Chapter 9
Yeast at the Forefront of Research on Ageing and Age-Related Diseases

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Abstract Ageing is a complex and multifactorial process driven by genetic, environmental and stochastic factors that lead to the progressive decline of biological systems. Mechanisms of ageing have been extensively investigated in various model organisms and systems generating fundamental advances. Notably, studies on yeast ageing models have made numerous and relevant contributions to the progress in the field. Different longevity factors and pathways identified in yeast have then been shown to regulate molecular ageing in invertebrate and mammalian models. Currently the best candidates for anti-ageing drugs such as spermidine and resveratrol or anti-ageing interventions such as caloric restriction were first identified and explored in yeast. Yeasts have also been instrumental as models to study the cellular and molecular effects of proteins associated with age-related diseases such as Parkinson's, Huntington's or Alzheimer's diseases. In this chapter, a review of the advances on ageing and age-related diseases research in yeast models will be made. Particular focus will be placed on key longevity factors, ageing hallmarks and interventions that slow ageing, both yeast-specific and those that seem to be conserved in multicellular organisms. Their impact on the pathogenesis of age-related diseases will be also discussed.

Keywords Ageing · Nutrient-sensing pathways · Autophagy · Chronological life span · Replicative life span · Yeast · Proteostasis

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Abbreviations

aSyn	Alpha-synuclein
AD	Alzheimer's disease
Aβ	Amyloid-β
AMPK	AMP-activated protein kinase
ATG	Autophagy gene
CLS	Chronological life span
CORE	Cross-organelle stress response
CR	Caloric restriction
DDR	DNA damage responses
ERCs	Extrachromosomal rDNA circles
HD	Huntington's disease
Htt	Huntingtin
IPOD	Insoluble protein deposit
INQ	Intranuclear quality control compartment
GTA	Genotoxin-induced targeted autophagy
JUNQ	Juxta nuclear quality control site
NQ	Non-quiescent
OXPHOS	Oxidative phosphorylation
PD	Parkinson's disease
PAS	Phagophore assembly site
PKA	Protein kinase A
Pho85	Phosphate metabolism protein 85
PolyQ	Polyglutamine
Q	Quiescent
ROS	Reactive oxygen species
RLS	Replicative life span
RNR	Ribonucleotide reductase
Snf1	Sucrose non-fermenting protein 1
TOR	Target of rapamycin
TORC1	Target of rapamycin complex 1
UPS	Ubiquitin proteasome system
VPS	Vacuolar protein sorting

9.1 Introduction

Ageing is a complex and multifactorial process driven by genetic, environmental and stochastic factors that lead to the cumulative incorporation of imbalances at the genomic and proteomic level in a multidimensional process resulting in the progressive decline of biological systems and decreased cellular fitness over time. In spite the fact that eukaryotic species have their own set of age-related diseases, the hallmarks

of cellular ageing are surprisingly conserved. These include primary events that trigger the progressively accumulation of cellular damage with ageing such as genomic instability, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing and mitochondrial dysfunction (reviewed in Lopez-Otin et al. (2013)). Due to the well-conserved hallmarks of cellular ageing, the budding yeast *Saccharomyces cerevisiae* has been widely used as a model of cellular and organismal ageing (Kaeberlein 2010; Longo et al. 2012; Sampaio-Marques et al. 2014a). The first study of yeast ageing, published more than 60 years ago, showed that yeasts have a finite replicative capacity (Mortimer and Johnston 1959). Mortimer and Johnston plotted their data and made the remarkable observation that the mortality curve for a yeast population resembles the mortality curves for many other organisms, including humans (Mortimer and Johnston 1959). Based on this observation, Replicative Life Span (RLS) was defined as the number of daughter cells produced by a single mother cell before dying. This definition underlies what has become a valuable model for studying ageing of mitotic cells. A second yeast model of ageing—the Chronological Life Span (CLS) model—was first proposed for budding yeast in 1980 (Muller et al. 1980). CLS is defined as the time that yeast cells can survive in a non-dividing state after exhaustion of the carbon source (Fabrizio and Longo 2003). Therefore, this single-celled organism provides a unique opportunity to study the ageing of both mitotic and post-mitotic cells (Kaeberlein et al. 2007). Not surprisingly, much of the advances on ageing research can be traced back to yeast that facilitates discovery of the evolutionarily conserved molecular and cellular mechanisms through which genetic and environmental interventions promote longevity. Yeasts were especially pivotal in the discovery of sirtuins and the TOR signalling pathway linking environmental nutrients to longevity. Different studies using these two yeast models of ageing have found that reducing glucose in the media can increase both RLS and CLS (Fabrizio and Longo 2003; Jiang et al. 2000). This paradigm linking environmental nutrients to longevity has been referred to as calorie restriction (CR) and is a major focus on ageing research.

This chapter intends to present some of the leading evidence and relevant advances on ageing and age-related diseases research in yeast models, with particular focus on longevity-promoting effects and proteostasis control.

9.2 Genes and Pathways Modulating Yeast Ageing: Replicative Versus Chronological Life Span

The two yeast models of ageing constitute important paradigms for the progressive accumulation of damage during ageing. While in the Replicative Life Span (RLS) damage is accumulated in mother cells due to the asymmetrical inheritance of damage, in the Chronological Life Span (CLS), the non-dividing cells cannot dilute the damage accumulated during ageing. Although both ageing models have different molecular and genetic determinants, they are not entirely independent. Indeed, there

is evidence that chronological aged cells have reduced RLS once they re-enter into the cell cycle (Ashrafi et al. 1999; Murakami et al. 2012; Piper 2006). Furthermore, some longevity-promoting interventions extend both CLS and RLS, while defects in protein quality control contribute to decreased survival in both ageing models (reviewed in Sampaio-Marques et al. (2014a)). This evidence gives strong support to damage-based theories of ageing but also recognizes that some molecular and genetic factors play a key role in ageing.

The key finding that defines the RLS is the fact that individual cells have a finite number of divisions (around 20–30), which is followed by cell death. Nearly, 100 yeast genes have been identified as involved in ageing and whose deletion enhances RLS (Kaeberlein et al. 2005; Longo et al. 2012). One of the best understood replicative ageing pathways involves the gene *SIR2* encoding a member of the sirtuin family of NAD^+-dependent deacetylases. Overexpression of *SIR2* was shown to extend yeast RLS (Kaeberlein et al. 1999) among other mechanisms, by suppressing homologous recombination of rDNA that leads to the formation of extrachromosomal rDNA circles (ERCs) (Fig. 9.1). These ERCs were thought to limit the mother's cells RLS due to their self-replicating capacity and asymmetrically segregation to the mother cells (Sinclair and Guarente 1997). Recently, this idea is being challenged by the suggestion that rDNA instability, rather than ERCs, is the primary cause of mother cells' senescence and death (Lindstrom et al. 2011). Importantly, mutations that suppress the rDNA instability such as deletion of *FOB1*, an rDNA replication fork block protein (Kaeberlein et al. 1999), are able to overcome the decreased RLS of *sir2Δ* mother cells (Longo et al. 2012). Several groups have later demonstrated that overexpression of Sir2 homologs, Sir2.1 in *Caenorhabditis elegans* and dSir2 in *Drosophila melanogaster*, extends life span (Rogina and Helfand 2004; Tissenbaum and Guarente 2001). Therefore, the first highly conserved determinant of ageing, Sir2, was discovered in yeast.

The role of Sir2 in the regulation of the RLS is not restricted to the formation of ERCs and rDNA instability. Deletion of *SIR2* results in a defect in the asymmetric retention of damage, particularly of oxidatively damaged cytoplasmic proteins in the mother cell leading to a shorter RLS of daughter cells (Aguilaniu et al. 2003). This defective asymmetric retention of damage promoted by deletion of *SIR2* can be overcome by the overexpression of *HSP104* (Erjavec et al. 2007) (Fig. 9.1).

Sir2 has also been shown to be fundamental to the regulation of the epigenetic modifications to histones. The best example is the increase in the H4K16 acetylation concurrently with a decline in Sir2 levels during ageing (Dang et al. 2009). Furthermore, Sir2 can also control RLS in an ERC-independent manner, by controlling cytoskeleton function and polarity (Liu et al. 2010). Importantly, overexpression of *SIR2* orthologs in worms and flies promotes longevity and activation of the mammalian Sir2-ortholog, SIRT1, can enhance health span in mice (reviewed in Finkel et al. (2009)). Although these results have been questioned by many, there appears to be a general consensus that SIRT1 interacts with important ageing-related pathways in mammals.

Mitochondrial function also plays a critical role in RLS determination, as mitochondrial oxidative phosphorylation (OXPHOS) deteriorates and mitochondrial ROS

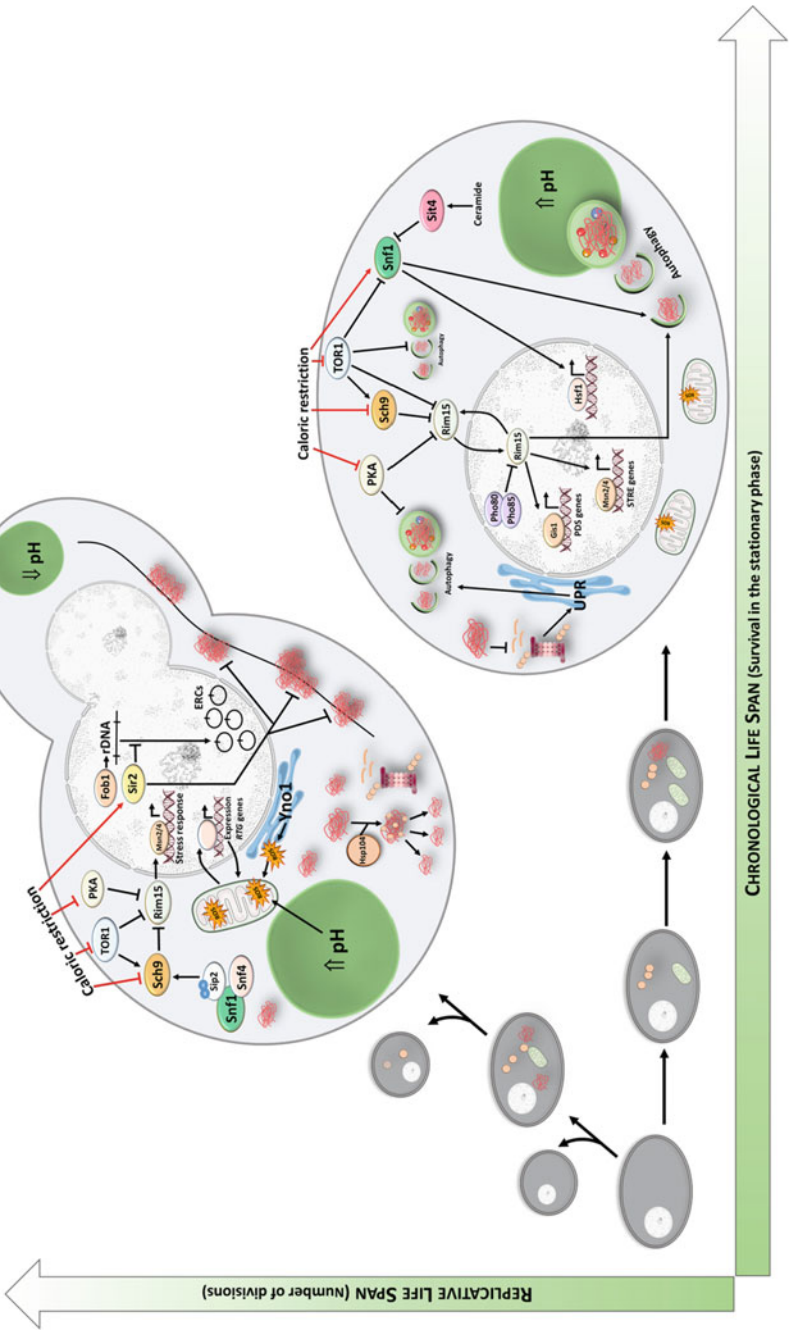

Fig. 9.1 Genes and pathways modulating replicative and chronological life spans. Shown are illustrated the main players and pathways that modulate ageing in the yeast *S. cerevisiae*. See text for details

generation increases with age. Jazwinski and colleagues have demonstrated that induction of the retrograde response pathway, which transmits signals of mitochondrial stress to the nucleus, extends RLS in certain genetic backgrounds (Kirchman et al. 1999). Therefore, RLS is extended by enhancement of mitochondrial biogenesis, correct mitochondrial segregation and inheritance, prevention of mitochondrial proteotoxic stress, and maintenance of proper nuclear–mitochondrial communication through activation of mitochondrial retrograde signalling pathways (Fig. 9.1; reviewed in Ruetenik and Barrientos (2015)). Recently, it was shown that the shortening of RLS due to mitochondrial dysfunction was not related to the accumulation of ERCs, but to increased ROS generation of the ER-localized NADPH oxidase Yno1 (Yi et al. 2018). Furthermore, mitochondrial function is also impaired due to the decline of vacuolar acidity that occurs with age. The age-dependent increase of pH promotes the storage of amino acids in the mitochondria that consequently affects membrane potential and mitochondrial function (Fig. 9.1) (Hughes and Gottschling 2012).

Recently, studies indicate that as cells age, damaged protein aggregates and oxidatively damaged organelles, as mitochondria, are predominantly sequestered in mother cells contributing as ageing factors (Aguilaniu et al. 2003; Denoth Lippuner et al. 2014; Erjavec and Nystrom 2007; Lam et al. 2011; Liu et al. 2010; McFaline-Figueroa et al. 2011). In contrast, disruption of endoplasmic reticulum diffusion barriers can result in the segregation of misfolded protein aggregates into daughter cells (Higuchi-Sanabria et al. 2014), see for review Smith and Schneider (2018).

The abovementioned detrimental age-dependent changes can be mitigated by anti-ageing intervention such as caloric restriction (CR). Mounting evidences demonstrated that CR extends life span and health span in several model organisms (Fontana et al. 2010). In yeast, CR is achieved by reducing glucose concentration from 2 to 0.5% or below (Lin et al. 2000). The *SIR2* gene and functional NAD^+ salvage genes were reported to be required for CR-mediated RLS extension (Anderson et al. 2002, 2003; Lin et al. 2000). Although it is still debated the role of Sir2 on the CR-mediated RLS extension, it has been accurately established that CR can also extend life span via Sir2-independent mechanisms and that Sir2 and CR work in parallel pathways (Kaeberlein et al. 2004). Additional discussions of the role of Sir2 and the other yeast sirtuins in RLS extension promoted by CR can be found in recent reviews of this topic Kaeberlein (2010), Kaeberlein and Powers (2007). Curiously, enhanced proteasome activity is able to increase RLS by a mechanism that is genetically distinct from both CR and Sir2 (Kruegel et al. 2011).

Accumulated evidence indicates that CR-mediated RLS extension is largely dependent on reduced Ras-PKA and TOR/Sch9 pathways signalling that play a concerted role in regulating growth, metabolism and stress resistance in response to nutrient availability (Kaeberlein et al. 2005). Importantly, Sch9 activity can be regulated independently of CR to influence RLS through acetylation of Sip2, a component of the yeast AMP-activated protein kinase complex, Snf1 (Lu et al. 2011). These nutrient and energy sensing pathways play a similar role in modulating yeast CLS, as well as longevity in worms, flies and mice, providing strong evidence for their conserved effects on ageing throughout eukaryotes (Fontana et al. 2010).

CLS defines the survival of stationary-phase cells after depletion of nutrients, and glucose is commonly the first limiting nutrient. Importantly, two main cell populations could be defined when glucose is exhausted at the diauxic shift. One of these populations corresponds to quiescent (Q) cells that are in G0, a non-proliferative state. Q cell population is mainly composed of unbudded daughter cells (Aragon et al. 2008; Li et al. 2013; Miles et al. 2013; Werner-Washburne et al. 2012) that are highly resistant to stress and present rigid cell walls and high accumulation of glycogen and trehalose and low accumulation of reactive oxygen species (ROS). These cells are able to re-enter mitosis when nutrients become available (Aragon et al. 2008; Leonov et al. 2017; Miles et al. 2013). Another cell population is composed of non-quiescent (NQ) cells, most or all of which are first-generation and higher generation mother cells (Aragon et al. 2008; Li et al. 2013; Miles et al. 2013; Werner-Washburne et al. 2012). These NQ cells can be metabolically active, with or without clonogenic capacity, or may exhibit hallmarks of apoptosis or necrosis (Aragon et al. 2008; Li et al. 2013; Miles et al. 2013; Werner-Washburne et al. 2012).

CLS is controlled by a complex signalling network including TORC1 (target of rapamycin complex 1), a highly conserved serine/threonine protein kinase complex that is the major regulator of the signalling network controlling cell growth; PKA (protein kinase A), a major regulator of metabolism, proliferation and stress resistance; and the protein kinase Sch9, a serine/threonine protein kinase that plays a central role in nutrient-mediated signalling (Smets et al. 2010). These three kinase complexes are the so-called ménage-à-trois that integrates inputs from several nutrient-sensing systems to regulate metabolism, intracellular trafficking, proteome integrity, autophagy, stress resistance, cell size, progression, growth and sporulation (reviewed in Deprez et al. (2018)). In addition to these regulators, other energy and sensing pathways play important roles in regulating longevity. This is the case for Snf1 (sucrose non-fermenting, protein 1), a member of the conserved AMP-activated protein kinase (AMPK) family that is a major sensor of cellular energy levels, and the Pho85 (phosphate metabolism, protein 85), which together with Pho80 forms a kinase complex with a major role on the cellular response to changes in extracellular and/or intracellular phosphate levels.

These nutrient-sensing pathways may overlap and create redundancy in the modulation of many downstream effector proteins including (among others) Rim15, a serine/threonine protein kinase that is essential for cell cycle arrest at G1 and entry of cells into quiescence. Rim15 is regulated by the TORC1, Sch9, PKA and Pho85 (reviewed in Leonov et al. (2017, Sampaio-Marques et al. (2014a), Smets et al. (2010)). Yak1 is another serine/threonine protein kinase under the control of PKA, and it is required for cell cycle arrest at G1 (reviewed in Leonov et al. (2017), Sampaio-Marques et al. (2014a), Smets et al. (2010)). TORC1 and PKA also control Mck1, a dual-specificity serine/threonine and tyrosine protein kinase. Msn2/4 and Gis1 are also downstream effectors of these two kinases. These transcription factors are controlled by TORC1 and PKA, as well as by Snf1—they activate the expression of genes involved in stress response and diauxic transition (reviewed in Leonov et al. (2017), Smets et al. (2010)). Another essential transcription factor is Hsf1, which is controlled by Snf1 and is involved in the expression of many genes, particularly those

related to control of proteostasis and energy generation. Snf1 and TORC1 regulate the transcriptional factor Gln3, which regulates genes involved in the metabolism of nitrogen, and eIF2α, a factor involved in the initiation of protein synthesis. It is also important to highlight the regulation of the Atg1–Atg13 complex, controlled by TORC1, PKA, Snf1 and Pho85, which initiates autophagy by enabling phagophore assembly site (PAS) formation (reviewed in Leonov et al. (2017)). Globally, these downstream effectors enhance several protective systems including glycogen and, glycerol and antioxidant enzymes and mechanisms related to the maintenance of proteostasis, such as HSPs and autophagy (Sampaio-Marques et al. 2014b).

Despite the different mechanisms of life span extension promoted by the inactivation of these signalling pathways, autophagy seems to be a common denominator. Reduced TOR signalling induces autophagy (Noda and Ohsumi 1998), while deletion of *SCH9*, a TOR effector that can function independently, has a minor impact on autophagy. Nevertheless, the role of Sch9 on vacuole acidification has to be considered, as deletion of *SCH9* could extend both RLS and CLS by contributing to the maintenance of vacuole acidification in aged cells (reviewed in Ruckenstuhl et al. (2014), Tyler and Johnson (2018a)). These nutrient-sensing pathways together with PKA are crucial for the regulation of pH homeostasis through their influence on the proton pumping activity of the V-ATPase, and possibly also on Pma1, from the plasma membrane ATPase (Deprez et al. 2018). pH homeostasis is a crucial regulator of autophagy, as the final step of autophagy is linked to vacuolar membrane integrity and acidification of the vacuolar lumen (reviewed in Deprez et al. (2018)). Besides PKA's role in pH homeostasis, inactivation of PKA also induces autophagy associated with RLS and CLS extension, albeit less efficiently than autophagy induction promoted by TOR inactivation (Budovskaya et al. 2004) or by deletion of both *SCH9* and PKA (Yorimitsu et al. 2007). In contrast, the positive regulator of autophagy, Snf1, promotes a reduction of CLS when deleted (Wang et al. 2001).

Regarding Sir2, its role on CLS is far more complex. Depending on the strain background and growth media, deletion of *SIR2* either has no effect or induces a moderate increase of CLS (reviewed in Wierman and Smith (2014)). Consequently, Sir2 has been mainly assigned a pro-ageing role in CLS (reviewed in Sampaio-Marques et al. (2014a)). Although Sir2 might antagonize CLS extension promoted by CR (Fabrizio et al. 2005), it was also shown that CR extends CLS independently of the sirtuins including Sir2 (Smith et al. 2007). We have shown that autophagy maintenance at homeostatic levels promoted by CR or *TOR1* deletion is achieved by decreasing Sir2 levels and activity (Guedes et al. 2017). Although *SIR2* deletion does not have a major effect on CLS, it does compromise the extension of CLS observed in *SCH9* deleted cells and in cells treated with the life span-promoting agent resveratrol (Fabrizio et al. 2005; Howitz et al. 2003). Furthermore, Sir2 plays an important role in autophagy regulation during CLS in certain scenarios. We have previously shown that Sir2, similar to mammalian SIRT1, activates autophagy and mitophagy through the transcriptional regulation of *ATG8* and *ATG32* under proteotoxic conditions (Sampaio-Marques et al. 2012). Therefore, it is tempting to speculate that Sir2 supports life span extension of *SCH9* deleted cells by maintaining autophagy.

Besides the activation of a general stress response, the pro-longevity effects linked to reduced activity of nutrient-sensing pathways appears to be also associated with ROS signalling and increased mitochondria function. Our studies have shown that abrogation of catalase activity or of nutrient-sensing pathways by CR extends CLS by producing hydrogen peroxide, which leads to the activation of superoxide dismutases that inhibit the accumulation of superoxide anions (Mesquita et al. 2010). These findings established a role for hormesis effects of hydrogen peroxide in promoting longevity. Later, it was reported that during exponential-growing phase, *TOR1* or *SCH9* deleted cells generate mitochondrial ROS, as an adaptive hormetic signal, which results in the reduction of ROS levels at stationary phase and extension of CLS (Pan et al. 2011). A few years ago, the beneficial effects of hormetic mitochondrial ROS on longevity signal that extends yeast CLS were shown to involve epigenetic alterations and the DNA damage responses (DDR) kinases, Tel1 and Rad53 (Schroeder et al. 2013). This hormetic pathway is independent and distinct from the nuclear DDR and involves histone modifications (Schroeder et al. 2013).

The existence of a nuclear pathway that senses mitochondrial ROS generation/accumulation points to the crucial role of mitochondrial function on ageing. In fact, the longevity-promoting effects of the global activation of general stress response by decreasing the nutrient-sensing pathways' activity appear to be associated with an increase in mitochondria function. In line with this, the lack of mitochondrial respiration severely impacts the longevity of stationary-phase cells (reviewed in Sampaio-Marques and Ludovico (2018)). In contrast, long-lived cells deleted on *TOR1* (Bonawitz et al. 2007; Ocampo et al. 2012; Pan et al. 2011) or *SCH9* (Lavoie and Whiteway 2008) presented an increased respiratory capacity. The promotion of longevity encompasses the activation of the Msn2/4 and Gis1 stress response (Fig. 9.1) (Ewald et al. 2016) and the Rph1-dependent epigenetic silencing by triggering a non-canonical activation of the DDR pathways (Schroeder et al. 2013).

More recently, novel ageing determinants were identified, as proteins involved in chromatin remodelling (Swr1, Arp6 and Swc3), Arv1, a lipid homeostasis factor that modulates autophagy, Tep1, the homologue of the human tumour suppressor PTEN, and proteins associated with phosphatidylinositol phosphate metabolism (Garay et al. 2014). A connection between sphingolipids and cell signalling through TOR, Sch9 and the ceramide-activated protein phosphatase Sit4 was recently shown to impact on mitochondria function, autophagy and CLS (Vilaca et al. 2018) (Fig. 9.1). Details on the link between sphingolipids signalling and CLS can be found in (Oliveira et al. 2017).

Numerous studies point to distinct determinants of yeast RLS and CLS. For example, deletion of *SIR2* or *RAS2* has dissimilar effects on RLS and CLS (reviewed in Smith and Schneider (2018)). In contrast, both RLS and CLS are extended in response to CR and other interventions that abrogate nutrient-sensing pathways. Nevertheless, it remains unclear whether similar downstream molecular events are common to both yeast ageing paradigms (Sampaio-Marques et al. 2014a). Importantly, both yeast ageing paradigms are connected. As briefly mentioned above, chronologically aged yeast cells show a proportional reduction in RLS (Ashrafi et al. 1999; Murakami et al. 2012; Piper et al. 2006) that is prevented by CR, suggesting that the metabolic

state and mitochondrial function of stationary-phase cells determine their replicative potential upon transfer to growth conditions (Delaney et al. 2013).

9.3 Proteostasis and Yeast Ageing

Ageing is driven by accumulation of damage in highly conserved cell-intrinsic processes such as chromosome structure/organization, transcriptional regulation, nuclear export/import, protein translation and quality control, recycling of damage/unnecessary organelles, maintenance of cytoskeletal structure and extracellular signalling (DiLoreto and Murphy 2015). These processes have the ability to communicate with each other resulting in an intricate interplay that governs cells' ageing. Therefore, the knowledge of the specific cellular and molecular mechanisms underlying ageing represents one of the most complex issues that have yet to overcome.

Several studies focused on molecular alterations occurring during yeast ageing revealed a series of progressive events that collectively contribute to ageing phenotypes. These events integrate damage and dysfunction with stress pathways, including oxidative stress associated with mitochondrial dysfunction and accumulation of ROS, genomic instability associated with nuclear DNA damage, mutagenesis and replication stress, metabolic alterations and loss of proteostasis. Importantly, DNA damage and error-prone DNA repair systems have been assumed as key for the mechanisms behind age-dependent genomic instability observed during ageing. The results of several studies consistently point to a role for oxidative damage that induces senescence and cell death as an important determinant of life span (reviewed in Weinberger et al. (2013)). However, the relationships between ROS, ageing and age-related diseases suggest increased complexity in this scenario (Ludovico and Burhans 2014; Weinberger et al. 2013).

Protein quality control systems as autophagy play a key role in the DDR by controlling the levels of proteins involved in cell cycle checkpoints and DNA synthesis/repair mechanisms. For example, in *S. cerevisiae*, DNA damage induces the autophagic degradation of ribonucleotide reductase 1 (Rnr1) (Dyavaiah et al. 2011), the large subunit of ribonucleotide reductase (RNR), which is a highly conserved enzymatic complex catalysing the formation of deoxyribonucleotides required for both DNA replication and repair. This DDR-dependent autophagic pathway in yeast was called genotoxin-induced targeted autophagy (GTA) and requires the involvement of the DDR kinases, Mec1 and Rad53, as well as a central component of the selective autophagy machinery, Atg11 (Eapen et al. 2017). Recently, it was reported that the kinase Mec1 plays a fundamental role in protein homeostasis (Corcoles-Saez et al. 2018). In agreement, it is becoming well recognized that one of the major determinants of ageing is proteostasis and that the other ageing hallmarks are intimately related to it. It is the example of the nutrient-sensing pathways that when inactivated mainly contribute to the maintenance of the proteome during ageing (Sampaio-Marques et al. 2014a). In this sense, it is proposed that early changes on protein homeostasis network that result in the cellular loss of proteostasis could

be one of the earliest events dictating ageing progression, affecting a multitude of downstream processes (Labbadia and Morimoto 2014). In fact, cells have multiple stress-responsive mechanisms to combat loss of proteostasis associated with cellular ageing as described below.

The ability of cells to maintain protein homeostasis, or proteostasis, in response to intrinsic cellular and environmental insults, which accumulate over time, is one of the main determinants of life span (Morimoto and Cuervo 2014). Proteostasis, referred as the healthy maintenance of the cellular proteome, comprises highly complex and interconnected pathways that govern the fate of proteins. Proteostasis is controlled by a multi-compartmental system that has the ability to coordinate protein synthesis, processing, trafficking, folding, localization, assembly/disassembly and degradation (Sampaio-Marques and Ludovico 2018). A major determinant of loss of proteostasis and protein aggregation is the overproduction and accumulation of unstable proteins (Lopez-Otin et al. 2013). For example, it was demonstrated that inhibition of protein translational machinery with cycloheximide blocks the formation of protein aggregates indicating that active protein translation is required for stress-induced protein aggregation in yeast (Zhou et al. 2014). Although the mechanisms underlying this observation remain unclear, a reduction in handling the burden of newly translated unfolded proteins and an increase in free molecular chaperones as well as in the activity of the degradation pathways could be simple explanations (Medicherla and Goldberg 2008).

Molecular chaperones assist in the folding/refolding of proteins (Fig. 9.2). Chaperones can be found in the cytoplasm but also in the ER and mitochondria. A recent study revealed that decline in chaperone activity in each cellular compartment triggers a response in other compartments that result in loss of respiration capacity, demonstrating the dependence of mitochondrial activity on cell-wide proteostasis. This phenomenon has been called cross-organelle stress response (CORE) and has a protective role by extending both CLS and RLS (Peric et al. 2016). Although several physical organelle contact sites exist in yeast, their involvement in CORE and the cell-wide proteostasis system is yet to be elucidated. Importantly, it was demonstrated that ER-formed protein aggregates are frequently associated with or are later captured by mitochondria (Zhou et al. 2014). In line with this concept, aged replicative cells exhibit a gradual decline of aggregate–mitochondria association decreasing mobility and leakage of aggregates from mother into the bud contributing to the decreased life span of daughter cells (Zhou et al. 2014).

The activity of ATP-dependent chaperones is greatly affected by the age-dependent reduction of cellular energy, due to reduced mitochondrial functionality and deregulation of lipid and glucose metabolism (Ma and Li 2015; Ritz and Berrut 2005). The activity of molecular chaperones could also be affected by their availability, which does not meet the needs of aged cells. These phenomena might be aggravated by protein modifications that are enhanced during ageing, such as accumulation of advanced glycation end products through non-enzymatic modifications that interfere with the chaperone's ability to recognize the target (Vanhooren et al. 2015), resulting in the accumulation and aggregation of the defective proteins (Kumar et al. 2007). Consistent with this possibility, it was shown that downregulation of

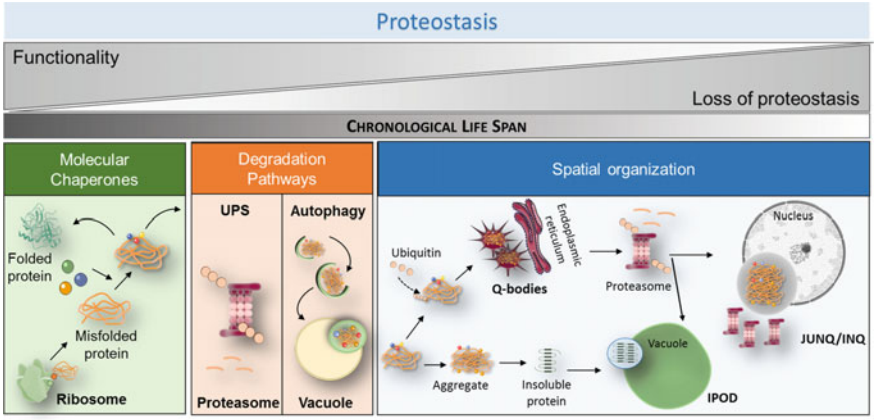

Fig. 9.2 Proteostasis and yeast ageing. Proteostasis is mainly maintained by the action of molecular chaperones and the two degradation pathways, the ubiquitin–proteasome system (UPS) and autophagy. In addition, yeast cells have developed a spatial quality control system where misfolded and aggregated proteins are sequestered into larger protective inclusions: the juxtanuclear quality control site (JUNQ)/the intranuclear quality control compartment (INQ) and the peripheral vacuole-associated, insoluble protein deposit (IPOD). See text for more details

yeast Hsp90 activity results in an increase in heat shock protein synthesis due to the inability to efficiently repress Hsf1 (Duina et al. 1998; Harris et al. 2001), which is correlated with increased viability over time. Thus, an enhancement of chaperone activity is associated with increased longevity. However, age-mediated alterations in proteostasis are due not only to decreased chaperones activity, but also to alterations in degradation pathways, the ubiquitin–proteasome system (UPS) and autophagy (Fig. 9.2). In yeast chronological ageing, proteasome dysfunction occurring over time induces the accumulation of protein aggregates and formation of inclusions that further obstruct proteasome function in a vicious cycle (Andersson et al. 2013). 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 during yeast ageing (Sampaio-Marques and Ludovico 2018).

Proteasome dysfunction might also be potentiated by the sequestration in protein aggregates of factors required for proteasome activity, such as ubiquitin ligases/proteases or proteasome activators (Andersson et al. 2013). For example, the production and accumulation of ROS lead to proteasome activity reduction and subsequent accumulation of carbonylated proteins and specific modifications in certain proteins, such as E1 and/or E2 enzymes, which results in the impairment of the ubiquitin-binding (da Cunha et al. 2011). In contrast, overexpression of key molecules, such as the proteasome chaperone Ump1, results in the proteasome-mediated protein degradation enhancement with the consequent longevity extension (Chen et al. 2006).

Accumulating evidence also suggests that damaged proteins are not randomly distributed in the cell during ageing. Yeast cells have developed a spatial quality control system where misfolded and aggregated proteins are sequestered into larger protective inclusions (Alvers et al. 2009a) (Fig. 9.2). This spatial compartmentalization of protein aggregates is a complementary protein quality control strategy that acts in parallel with temporal quality control. The presence of these inclusions is not essential for their degradation, but it may facilitate refolding/degradation by increasing the proximity of chaperones and their substrates limiting the toxic interactions of misfolded proteins. Several studies suggest that immediately upon misfolding, an active chaperone-dependent transport of damaged proteins to dynamic compartments called Q-bodies, which are attached to the ER, takes place (Escusa-Toret et al. 2013). The damaged proteins inside of these Q-bodies are rapidly cleared through the UPS—however, if clearance is impaired, these misfolded proteins concentrate in one of the two major protein quality control compartments, the juxta nuclear quality control site (JUNQ) and the peripheral vacuole-associated, insoluble protein deposit (IPOD) (Kaganovich et al. 2008) (Fig. 9.2). More recently, JUNQ was found inside the nucleus, where it serves as a new intranuclear quality control compartment (INQ) for the deposition of both nuclear and cytosolic misfolded proteins, irrespective of ubiquitination (Miller et al. 2015). Proteins are targeted to the JUNQ by a ubiquitin-based sorting mechanism, while they are sent non-ubiquitinated to IPOD (Kaganovich et al. 2008) (Fig. 9.2). Recently, it was found that the small Hsp42 can assemble into versatile dynamic oligomers—the Hsp42-containing stationary-phase granules (Hsp42-SPGs), which contain protein components including molecular chaperones, metabolic enzymes and regulatory proteins (Lee et al. 2018). These Hsp42-SPGs are enriched in long-lived quiescent cell populations, suggesting that these granules may help quiescent cells to combat various stresses during stationary phase, by mechanisms that remain unclear (Lee et al. 2016). Furthermore, Hsp42-SPGs may work as centres that control both protein quality and quantity in stationary-phase cells (Lee et al. 2018).

Like the other proteostasis pathways, spatial quality control also declines with age, and cells that lack this ability show accelerated ageing (Escusa-Toret et al. 2013). During ageing, the increase in the load of aggregated proteins and the inactivation of cellular chaperones could provide one explanation for the age-associated loss of spatial protein quality control (Hill et al. 2017). Furthermore, the reduction on efficiency of this spatial protein quality control system might be also related with the failure of organelles function. For example, vacuolar pH alterations could cause a breakdown in vesicle trafficking and fusion to the vacuole, an important process for spatial sequestration of aggregated proteins. Furthermore, disruption of a functional actin cytoskeleton might also affect vesicle trafficking and fusion, mitochondrial inheritance, and increase ROS accumulation—this could explain the inefficient inclusion formation observed in ageing cells (reviewed in Hill et al. (2017)).

Surveillance of proteostasis is mainly played by the chaperones and the main protein degradation pathways, the UPS and the autophagy. Although UPS is the primary cellular route for protein degradation, it does not allow for the degradation of unfolded or large protein complexes. Therefore, larger substrates, such as

large protein inclusions, can be directly degraded by autophagy. In the budding yeast *S. cerevisiae*, macro(autophagy) occurs through the formation of a double-membrane vesicle—autophagosome—that sequesters cytosol and organelles and fuses with the vacuole releasing the content to be degraded and recycled. Besides bulk unspecific degradation, autophagy can occur by selective mechanisms encompassing the degradation of specific cargos such as organelles (reviewed in Galluzzi et al. (2017)). Selective autophagy requires functional actin cytoskeleton for specific degradation of mitochondria, peroxisomes, mature ribosomes, and cytosolic proteins such as acetaldehyde dehydrogenase Ald6 (reviewed in Smith and Schneider (2018)). The functioning of autophagy is supported by two main groups of genes, autophagy-related genes (ATG) and vacuolar protein sorting (VPS) genes (Reggiori and Klionsky 2013; Tyler and Johnson 2018b). Collectively, the processes underlying autophagy are highly complex and beyond the scope of this chapter and have been extensively reviewed elsewhere Yin et al. (2016).

Similar to UPS, autophagy activity decreases during ageing, as reported in different model systems (reviewed in Rubinsztein et al. (2011)). However, due to the complexity of the autophagy process and the stochastic nature of ageing, the mechanisms underlying decreased autophagy remain largely unclear. The decline of autophagy activity promoted by ageing enhances the accumulation of aberrant proteins/aggregates, causing additional molecular and cellular damage, as a vicious cycle. Furthermore, an age-associated increase of vacuolar pH (Fig. 9.1), which limits the activities of vacuolar proteases and results in the loss of vacuolar homeostasis, may contribute to autophagy impairment during ageing (Nakamura et al. 1997). Autophagy deregulation in aged cells can also be a consequence of persistent activity stimulation. Although increased autophagy might initially have a favourable outcome, if maintained at a high rate, it can promote the depletion of functional organelles/proteins and essential autophagic molecules, contributing to cell death and thus shortening of life span (Meijer and Codogno 2007; Sampaio-Marques et al. 2012). In agreement, we showed that heterologous expression of human alpha-synuclein (aSyn) in yeast cells results in aberrantly high activation of autophagy associated with shortening of CLS (Sampaio-Marques et al. 2012). Furthermore, all the genetic and environmental manipulations reducing aSyn toxicity resulted in decreased autophagy activity (Guedes et al. 2017; Sampaio-Marques et al. 2012) indicating that autophagy should be maintained under homeostatic levels. It is, nonetheless, well recognized that autophagy is required for maximal CLS and has been implicated in almost all the CLS promoting interventions, as discussed herein and reviewed in Sampaio-Marques et al. (2014a), Tyler and Johnson (2018a). Different genetic studies have demonstrated that genes encoding proteins involved in autophagy machinery as *ATG1*, *ATG2*, *ATG7*, *ATG8*, *ATG16* or *VPS21* are required for life span extension (Alvers et al. 2009a; Aris et al. 2013; Fabrizio et al. 2010; Matecic et al. 2010). Dietary interventions such as caloric or methionine restriction were also shown to be dependent on autophagy (Aris et al. 2013; Ruckenstuhl et al. 2014). As we have discussed in the previous section, nutrient-sensing pathways also have profound effects on autophagy and longevity, as well as other relevant players linking metabolism, autophagy and longevity. Notably, accumulation of acetyl-

CoA, a critically important molecule in metabolism, has been shown to result in the hyperacetylation of histones that transcriptionally repress autophagy genes and negatively impact ageing (Eisenberg et al. 2014). The Esa1 and Rpd3 antagonistic acetyltransferase–deacetylase pair has also been shown to transcriptionally regulate autophagy—implicating epigenetic regulation of autophagy also in RLS (Yi et al. 2012). Together, these studies clearly demonstrate a bona fide role for epigenetics in the regulation of autophagy and yeast life span.

Lipid metabolism can also engage the autophagy machinery to positively regulate longevity, as referred above. The Arv1, a protein involved in sterol and sphingolipids metabolism, was identified as involved in the regulation of CLS through autophagy (Garay et al. 2014). An additional study demonstrated that supplementation of nutrient medium with phosphatidylethanolamine (PE) or genetic interventions that result in increased PE levels activate autophagy and extend CLS (Rockenfeller et al. 2015).

Interestingly, due to the large number of conserved ageing-related genes and ageing mechanisms in yeast and humans, testing candidate anti-ageing molecules in yeast has proven highly successful in the search for potential anti-ageing therapies. Yeast-based studies have helped to understand the mode of action of anti-ageing molecules such as rapamycin, spermidine or resveratrol. Rapamycin is a macrolide antibiotic with antifungal and immunosuppressive properties, which inhibits the TOR signalling pathway. Autophagy activation was shown to be essential for rapamycin-mediated life span extension in yeast (Alvers et al. 2009b). The polyphenol resveratrol and the natural polyamine spermidine are currently the most promising potential anti-ageing agents that were discovered in yeast and shown to activate autophagy and extend both RLS and CLS (Eisenberg et al. 2009; Morselli et al. 2011). Collectively, these observations are consistent with results from the large number of studies that point to general anti-ageing properties of autophagy. In spite of the role of autophagy in CLS, autophagy does not appear to contribute to RLS under normal growth conditions. Indeed, deletion of most of the *ATG* genes has negligible effects on RLS, and in some cases even results in extension of RLS (Ghavidel et al. 2015; McCormick et al. 2015).

Altogether, these studies demonstrate that yeast is an invaluable tool for the identification and characterization of conserved mechanisms that promote cellular longevity, and that autophagy plays an important role in nearly all known longevity-promoting interventions, as reviewed elsewhere Tyler and Johnson (2018a).

9.4 Modelling Age-Related Diseases in Yeast

Deregulation of protein network functionality is correlated with ageing and is a major risk factor for the development of a wide spectrum of age-related protein diseases (Morimoto and Cuervo 2014). The budding yeast *S. cerevisiae* is a simple unicellular eukaryotic organism that shares well-conserved molecular and cellular mechanisms with higher eukaryotes and has been particularly useful as a biological model for ageing and age-related diseases (Tenreiro et al. 2013). Thus, *S. cerevisiae*

has played an extremely important role in the discovery of key molecular events associated with neurodegenerative diseases, including Parkinson's (PD), Alzheimer's (AD) and Huntington's (HD) diseases. These protein misfolding disorders are age-related degenerative diseases in which misfolded proteins are prone to form intra- or extracellular aggregates with specific composition and localization for each disease. While intracellular aSyn and huntingtin (Htt) aggregates are hallmarks of PD and HD, respectively, extracellular aggregates of tau protein and amyloid-β (Aβ) peptide are characteristic of AD. Depending on the disease, the resulting aggregates might result in the loss of protein function and/or in the gain of a cytotoxic function. Mitochondrial dysfunction, transcriptional dysregulation, trafficking defects and loss of proteostasis are some of the molecular and cellular mechanisms conserved from yeast to human that underlie the pathogenesis of these diseases (reviewed in Tenreiro and Outeiro (2010)).

It is estimated that around 25–30% of the genes linked to human diseases have yeast orthologues (Bassett et al. 1996). Thus, if a gene related to a human disease has a yeast homologue, its role can be investigated by simply deleting or overexpressing this gene in yeast. For example, yeast-based studies on the *SOD1* and *YHF1* genes, homologs of the human genes involved in Friedreich's ataxia and amyotrophic lateral sclerosis, respectively, contributed greatly to our understanding of these disorders. Furthermore, even if a human gene is absent from the yeast genome, its role in disease can be modelled by the heterologous expression of the human gene in yeast cells. Yeast models for PD, HD and AD, which are examples of this strategy, have been extensively exploited and have greatly contributed to the elucidation of the molecular and cellular aspects of these disorders, as detailed below (Miller-Fleming et al. 2008; Sampaio-Marques and Ludovico 2015; Sampaio-Marques and Ludovico 2018).

A yeast HD model can be generated by the heterologous expression of mutant human Htt exon 1 with different polyglutamine (polyQ) expansions (more than 35 glutamine residues) in yeast cells, reproducing many of the cellular and molecular features of HD pathology. For example, expression of mutant fragments of Htt resulted in polyQ length-dependent aggregation and toxicity, endocytosis impairment, transcriptional dysregulation, mitochondrial dysfunction, oxidative stress and apoptosis (reviewed in Tenreiro and Outeiro (2010)). Furthermore, genetic screens using yeast HD models have identified different modulators of mutant Htt aggregation and toxicity. Notably, chaperones members of Hsp40 and Hsp70 families have been identified as potential therapeutic targets (reviewed in Tenreiro and Outeiro (2010)). Yeast HD models have also been useful in screening drugs.

Studies on AD primarily make use of human cell lines and transgenic mouse models. However, yeast AD models are becoming increasingly important to unravel fundamental molecular aspects of AD. Pathological hallmarks of this disease include the presence of extracellular plaques of Aβ and intracellular neurofibrillary tangles of phosphorylated tau protein. Tau and Aβ have no functional yeast orthologues and thus, different yeast models have assessed the cellular consequences of expressing Aβ peptides or Tau. Different yeast models have been used, and some of them fuse Aβ40 or Aβ42 to C-terminal part of Sup35, a translation termination factor without the prion domain, to create an oligomerization assay to find specific point mutations able to

inhibit Aβ oligomerization (Bagriantsev and Liebman 2006). These models have been used in high-throughput screens resulting in the identification and validation of two compounds with anti-oligomeric effects (Park et al. 2011). In other yeast AD models, the Aβ42 peptide is directed to the secretory pathway (D'Angelo et al. 2013; Treusch et al. 2011).

Although only a few studies have employed yeast to study the biology of Tau, the data obtained revealed that yeast cells have an enormous potential to disclose key aspects of Tau pathophysiology, since these models recapitulate central features of the AD, including Tau hyperphosphorylation at pathological residues, conformational changes and aggregation (reviewed in Verduyckt et al. (2016)). For example, in yeast, Tau phosphorylation is regulated by the kinases Mds1p and Pho85p, the orthologues of human GSK3b and CDK5, respectively, at the same residues that Tau is hyperphosphorylated in neurons (Vandebroek et al. 2005). Downregulation of Pho85 increases Tau phosphorylation and aggregation, while deletion of *MDS1* is associated with reduced Tau phosphorylation (Vandebroek et al. 2005). Furthermore, oxidative stress and dysfunctional mitochondria exacerbate Tau aggregation, although Tau is less phosphorylated under those conditions, suggesting that other mechanisms are involved in Tau aggregation (Vanhelmont et al. 2010) (for review see Seynnaeve et al. (2018)).

Insoluble aggregates of aSyn are found in synucleinopathies including idiopathic and familial forms of PD. Duplication or triplications of *SNCA* gene, which encodes aSyn, are linked to sporadic PD, while aSyn point mutations (A30P, E46K, H50Q, G51D, A53T and A53E) are associated with familial PD forms, with early onset (reviewed in Sampaio-Marques and Ludovico (2015)). Cell-based models for PD include yeast models, immortalized cell lines, primary neuronal cultures, stem cells and patient-derived cell models. These cellular models have been widely explored to dissect molecular mechanisms behind pathology using unbiased genetic screens, as well as multi-omic approaches to identify relevant genes and proteins. In addition, they can be easily manipulated genetically and pharmacologically at a reduced cost and in the absence of ethical issues. Nevertheless, these cellular models cannot reproduce several features of disease related to multicellularity and require validation in animal models.

The yeast *S. cerevisiae* is one of the best characterized eukaryotic organisms that provides a relevant biological context for the study cellular pathologies associated with PD (reviewed in Tenreiro et al. (2017)). Several molecular aspects of PD have been modelled in yeast, even though yeast lacks orthologs for aSyn. The first PD yeast model was reported in 2003 (Outeiro and Lindquist 2003), and since then a number of different humanized yeast PD models have been developed and employed to investigate PD. Similar to observations in other PD models, aSyn heterologous expression in yeast inhibits cell growth and promotes cell death in a concentration-dependent manner (Outeiro and Lindquist 2003). Independent studies identified mitochondrial dysfunction associated with oxidative stress (Buttner et al. 2008; Sampaio-Marques et al. 2012; Sharma et al. 2006), proteasome impairment (Chen et al. 2005; Sharma et al. 2006), autophagy and mitophagy dysfunction (Petroi et al. 2012; Sampaio-Marques et al. 2012), vesicular trafficking defects (Outeiro and Lindquist 2003) and

ER-to-Golgi trafficking impairment (Cooper et al. 2006) as relevant features of PD (Fig. 9.3). Furthermore, several post-translational modifications such as phosphorylation, ubiquitination, sumoylation and acetylation appear to influence aSyn toxicity and inclusion formation (reviewed in Tenreiro et al. (2017)). Our group was the first to develop a yeast model to study aSyn toxicity during ageing. Most yeast PD models are based on the heterologous expression of human aSyn under the control of a strong *GAL* promoter. Nonetheless, to avoid metabolic manipulations during chronological ageing, the *GAL* promoter was replaced by the *TPI1* promoter, which results in aSyn expression at lower levels when compared to *GAL* promoter, but allows for constitutive expression of aSyn during growth and ageing (Fig. 9.3).

Ageing constitutes a major risk factor for neurodegenerative diseases including PD and other synucleinopathies. By exploring the pathobiology of aSyn during yeast ageing, we observed that aSyn-expressing cells display a dramatic increase of autophagy and particularly of mitophagy that is deleterious for cells and shortens life span (Sampaio-Marques et al. 2012). Although increased autophagy can help aSyn clearance in functionally competent cells, it might also affect autophagy efficiency and selectivity in aged cells that have lost proteostasis. Studies in other cellular models have associated aSyn toxicity with aberrantly high activation of autophagy (Choubey et al. 2011; Stefanis et al. 2001; Xilouri et al. 2009). In addition, our experimental results showed that impairment of mitophagy by deletion of the yeast mitophagy-specific genes, *ATG11* and *ATG32*, resulted in CLS extension, further implicating mitophagy in aSyn toxicity. When exploring the pathways underlying autophagy and mitophagy after they have been aberrantly stimulated, we found that deletion of the *SIR2* gene alleviated aSyn toxicity as evidenced by CLS extension, and this phenomenon is linked to a drastic inhibition of autophagy and mitophagy (Sampaio-Marques et al. 2012) (Fig. 9.3). Notably, Sir2 was determined to be essential for the transcriptional regulation of *ATG8* and *ATG32* in stationary-phase cells expressing aSyn toxic variants (Sampaio-Marques et al. 2012). Our work emphasizes the fact that increased autophagy/mitophagy activity mediated by Sir2-mediated transcriptional regulation of *ATG* genes is an important phenomenon linked to aSyn toxicity during ageing.

In support of an association between exacerbated autophagy and aSyn toxicity, we have also shown that interventions that extend longevity and are associated with autophagy regulation, such as CR and inactivation of the TOR signalling pathway, are able to abolish aSyn toxicity and restore normal chronological longevity by maintaining autophagy at homeostatic levels (Guedes et al. 2017). In general, our results strongly suggest that it is important for life span extension to maintain autophagy under homeostatic levels, as has been reported in other biological systems. Furthermore, together with other findings, our data clearly indicate the relevance of proteostasis control in this age-related disease and confirm the utility of yeast as a model system for investigating different aspects of aSyn toxicity.

In conclusion, yeast cell-based models for neurodegenerative diseases provide valuable tools for deciphering the biological mechanisms of pathogenesis of these diseases as well as the discovery of novel therapeutic targets for treating them. Notably, yeast is also a well-recognized cellular ageing model that makes it possible to inves-

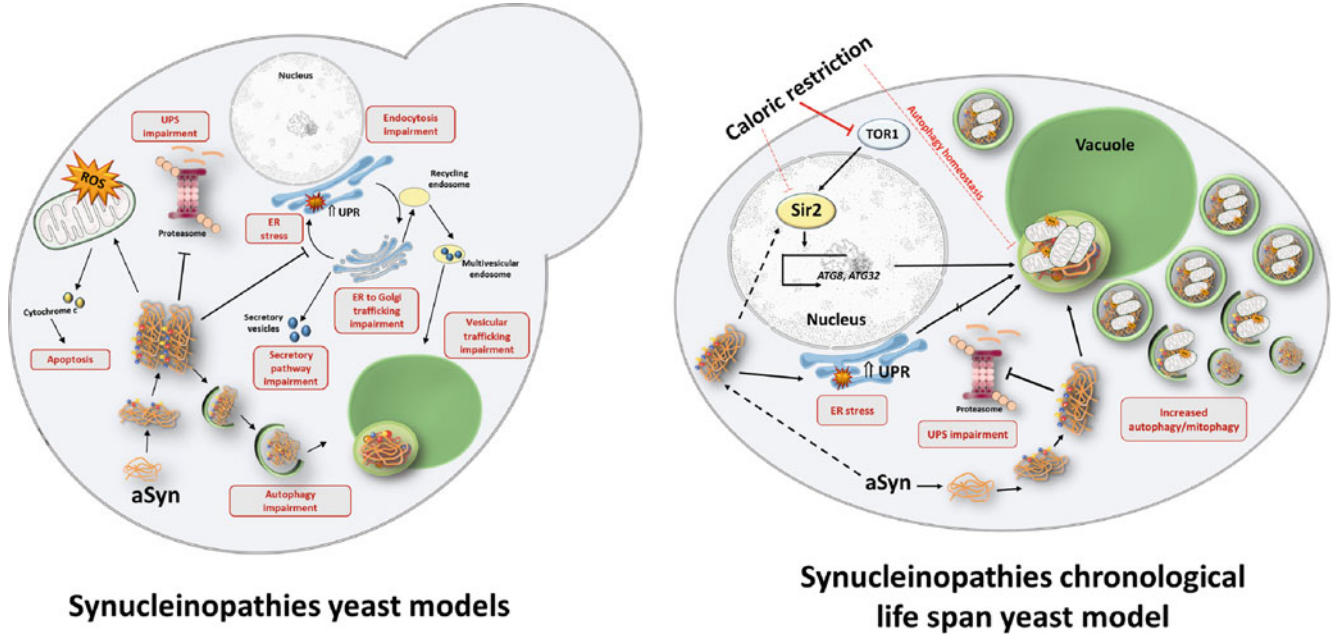

Fig. 9.3 Yeast models for synucleinopathies including Parkinson's disease. Shown are the pivotal pathways associated with aSyn-mediated toxicity in yeast. See text for additional details

tigate ageing as a component of neurodegenerative and age-related diseases in a manner that may not be possible in other cellular and animal models. To date, yeast ageing models have not been used to assess the toxicity of different factors in either HD or AD. This should provide fruitful avenues of investigation of these diseases in the future, as has been the case for Parkinson's and other synucleinopathies.

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