpha subunit of pyruvate dehydrogenase and *TDH2*-isoform 2 of glyceraldehyde-3-phosphate dehydrogenase) were used as internal standards and for the normalization of mRNA expression levels. Data represent mean ± SEM of three biological independent replicas. The error bars represent the standard error of the mean (SEM). Significance of the data was determined by one-way ANOVA (****$P < 0.0001$; **$P < 0.01$).

the normal cell functioning converging in the different aspects of the ageing phenotype. Thus, this age-dependent decline of autophagy may cause the build-up of severely accumulation of aberrant proteins/aggregates, further causing additional molecular and cellular damage with age, as a vicious cycle.

Deregulation of autophagy in old cells can also be a consequence of persistent activity stimulation. Although increased autophagy might initially have a favorable outcome, its maintained activation can lead to the depletion of functional organelles/protein and essential autophagic molecules contributing to cell death and shortening of lifespan (Meijer and Codogno 2007; Sampaio-Marques *et al.* 2012).

The cell's capacity to maintain the spatial protein quality control and to form the above described JUNQ/INQ or IPOD inclusions declines with ageing (Yang *et al.* 2016). Furthermore, the nature of the inclusions formed during ageing is apparently distinct from JUNQ/INQ or IPOD, representing another form of quality control site (Saarikangas and Barral 2015). The cause of the impairment of the spatial protein quality control is not well disclosed, but apparently it could be the result of the collapse of multiple systems that also fails during ageing (Dillin, Gottschling and Nystrom 2014). Particularly, an enhancement of aggregated proteins and the decline of the proteostasis network could underlie the age-associated loss of spatial protein quality control (Shiber *et al.* 2013). In addition, the homeostasis changes that some organelles experience could be also linked. The increased cytosolic acidification, due to the alteration on the vacuolar pH, might impact on the protein stability, ultimately causing the collapse of the spatial protein quality control. The loss of vacuole acidification detected during ageing can lead to the breakdown of the vesicle trafficking and fusion with the vacuole (Baars *et al.* 2007), central processes for spatial sequestration of aggregated proteins (Kumar, Nawroth and Tyedmers 2016). Furthermore, the disruption of a functional actin cytoskeleton that occurs during ageing can also impair the trafficking and fusion of vesicles, promoting the inefficient inclusion formation that is observed in ageing cells (Gourlay *et al.* 2004).

CONCLUSIONS

Mounting evidence suggests that maintenance of proteostasis is fundamental to delay ageing. Loss of proteostasis accelerates ageing—thus it is expected that genetic or pharmacological

enhancement of the proteostasis network might extend lifespan and suppress age-related phenotypes. Indeed, the genetic enhancement of the expression or activity of individual proteostasis components is associated with the suppression or the delays of the ageing and age-associated diseases onset. The pharmacological development and use of small molecules that can enhance the expression of molecules or pathways of proteostasis network might provide effective strategies for promoting proteostasis. One of the previously employed strategies is the activation of Hsf1, which induces the simultaneous expression of multiple molecular chaperones. This approach is usually accomplished through the pharmacologic inhibition of Hsp90 with radicicol, geldanamycin, or 17-AAG (Sittler *et al.* 2001; Hay *et al.* 2004; Waza *et al.* 2005; Fujikake *et al.* 2008). In fact, this strategy was previously shown to assist in the suppression of polyQ aggregation and toxicity several models, including *Drosophila melanogaster*, *C. elegans* and mouse models of disease (Sittler *et al.* 2001; Auluck and Bonini 2002; Fujikake *et al.* 2008; Labbadia *et al.* 2011). Nevertheless, inhibition of Hsp90 is not a viable option due to the central role of Hsp90 in numerous essential cellular processes (Taipale, Jarosz and Lindquist 2010). Therefore, new strategies target to the suppression or the delay of ageing and age-associated diseases are urgently needed. Part of the molecular mechanisms responsible for proteostasis network remodeling and the signals that promote the age-associated changes remain unknown. Therefore, a more complete understanding of the age-dependent repertoire of proteostasis network alterations as well as the signaling pathways responsible for those alterations could have a tremendous impact on our ability to modulate the ageing process and allow the development of new strategies to successfully improve health ageing.

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