

Chapter 2

Candidate Genes That Affect Aging Through Protein Homeostasis

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Introduction

Aging is a multifactorial and pleiotropic process with many interacting mechanisms contributing to the decline. Because of this complexity, the candidate gene approach to understanding aging often relies on identifying human genes whose function during aging can be predicted based on animal models of aging. Then, association to longevity in humans is determined statistically if a variant of the candidate gene is prevalent among people who live healthy, long lives compared to people with average health span and lifespan. Many of the longevity genes identified thus far influence one of the following pathways: the insulin/insulin-like growth factor pathway, TOR (target of rapamycin) signaling, DNA repair, lipid metabolism, mitochondrial activity and nutrient intake. Understanding how these genetically distinct mechanisms interact at the molecular level to control longevity is a fundamental and fascinating problem, which has been subject to many reviews, e.g. [1–3]. In some cases, as for telomerase, the role in longevity is rather clear—maintaining telomere length [4, 5]. In others, such as mitochondrial activity, the connection is vague, because redox state and reactive oxygen species (ROS) encompass so many aspects of biology. In the case of other candidate genes, for example apolipoprotein E alleles that are prominently identified by genomic association studies [6], their mechanistic contribution to longevity is related to their different biochemical activities and in particular to their differential binding to damaged protein (reviewed recently by

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[7, 8]). Common to all these pathways is their role in cytoprotection against various cellular stresses. Clearly, maximal lifespan extension demands the co-activity of multiple cytoprotective pathways, as emphasized below. In this chapter we focus on the processes that guard the proteome during aging, an aspect that had been considered less than DNA repair or lipid metabolism.

Even at a simple-minded level, it is clear that the various types of stress impact or damage the proteome. Loss of enzymatic activity and translational fidelity, accumulation of somatic mutations, altered post-translational modification, are all ways in which the protein homeostasis network is functionally eroded upon alteration the conditions in either the cytosol, the mitochondria or the endoplasmic reticulum (ER) (Fig. 2.1). Damage to the proteome is intimately linked to the body's stress responses, which deal with altered or damaged proteins by either attempting to repair the damage, dispose of the damaged molecules and/or compensate by increasing expression of the active one.

Cumulative Protein Damage and Aging

Cumulative damage is an often-discussed concept in the context of the genome, but it is clearly also important in the context of the proteome. Sustained exposure to environmental factors like UV radiation or free radicals causes incre-

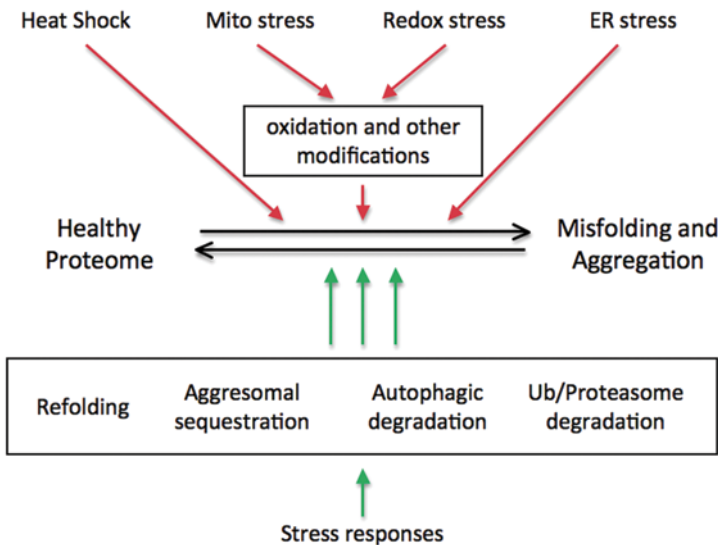


Fig. 2.1 Proteotoxic stresses and stress responses in maintaining a healthy proteome. While heat shock and ER stress largely cause protein misfolding, mitochondrial and other redox stresses mostly cause damaged proteins indirectly, through oxidation and other post-translational modification (*red arrows*). Stress responses, located at the various cellular compartments, control protein misfolding either by refolding, sequestration or degradation (*green arrows*)

mental damage that is proportional to age and is problematic in particular with long-lived proteins. Even normal metabolism associated with daily activities, such as respiration and signaling, interface with stress responses and can cause cumulative protein damage (Fig. 2.1). The late onset of many degenerative diseases is commonly interpreted as reflecting cumulative damage, for example by accumulation of misfolded or aggregated proteins. In addition, proteome analysis by global methods often report on progressively damaged proteins through post-translational modifications that increase with age [9–11]. A remarkable example was provided by two species of bats, which generally live far longer than predicted by their size, relative to other organisms. The proteomes of these bats exhibit significantly lower protein carbonylation, resistance to protein oxidation under conditions of acute oxidative stress, low levels of ubiquitination and reduced proteasome activity [12]. Thus, diminished protein damage and removal are related to longevity, yet much more needs to be learned about the process of cumulative protein damage and how the thresholds are set so as to initiate disease late in life.

The accumulation of damaged proteins with aging is coupled to a progressively decreased ability of the normal quality control mechanisms, as discussed further in subsequent sections. The ability of quality control systems to detect and deal with damaged proteins is important at all times, but becomes particularly critical when the quality control capacity is reduced. For example, the level of expression of molecular chaperones, which assist other proteins to attain or maintain a folded conformation, itself changes with age.

Perhaps the most consistently identified ‘aging gene’ in humans that affects both long lifespan and susceptibility to certain age-related diseases is the apolipoprotein E gene (ApoE) [7, 8]. The inheritance of the three alleles of ApoE correlates with protection or susceptibility to aspects of aging [7, 8, 13]. This correlation has been appreciated since the early 1990s, when a case-control studies found the $\epsilon 4$ allele, a previously known risk factor for cardio-vascular diseases and Alzheimer’s disease, to be significantly less frequent in centenarians than in controls. The ApoE allele most frequent in centenarians was $\epsilon 2$, suggesting that it was protective [14]. The nature of the protective effect of the $\epsilon 2$ allele is not entirely clear, as it has been associated with risk for certain types of ischemic heart disease and hyperlipoproteinemia [15], or found to have no association, positive or negative, with heart disease or stroke [16, 17].

Multiple mechanisms have been proposed to explain the effects of the $\epsilon 4$ isoform of the protein, which differs by 2 amino acids from the $\epsilon 2$ isoform (arginines instead of cysteines at positions 112 and 158). First, hyperlipidemia and hypercholesterolemia resulting from lipid transport abnormalities can directly contribute to heart disease, atherosclerosis, and stroke (reviewed in [7, 8]). In Alzheimer’s disease, ApoE is thought to be involved in binding and clearance of amyloid β (A β). ApoE $\epsilon 4$ has decreased interaction with A β [7, 8] and its deposition into senile plaques is higher in patients carrying the $\epsilon 4$ allele. Its direct role in developing the disease is still not clear, though, as increased deposition of amyloid is also seen in cognitively healthy $\epsilon 4$ carriers [18]. Soluble A β is another potential target of ApoE: in a mouse

model, clearance of soluble A β from the brain depended on the isoform of human ApoE expressed [19]. Alternatively, ApoE ϵ 4 may directly promote A β oligomer formation, more so than ϵ 3 or ϵ 2 [20]. Another hypothesis suggests a negative effect of ApoE ϵ 4 on recycling of the ApoE receptor and thus on neural function [21]. Other possibilities include disruption of neural connectivity, independent of A β ; increased tau hyperphosphorylation, cytoskeletal disruption and mitochondrial dysfunction by the proteolytic fragments of ApoE ϵ 4, decreased protection from excitotoxicity, decreased synaptic function, and other deficiencies [22]. How all these phenotypic changes are caused by the two cys/arg substitutions is not known. One molecular consequence of the substitutions is decreased interaction of N-terminal and C-terminal domains of the ApoE protein, leading to decreased binding to the receptor and possibly to other interacting proteins [7].

It is worth noting that the association of specific alleles of ApoE with the detrimental cognitive phenotypes appears to be age-dependent [23]. For example, the presence of ϵ 4 allele has been associated with better, not worse, memory and neural efficiency in young healthy people [24]. The differential effect of ϵ 4 allele on memory performance depending on age was suggested to indicate antagonistic pleiotropy, a theme common for longevity genes: ApoE ϵ 4 may be beneficial during development and early adulthood, at the expense of accelerated decline in cognitive function with ageing [25].

The action of ApoE exemplifies one type of coping with protein damage, systemic mechanisms that react to extracellular damaged proteins. The main focus of this chapter addresses a different type—intracellular mechanisms that monitor aging-dependent protein damage.

Proteostasis

Proteostasis, or protein homeostasis, is the state of a healthy proteome that is maintained by many sets of interactions among proteins and between proteins and metabolites, which enable the proteome to react to perturbations [26]. Like aging itself, proteostasis is highly complex and involves many hundreds of genes whose products engage in diverse protein complexes and function in many distinct metabolic pathways [26]. The interacting networks include protein synthesis, machineries of post-translational modifications, chaperone machines that assist in proper folding and various disposal systems. These protein networks are usually thought of as ‘stress responses’ (Fig. 2.1), but it is important to emphasize that they operate during normal physiology, even without external stress. Of course, since proteins are made and fold in different cellular compartments that present distinct environments, proteostasis networks in the cytosol, the endoplasmic reticulum and in other organelles have distinct functions and components, which evolved to address the distinct environmental conditions [27].

Also like aging, proteostasis is by definition tissue-specific, and while it has been studied in single cells for a long time, the molecular tools for studying it in whole

organisms are still evolving. Here, we focus on some concepts and challenges linking these longevity pathways to the homeostasis of proteins.

Caloric Restriction

One of the most basic and general factors in determining lifespan is the diet. Much experimental work in animal models has shown that caloric restriction (CR) enables extension of lifespan, and studies in human populations often emphasize the inverse relationship between caloric intake and healthy aging [28]. Much of our understanding of the genetic factors that favor long lifespan also relates the function of these genes to their effects on the metabolism. A simplistic view of caloric restriction is that increased food intake leads to metabolic demands, which over time stress the organism and thus translates to shorter maximal lifespan or to poorer quality of aging. Clearly, sensing the intracellular nutrient and energy status involves many of the arms of the proteostasis signaling pathways in the cytosol, the secretory apparatus and the mitochondria. Caloric restriction has been shown to affect many genes involved in preserving cellular homeostasis during stressful conditions. Such genes, sometimes termed ‘vitagenes’ [29], encompass heat shock proteins (HSP), the thio-redoxin and sirtuin protein systems, nutrient sensing systems like mTOR and redox systems. As discussed below, all these systems affect protein homeostasis.

Protein Misfolding During Aging

Many diseases whose molecular basis is misfolding of proteins are associated with aging and affect almost every tissue. A typical feature of diseases such as Huntington disease, ALS, Alzheimer’s disease or systemic amyloidosis is their onset late in the life of adults. An illustrative example of such diseases is cataract, where most commonly progressive loss of function of the crystallins in the lens leads to visual degeneration. Lens cells lack the machinery for folding and degradation of proteins and are therefore thought to depend on “holdase” chaperone function to prevent protein aggregation. α -crystallin, one of the small heat shock proteins, is the built-in lens chaperone. It is present throughout life in the protected environment of the lens and over time it accumulates damage from oxidation, non-enzymatic modifications and proteolysis. This damage partially unfolds the crystallins and creates aggregation-prone intermediates. In the young lens, α -crystallin sequesters such intermediates effectively, but in an old lens α -crystallin’s capacity to bind polypeptides is saturated, and lens proteins are able to aggregate, promoting light scatter and loss of visual acuity [30].

The presence of insoluble proteins is a common feature of most aging-associated protein misfolding diseases (Fig. 2.1). Alzheimer’s and Parkinson’s diseases, type II diabetes and a host of lesser-known, but often equally serious conditions

such as fatal familial insomnia are characterized by insoluble protein aggregates. These aggregates are found either in the cytoplasm, the nucleus or the endoplasmic reticulum (ER) and are often used in the diagnosis of the diseases [31]. Nonetheless, all too often the data only correlate the aggregates with aging; relatively few studies address whether the misfolded protein itself causes accelerated aging through cellular dysfunction, or whether its misfolding and aggregation are consequences of the progressive decline of proteostasis [32–34]. The increased lifespan of *C. elegans* when aggregation-prone proteins are knocked down [35], or when proteostasis-stimulating compounds are used [36–38], supports the causative role of misfolding/aggregation. However, these approaches do not distinguish between the formation of protein aggregates themselves and accumulation of earlier misfolded intermediates. They also do not distinguish between genetic factors that change the aggregation behavior of the misfolded protein, and factors that change the organismal response to the misfolded protein; these can modify the lifespan independently of each other [39] and further work is needed to distinguish between these mechanisms. Furthermore, more data on the relation between protein aggregation and longevity in mammals is still required to demonstrate evolutionary conservation of the mechanisms that operate in the model genetic organisms [40].

Oxidative Damage to Proteins

Many candidate aging genes involved in the control of redox states and the response to oxidative stress have been highlighted and explored, no doubt because of the impact of the long-standing free radical theory of aging [41]. Oxidative damage to proteins, like damage to nucleic acids and lipids, is mediated by reactive oxygen species (ROS) and increases with age. ROS formation is a highly regulated process controlled by a complex network of intracellular signaling pathways. The intracellular nutrient and energy status of the cell, the functional state of mitochondria, and the concentration of ROS produced in mitochondria, in the cytosol and the ER are all sensed during normal physiology. CR appears to prolong life by reducing reactive oxygen species (ROS)-mediated oxidative damage. However, the correlation between ROS tolerance and lifespan is complex, which at the minimum contradicts the existence of a simple relationship between ROS and aging [41].

Many long-lived mutants are resistant to treatment with ROS, and mutants selected for resistance to ROS are also long lived, but an exhaustive study on the effect of under- or over-expressing a wide variety of mouse genes coding for antioxidant enzymes showed that only the deletion of the *Sod-1* gene affects lifespan [42]. Consistent with this, *C. elegans* devoid of all 5 SOD enzymes have normal lifespan under non-stressful conditions [43].

Numerous antioxidative enzymes (catalases, superoxide dismutases, glutathione enzymes, metal-binding proteins) have provided promising avenues for mechanis-

tic analyses of ROS defenses, but inactivation of such candidate genes has shown at best a modest effect on lifespan. The current opinion is that ROS are not the sole determinant, but only a contributor to aging [41, 44].

Age-Related Decline in the Proteostasis Network

The proteostasis network is designed to react to the stresses that lead to misfolding, to the misfolded protein species, and to the protein aggregates that form [26]. Each of these processes can be countered by different arms of the network: Transcription and synthesis of anti-stress proteins such as folding enzymes or molecular chaperones are activated through the heat shock response (HSR), the unfolded protein response (UPR) in the ER and the mitochondrial UPR; Activities of chaperones are involved in recognition of misfolded molecules; Disposal systems such the proteasome or autophagy are activated to rid the tissues of the toxic misfolded species; Toxic proteins can be segregated to prevent cytotoxic effects.

The cytosolic stress-responsive machinery Since misfolded proteins usually accumulate in the cytosol, even if they originate from the secreted proteome [31] they occupy and titrate cytosolic HSPs, and thereby activate HSF1, the main regulator of the HSR [45]. The activation of HSF1 involves hyper-phosphorylation and deacetylation, allowing HSF1 to trimerize, translocate into the nucleus, and bind to heat-shock elements in the promoter regions of the many HSR target genes. During aging, there is clearly a decline in the capacity of the HSR to counter stress. Up-regulation of the stress-inducible HSP70 by heat shock is reduced approximately 50% in hepatocytes from old rats [46] and this decrease in HSP70 expression is also seen in other cells, such as human mononuclear cells and lymphocytes [47]. Similarly, expression of the other major cytosolic chaperone, HSP90, is reduced in fibroblasts from old rats [48]. The ability of HSF1 to bind the promoters of heat-shock genes in response to increased temperature was absent in the brains of 36 month old rats, while its levels were not affected [49]. In *C. elegans*, the inducibility of HSR becomes impaired in early adulthood [33], and this sharp transition seems to be mediated by the germline stem cell signaling at the onset of reproduction [50].

Expression of many HSPs differs significantly between tissues of ‘normal’ and long-lived Pit1(dw/dw) Snell dwarf mice. The magnitude and direction of the expression changes are tissue-specific and where HSP expression declines, it is often related to GH and/or IGF-I signaling [51]. These observations in mice parallel the role of IGF/insulin signaling in determining worm lifespan. On the other hand, forced overexpression of molecular chaperones is deleterious to growth of normal mammalian cells [52, 53], while the transformed phenotype of cancer cells depends on HSF1 and on an increased expression of chaperones [54–56]. A related example is the expression of the XBP-1 UPR transcription factor. Ectopic expression of the active form of XBP-1 specifically in *C. elegans* muscle reduced lifespan while expression in neurons prolonged longevity [57]. It appears then that the precise

regulation of proteostasis networks is essential for the health of the organism, while accumulating evidence points to its dysregulation during aging as one of the main causes of cellular dysfunction and disease onset (Fig. 2.2).

Molecular chaperones work in transient complexes with co-chaperones and catalytic factors and diminished chaperone capacity is brought about by down-regulation of these components, not only HSP70 and HSP90. For example, HSP110, which suppresses the important pro-apoptotic p38 MAPK stress signaling [58], is down-regulated in livers of three longevity mouse models (Pit1(dw/dw), Prop1(df/df), Ghr^{-/-}), and in both kidney and heart of Pit1(dw/dw) and Ghr^{-/-} [51]. Hsp1 expression is likewise reduced in many tissues of mice exposed to the anti-aging effects of caloric restriction, including liver, heart, white adipose tissue, hypothalamus and colon [59].

Perhaps more importantly than the level of expression, the functionality of HSP70 from old rat livers was half that of HSP70 from young livers, as measured by *in vitro* chaperone assays [60]. The age-associated lower activity is important, because even if there is reduced level of HSP70 or HSP90, they are still highly abundant, accounting for more than 1 % of total cell protein even in aged tissues. Thus, reduced activity attests to reduced chaperone capacity, which may prevent repair of protein damage, leading to degeneration and cell death.

The ER stress-responsive machinery Reduced inducibility of stress responses and reduced levels of chaperones with age are not restricted to the cytosolic proteostasis network. Several studies had documented age-related decline in UPR function, another important arm of the proteostatic network that reacts to stress in the ER. In *C. elegans*, the capacity to activate UPR signaling is strikingly reduced during early adulthood [50, 57] coinciding with the decline of HSR [33], and when the ability to respond is diminished, the extension of lifespan is also diminished.

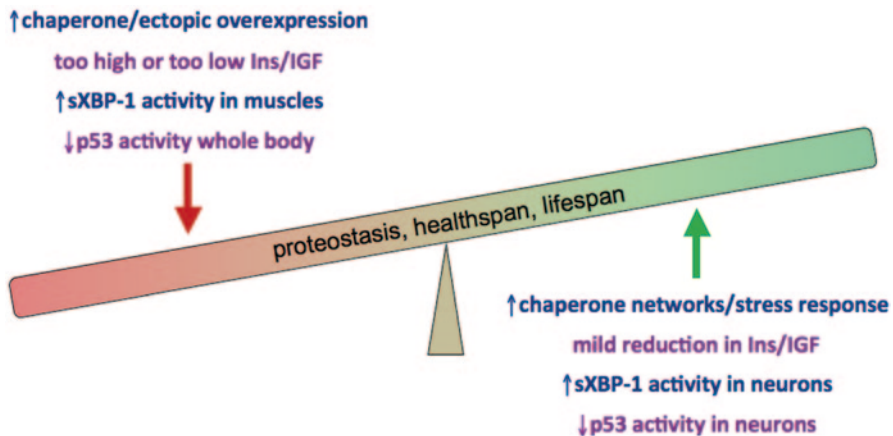


Fig. 2.2 Complexity of the effects of longevity pathways on aging. Examples of how intervention/regulation of transcription factors and chaperones either increase or decrease longevity and its quality

Constitutive expression of ER chaperones and folding enzymes also declines with age. Hepatic expression of BiP, calnexin, ERp57 and ERp72 declined 30–50% in aged rats [61]. Liver expression of the ER gene *dnajc3* (encoding p58IPK) was diminished by all three of the above murine longevity models and was also lower in kidney, muscle, and heart of the *Pit1(dw/dw)* mice. *Dnajc3* is a stress-inducible co-chaperone for the UPR chaperone BiP critical for survival in response to ER stress [62], and there is evidence that this role of *Dnajc3* in cellular stress response can have widespread physiological effects. For instance, mice that lack p58IPK have severely reduced body fat levels, low body weight and are diabetic, most likely due to apoptosis of pancreatic β cells [63]. Since 1/4–1/3 of the proteome is secretory and originates in the ER, and considering the preponderance of membrane and secreted protein among aging-related protein misfolding diseases, these data support the concept that diminished chaperone capacity contributes to the physiological decline associated with aging.

The UPR is implicated in many of these neurodegenerative and aging-related protein folding diseases. Activation of the UPR signaling by accumulation of misfolded proteins (not necessarily aggregates) is well established [64], but the outcomes of this activation are varied. For example, response to most of the misfolded mutants of $\alpha 1$ anti-trypsin results in induction of ER-associated proteasomal degradation (ERAD), but in response to accumulation of the *PiZ* mutant the UPR induces mostly the autophagy mechanism of disposal [65]. In addition, the effects of individual UPR pathways are quite complex and can be disease-specific. For example, deletion of XBP1, the central mediator of the IRE1 pathway, did not influence prion disease progression in animal models [66], but did delay onset and progression of ALS and HD in respective mouse models by stimulating autophagy [67].

Proteasomes The proteasome is a multi-catalytic enzyme complex that mediates the degradation of both normal and damaged proteins. An age-related decline in proteasomal activity has been implicated in various age-related pathologies. Tomaru *et al.* created a transgenic mouse model with decreased proteasomal chymotrypsin-like activity, by over-expression of a thymus-specific subunit $\beta 5$ that has weak chymotryptic activity [68]. These mice exhibited a shortened life span and developed age-related phenotypes. Poly-ubiquitinated and oxidized proteins accumulated in these mice, and the expression levels of cellular proteins such as Bcl-xL and RNase L were altered. When fed a high-fat diet, the $\beta 5$ transgenic mice developed more pronounced obesity and hepatic steatosis than did wild-type mice. These results provide *in vivo* evidence that decreased proteasomal chymotrypsin-like activity affects longevity and aggravates age-related metabolic disorders [68]. In *C. elegans*, adaptation of peptide accessibility to the proteasome's active site in response to environmental stress, through modifying its 19 S regulatory particle, is necessary for both resistance to proteotoxic insults and maintenance of animal life span under normal conditions [69].

Although proteasome activity is diminished when poly-ubiquitinated substrates accumulate, Hipp *et al* showed that an aggregated ubiquitinated substrate is not a direct inhibitor of proteasomes, but rather impairs degradation by saturating some as-yet-unidentified component [70].

At least one signaling pathway for proteasome dysfunction is through reactive oxygen species that originate from malfunctioning mitochondria and trigger an Nrf2-dependent up-regulation of the proteasomal subunits. This is a tissue- and age-specific regulatory circuit that adjusts the cellular proteasome activity according to temporal and/or spatial demands [71].

Proteasomes and aggresomes are likely complementary quality control pathways, since aggresome formation/growth is promoted when proteasomes are inhibited [72]. Each of these arms of the proteostasis network declines with age, thus diminishing the response to metabolic stress or to accumulating misfolded proteins and reducing the chances of return to homeostasis.

Autophagy An independent way to dispose damaged proteins is the process of autophagy, where whole organelles can be engulfed by membrane, the contents of which then becomes an acidic degradative compartment. The formation of autophagosome requires a dedicated machinery that includes chaperones, and their relevance to aging is demonstrated by mutational inactivation of autophagy genes, which accelerates tissues aging in *C. elegans* and *Drosophila*. [73]. Inactivation of autophagy also suppresses life span extension by CR, aberrant insulin/IGF-1 or mTOR signaling, and lowers mitochondrial respiration. These findings suggest that the different longevity pathways converge on the autophagy pathway to slow down aging and increase life span. Thus, autophagy is one central effector mechanism of animal aging [74].

Aggresomes One way to cope with accumulated misfolded proteins is to segregate them in cytosolic protein inclusions, termed ‘aggresomes’, that are usually perinuclear, near the microtubule organizing center and whose formation and maintenance depends on microtubule-based protein traffic [75]. Aggresomes often contain aging-relevant misfolded proteins and were suggested as a coping mechanism to protect cells from the cytotoxic effects of misfolded proteins [31]. One line of evidence supporting this notion is the enhanced proteo-toxicity in a yeast poly-Q model when aggresome formation is inhibited [76]. Targeting misfolded proteins to aggresomes seem to depend on recognition signals, like proline-rich domains, present on some aggregation-prone proteins. Another signal that is likely involved in the formation of aggresomes is the ankyrin-like repeat in synphilin 1, a protein related to Parkinson Disease [77]. It is not currently known how the aggresome formation machinery changes with age.

Proteostasis in Somatic Tissues Vs. the Gonads

Hallmarks of somatic tissues of aging flies are gradual accumulation of ubiquitinated and carbonylated proteins and reduction of proteasome expression and activity. In contrast, gonads of aging flies were relatively free of proteome oxidative damage and maintained substantial and highly active proteasomes. Exposure of flies to oxidants induced higher proteasome activities in the gonads, which were,

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