AN ABSTRACT OF THE DISSERTATION OF

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Title: Long-lived species are more resistant to polyQ82 toxicity than short-lived species: Role of robust protein quality control mechanisms and aggresome-like inclusions

Abstract approved:

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Aging is a multifactorial process that affects most if not all-living organisms. It is characterized by a progressive loss of physiological function leading to increased vulnerability to death. In humans, aging is the largest risk factor for many of chronic diseases. For decades many hypotheses have been proposed to explain the process of aging. With the purpose to unify or categorize the cellular mechanisms that contribute to the aging phenotype, a set of hallmarks (or pillars) of aging has been proposed. These hallmarks are conserved longevity pathways identified across disparate eukaryotic species, and have to fulfill some requirements; i.e., the activity of these pathways should decline with normal aging, their experimental aggravation should accelerate aging,

and their experimental activation retard the normal aging process (Kennedy et al., 2014; López-Otín et al., 2013).

One of these hallmarks of aging is protein homeostasis or proteostasis. Proteostasis involves mechanisms that work together to ensure the health of the cell's proteome. The extensive work done in several animal models, such as *C. elegans, Drosophila,* and mice, has shown that the capacity of cells to maintain proteostasis declines with age, thereby giving rise to a rapid accumulation of damaged proteins and protein aggregates that are harmful to cells function (Koga et al., 2011; Martinez-Vicente et al., 2005). However, all such studies have been conducted in short-lived species, and not much is known about the importance of proteostasis in long-lived species, species which live longer than expected for their body size.

Previous evidence found that liver tissue from the long-lived naked mole-rat (MR) showed less protein ubiquitination and higher proteasome activity compared to mice and that their proteome was more resistant to urea-induced unfolding (Pérez et al., 2009a). These were the first studies indicating that protein homeostasis could be an important mechanism to explain successful aging in long-lived species. Based upon these research findings, my Ph.D. dissertation expands this work and critically evaluates the role of protein homeostasis in species longevity.

This dissertation presents two chapters of research where a comparative biology approach is used to study the differences in proteostasis between

short-lived and long-lived mammalian species. My research expands the previous work to three phylogenetic clades to ensure generality of our conclusions for differences in long-lived and short-lived species for entire mammalian class.

In chapter 2 the experiments were performed to study differences in protein quality control processes between short- and long-lived mammals from three phylogenetic clades. A clade is a group of organisms that consist of a common ancestor and all its lineal descendants (Assembling tree of life, 2004). The three phylogenetic clades and the animals in each clade were chosen based on the following criteria: a) longevity record that had been determined from a large number of individual records; b) species which are exceptionally long- or short-lived for their body size; c) species with a broad phylogenetic coverage within mammals to ensure generality of my conclusion; d) similar body size because body size can independently affect some biological parameters (Lindstedt and Calder, 1981). I chose the cell culture model because this system allows to measure directly the characteristics of aggregation and the rates of protein degradation in these different species. I recognize that the skin fibroblasts may have limitations in terms of being representative of other cell types in the body; however, the ease of isolating fibroblasts, ready standardization of culture procedures, and widespread use of fibroblasts in research (including studies in comparative biology of aging) makes it a good choice for my experiments. The results obtained in chapter two showed that in

general, long-lived species have much higher protein quality control when compared to their respective short-lived counterparts.

In chapter 3, we tested whether the robust protein quality control activities in long-lived species confer more protection against toxic misfolded protein. For this purpose, poly-glutamine 82-YFP (polyQ82-YFP), a well-established model of protein aggregation, was used. I found that fibroblasts from long-lived species are more resistant to proteotoxicity induced by polyQ82-YFP compared to those of short-lived species. Surprisingly, I found that the protein aggregation (inclusions) in fibroblasts of long-lived species was not any lower when compared to those of short-lived species. In addition to this, these inclusions were located at the juxta-nuclear region and formed at a lower mean cell fluorescence (lower concentration), indicating that inclusion formation could be serving a protective function in long-lived species. I also identified that polyQ82 inclusions in MR fibroblasts co-localize with HSP27, suggesting a possible role of this small heat shock protein in providing protection against misfolded protein toxicity.

Here I identified that protein quality control mechanisms in general are more robust in fibroblasts of long-lived species than short-lived species. In addition, I discovered that fibroblasts from long-lived mammals are more adept at resisting misfolded protein toxicity. Based on my data, I propose a candidate mechanism that can be responsible for this protection, the aggresome–like inclusions, where inclusions in long-lived species form at lower fluorescence, are predominantly juxtanuclear and co-localize with HSP27 unlike those of

short-lived species. Overall, my work is a stepping-stone in identifying key players that confer protection against misfolded proteins in long-lived species.

Long-lived species are more resistant to polyQ82 toxicity than short-lived species: Role of robust protein quality control mechanisms and aggresome-like inclusions

by
Bharath Sunchu

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Bharath Sunchu, Author

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CONTRIBUTION OF AUTHORS

Chapter 2: Pride, H., Sunchu, B., and Perez, V.I. designed experiments. Pride, H., Yu, Z., Sunchu, B., Mochnick, J., Coles, A., and Perez, V.I. performed experiments. Sunchu, B., Yu, Z., Mochnick, J., and Perez, V.I. analyzed the data. Sunchu, B., and Perez, V.I. wrote the manuscript.

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Chapter 1

Introduction

1.1 Aging

1.1.1 Lifespan and healthspan

Human lifespan has increased tremendously over the last few decades, from an average of 47 years in 1910 to 78 years in 2010 (Crimmins, 2015; Oeppen and Vaupel, 2002). This increase in longevity is because of a decline in infant mortality, improved sanitation and the discovery of antibiotics in the first half of the 20th century, and improvements in health care for the elderly in the second half (Lunenfeld, 2008). Although healthspan, the number of years lived healthily without any chronic disease, has also increased, it didn't keep pace with increase in lifespan. This is because chronic diseases became more prevalent as humans are living well into old age (Crimmins, 2015).

1.1.2 Aging- a major risk factor for chronic diseases

This increase in incidence of chronic diseases is because aging is the biggest risk factor for many, if not all, chronic diseases (Kennedy et al., 2014; Niccoli and Partridge, 2012). This in combination with population aging, the shift towards an increased proportion of older persons in the population, poses a huge economic problem. Population aging is a global phenomenon in the 21st century resulting from reduction in mortality coupled with increased longevity, and a decrease in the proportion of young individuals due to decrease in reproductive fertility rates (Lunenfeld, 2008; Lutz et al., 2008). The share of the population aged 60 and over is projected to increase in nearly

every country in the next few decades (Bloom et al., 2010). Therefore, in the coming decades it is expected that there will be a significant increase in the proportion of population suffering from one or more chronic illnesses. Thus the demand on the health care to help manage the chronic conditions of an aging population and loss of revenue associated with a less productive population poses a world economic problem (Chatterji et al., 2008; Hashimoto and Tabata, 2010). Hence it is necessary to understand the molecular mechanisms of the aging process, to delay the onset of chronic diseases and decelerate the decline of health triggered by aging.

1.2 Hallmarks of aging

Significant developments in aging research only began in the 20th century, when the first long-lived strains in *Caenorhabditis elegans* was identified (Klass, 1983). Well before this in 1935, Clive McCay and his colleagues demonstrated life span extension in rats by caloric restriction (McCay et al., 1989). These discoveries suggested that the process of aging is malleable and therefore can be extended. With a rapid increase in knowledge about different processes that modify aging, measured as maximum longevity, it was essential to identify a set of hallmarks of aging (figure 1.1). The idea is to help classify or unify all identified candidate pathways within biological categories that lead to and facilitate studying the relation between each of these processes and their relative role in contributing to aging. These hallmarks of aging are mechanisms that are shown to be important in contributing to the aging

process. In an extraordinary effort to put together these hallmarks, two groups independently, one from Europe and one from the United States, published seminal papers where a set of hallmarks of aging are identified (Kennedy et al., 2014; López-Otín et al., 2013). To be classified as a hallmark of aging, a cellular and molecular process has to a) alterations or loss in function that should manifest with age b) disrupting its activity experimentally should accelerate aging and c) enhancing its activity or intervening in its decline should increase health span. In these two articles, a set of 7 and 9 hallmarks of aging are proposed (Kennedy et al., 2014; López-Otín et al., 2013, respectively) (figure 1.1). One of the common hallmarks of aging mentioned in both papers is a loss of protein homeostasis or proteostasis.

1.3 Proteostasis

Proteostasis involves many processes that together maintain the stability and functionality of the cellular proteome and hence these systems are crucial for cellular and organismal health. Proteostasis meets all the criteria to be a hallmark of aging. Proteostasis is shown to decline with age in several organisms (Bulteau et al., 2002; Hayashi and Goto, 1998; Keller et al., 2000a; Koga et al., 2011; Martinez-Vicente et al., 2005; Terman, 1995). Moreover, many studies have shown that inhibition of proteostasis processes accelerates aging and its activation increases lifespan and/or health span (Bedford et al., 2008; Bergamini et al., 2004; Kruegel et al., 2011; Lee et al., 2010; Massey et al., 2008; Morley and Morimoto, 2004).

Proteins are structurally and functionally very diverse macromolecules. They are structurally complex and participate in almost every biological process. The cell's "proteome" consists of more than 10,000 different proteins. Most of these proteins must be folded properly into their native state, an ensemble of a few closely related three-dimensional structures (3D). In addition to this 20 to 30% of proteins are intrinsically devoid of any 3D structure and abnormal behavior of such proteins could be lethal to cell and organism (Hartl et al., 2011).

Proteostasis Network (PN) involves translation (synthesis of a nascent peptide), protein folding, protein trafficking and protein degradation (Powers et al., 2009). PN works to maintain and renew the cellular proteome. Under stress conditions, when proteins get unfolded or damaged, these machineries work to either refold or degrade the proteins to prevent accumulation of toxic misfolded proteins and aggregates. Within the PN, much research and lot of emphasis in literature has been placed on molecular chaperones, the ubiquitin-proteasome system, and autophagy, that are key components of protein quality control and are referred collectively as "Protein Quality Control Processes" (PQCPs). However more recently, other mechanisms, such as AAA+ proteases and unfolded protein response (UPR) has been recognized as part of this PQCPs (Kaushik and Cuervo, 2015).

1.3.1 Chaperones

Chaperones are small proteins that play key roles in maintenance of protein homeostasis. They help with protein folding, refolding of damaged proteins, protein trafficking, and protein degradation (Becker and Craig, 1994; Ellis, 1987; Hartl et al., 2011). Most proteins to function properly should be in their 'native state', a group of few closely related three-dimensional structures (Bartlett and Radford, 2009). The amino acid sequence of the protein provides the information for folding of the nascent polypeptide chain (Dobson et al., 1998). However many proteins require molecular chaperones to fold properly and fast (Hartl, 1996). It is known from *in vitro* studies that proteins, especially large ones, take minutes to hours to fold in dilute buffer solutions and some proteins even fail to reach their native state (Herbst et al., 1997; Kubelka et al., 2004). Partially folded states need to traverse the free energy barriers to reach native state, and molecular chaperones help in accelerating this process (Hartl et al., 2011). Furthermore, folding of the polypeptide chain becomes more challenging in a crowded cellular environment, where there is a constant danger of unwanted inter- and intra-molecular interactions via hydrophobic regions in the non-native and structurally flexible proteins (Ellis and Minton, 2006). Chaperones shield these hydrophobic regions to prevent protein aggregation and maintain proteins in a soluble yet conformationally dynamic state(Hartl and Hayer-Hartl, 2009; Hoffmann et al., 2010).

The most studied and well-known type of chaperones are those chaperones whose expression are induced by stress, or commonly called stress proteins or

heat shock proteins (HSPs). HSPs were first discovered after their expression under heat stress, hence the name heat shock proteins. However, now is it known that these chaperones are also induced by other types of cellular stress such as anoxia, ischemia, heavy metal ions, ethanol, nicotine, surgical stress, and viral agents (Whitley et al., 1999). Chaperones are usually identified based on their molecular weight. For example, the 70 kDa protein is referred to as HSP70 (Brandvold and Morimoto, 2015). Chaperones such as HSP70s and HSP90s are multi-component molecular machines that promote folding through ATP- and cofactor- regulated binding and release cycles. The main function of chaperones such as HSP90 and HSP70 is to help in *de novo* protein folding and refolding. They work through ATP-regulated binding and release cycles (Lotz et al., 2010; Rüdiger et al., 1997; Taipale et al., 2010). They typically recognize hydrophobic amino acid chains exposed by non-native proteins and functionally cooperate with ATP-independent chaperones, such as small HSPs such as HSP27, which function as 'holdases', buffering aggregates. In the ATP-dependent mechanism of chaperone action, *de novo* folding, and protein refolding is promoted through kinetic partitioning. Chaperone binding (or rebinding) to hydrophobic regions of a non-native protein blocks aggregation; ATP-triggered release allows folding to proceed (Frydman et al., 1994). Small heat shock chaperones that serve as "holdases", bind to hydrophobic regions of non-native protein substrates and prevent their irreversible aggregation. Small HSPs are ATP-independent molecular chaperones and assist in managing of unfolded proteins, independently or

under the help of other ATP-dependent chaperones. The mechanism of these sHSPs are not yet clear (Fu, 2014). Thus most of the proteins are thermodynamically unstable and need assistance to retain their native form. Overall molecular chaperones are important for protein folding, refolding and in preventing protein aggregation (Hartl et al., 2011; Kopito, 2000; Kubelka et al., 2004).

1.3.2 Protein degradation mechanisms

If chaperones are unable to rescue a misfolded/unfolded protein, they have to be degraded to prevent toxicity and accumulation of protein aggregates (Cuervo, 2008; DeMartino and Gillette, 2007). There are two main protein degradation mechanisms: the ubiquitin proteasome system (UPS), and autophagy (Martinez-Vicente et al., 2005). They help in recycling of functionally expired proteins, degradation of damaged and/or misfolded proteins and degradation of protein aggregates. As proteins are involved in almost all biological processes, it is important that the unused proteins are recycled promptly so that the amino acids can be used to synthesize new proteins. Proteins are also degraded to meet energetic needs of the cell. *De novo* synthesis of amino acids are energetically expensive; hence recycling of amino acids through protein degradation mechanisms results in less energy expenditure (Rabinowitz and White, 2010). Protein aggregates can be degraded mostly by a process called autophagy, although in certain cases proteasomes also degrade protein aggregates with the help of disaggregases

(Iwata et al., 2009). Ubiquitin proteasome system degrades 80% of cellular proteins whereas autophagy degrades long-lived proteins in addition to protein aggregates, and organelles like mitochondria, ribosomes, and peroxisomes (Martinez-Vicente et al., 2005).

1.3.2.1 Ubiquitin Proteasome System (UPS)

Proteolysis by UPS involves two major steps: 1) Ubiquitination of the target proteins, and 2) degradation of the ubiquitinated proteins by the 26S proteasome (figure 1.2). At least four molecules of ubiquitin (ub) forming a tetra-Ub chain need to be attached to the target protein for it to be recognized by the proteasome (Cook et al., 1994). Ubiquitin is a highly conserved small protein with 76 amino acids. Three enzymes are involved in protein ubiquitination, Ub-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3). E1 activates ubiquitin in an ATP-dependent manner by forming a thiol ester bond at its c-terminus. Activated ubiquitin is then transferred to several distinct E2 enzymes. Finally, the ubiquitin is transferred to lysine of the target protein with the help of E3. Such ubiquitinated targets are degraded by the 26S proteasome. The 26S proteasome is made up of a 20S catalytic core which is bound on either side by 19S regulatory caps (DeMartino and Gillette, 2007; Pickart and Cohen, 2004). The 20S proteasome has four stacked rings that form a barrel shaped structure with a central cavity. The outer alpha rings are non-catalytic and inner beta rings are catalytic (DeMartino and Gillette, 2007). The 20S proteasome has three proteolytic activities: 1) chymotrypsin-like

(cleavage after hydrophobic amino acids), caspase-like (cleavage after acidic residues) and trypsin-like (cleavage after basic residues). The 19S regulatory cap is involved in binding and unfolding of ubiquitinated proteins and opening of the alpha sub-unit gate of the 20S proteasome to allow for entry of de-ubiquitinated proteins into the catalytic core. The protein is degraded in the catalytic core and small peptides and/or amino acids are ejected out of the proteasome (Chen and Dou, 2010; Collins and Goldberg, 2017).

1.3.2.2 Autophagy

The term autophagy means “eating of one self”, derived from the Greek words *auto*=self, and *phagos*=eat. It involves degradation of cytoplasmic contents through a lysosomal pathway. It recycles long-lived proteins, macro molecules, and organelles including mitochondria, ribosomes, peroxisomes and endoplasmic reticulum (Cuervo, 2008; Mizushima et al., 2008). There are three forms of autophagy; microautophagy, chaperone-mediated autophagy (CMA, and macroautophagy (Cuervo, 2004) (figure 1.3). Microautophagy involves direct ingestion of cytoplasmic contents by the lysosome through either membrane invaginations or projected arm-like protrusions(Li et al., 2012). These invaginations or protrusions differentiate into the autophagic tube to enclose small portions of cytoplasm. Vesicles which form at the top of these tubes fuse, completing the engulfment (Li et al., 2012). CMA involves direct translocation of proteins which contain a KERFQ sequence by protein chaperones to the lysosomes (Dice, 2007). The substrate/chaperone complex

binds to the lysosome-associated membrane protein type 2A (LAMP-2A), a receptor for CMA on the lysosome surface. Once bound, the substrate unfolds and crosses the lysosomal membrane into the lysosome where it is degraded (Dice, 2007). In macroautophagy, quantitatively the most important process, entire regions of cytosol are engulfed by *de novo*-synthesized vesicle, the autophagosome, which fuses with the lysosome acquiring the required proteases and other degradation enzymes. The autophagosome is a double membraned structure which sequesters cytoplasmic contents. Macroautophagy starts with a *de novo* formation of the isolation membrane or phagophore. This phagophore expands to surround a portion of cytoplasm resulting in the formation of autophagosome. This autophagosome then fuses with a lysosome to form an autolysosome with which lysosome enzymes degrade the contents (Rajawat et al., 2009).

1.3.3 Protein aggregation

Protein aggregation is triggered by inter-molecular interactions of surface exposed hydrophobic residues on misfolded/unfolded proteins, which are normally buried inside in their native form (Kopito, 2000; Tyedmers et al., 2010). If chaperones cannot help in refolding the protein into its native form, and the degradation systems are unable to degrade such protein, then misfolded/unfolded proteins tend to form aggregates (Ellis and Minton, 2006). Protein aggregation is a sign of loss of protein homeostasis. Protein aggregation is well known to be associated with various diseases where the loss

of proteostasis take place, such as neurodegenerative diseases, diabetes-2, cystic fibrosis, cancer, etc. (Dillin and Cohen, 2011; Ross and Poirier, 2004). For all these diseases, aging is the major risk factor (Niccoli and Partridge, 2012). Furthermore, recent research has revealed that even in the absence of any chronic disease, there is an increase in insoluble protein or protein aggregate content with normal aging. Thus, there is a strong relationship between aging, loss of proteostasis, and chronic diseases, and therefore it is important to study it.

1.4 Proteostasis and aging

The interest on studying proteostasis with respect to aging was developed with discovery of and increasing knowledge about protein quality control processes (Deter et al., 1967; Ellis, 1987; Tanaka et al., 1983). This was followed by observations that the rate of protein degradation decreases with age which was later related to the decline in activity of autophagy and ub-proteasome systems (Bergamini et al., 2004; Bulteau et al., 2002; Carrard et al., 2002; Cuervo and Dice, 1998, 2000a; Hayashi and Goto, 1998; Keller et al., 2000a; Makrides, 1983; Martinez-Vicente et al., 2005; Terman, 1995; Terman and Brunk, 2004; Viteri et al., 2004). Similar observations where decline in levels of molecular chaperones with age were made in *C. elegans* and rodents (Hall et al., 2000; Heydari et al., 2000; Yang et al., 2014). Studies involving pharmacological and genetic manipulations of PQCPs further support the notion that chaperones and protein degradation pathways play a key role in health and

longevity (Eisenberg et al., 2009; Hsu et al., 2003; Lee et al., 2010; Min et al., 2008; Morley and Morimoto, 2004; Morrow et al., 2004; O'Rourke et al., 2013; Vilchez et al., 2012; Walker and Lithgow, 2003; Zhang and Cuervo, 2008). For example, Eisenberg et al., have shown in that spermidine treatment enhanced longevity in yeasts by inducing autophagy (Eisenberg et al., 2009). In another example, overexpression of HSF1 extended life span in *C. elegans* (Hsu et al., 2003). There is also evidence in mouse models, for example, when CMA activity which declines with age, was maintained in a transgenic mouse model there was improvement in organ function (Zhang and Cuervo, 2008).

Although protein aggregates were long known to be characteristics of many age-related diseases (i.e. neurodegenerative diseases), the accumulation of protein aggregates with normal aging was also observed in Drosophila, *C. elegans*, and mouse animal models (David et al., 2010; Demontis and Perrimon, 2010; Reis-Rodrigues et al., 2012), suggesting that the decline in protein homeostasis is a general phenomenon with age, and not linked only to disease state. There is also ample evidence indicating that protein aggregation plays an important role in longevity. For example, genetic manipulations in *C. elegans* that decrease protein aggregation correlates with increased longevity (Cohen et al., 2006; Hsu et al., 2003; Morley et al., 2002; Morley and Morimoto, 2004).

In summary, there is meaningful scientific evidence that support the role of protein homeostasis in longevity. However, all the above observations were made using the most common animal models for aging research (*C. elegans*,

drosophila, mice); each of which have a considerably short lifespan. However, increase in longevity resulting from experimental manipulations in these short-lived species is much more than the variations in maximum life span (MLS) observed among species in nature. More importantly, we humans live longer than predicted for our body size. Hence it is important to investigate whether protein homeostasis mechanisms are important processes that are common to evolution of long-lived species or if it is only favorable in species that have a short life.

1.5 Comparative biology

As I described in the section above, a significant amount of research has been done to study the role of proteostasis in aging. It has been shown that manipulations which enhance activity of PQCPs result in increased longevity, and decreased protein aggregation (Hsu et al., 2003; Morley and Morimoto, 2004; Zhang and Cuervo, 2008). However, a major share of this entire body of research has used short-lived animal models such as *C. elegans*, mice, and fruit flies. But, there are very little data on the role protein homeostasis plays in species longevity. Maximum species lifespan increases in a predictable manner as species increase in body size (Lindstedt and Calder, 1981). Long-lived species are those that show exceptional longevity than predicted for their body size (Buffenstein, 2005).

The two major reasons why studying the role of proteostasis (or any process that alters aging) of long-lived species is important are: 1) Studying long-lived

species we will get access to the pathways that evolution picked to increase lifespan/health span in long-lived species, and 2) As humans are long-lived, information obtained from other long-lived species will allow such evolution-tested information to be more useful and easy to apply in humans. Comparative biology of aging involves comparing short-lived and long-lived species with an objective to identify process that are more robust and contribute to longevity of long-lived species. Average lifespan of species increases with increase in their body size. However, there are some exceptions to this rule and some species live well beyond their expected lifespan based on their body size. Naked mole rats, MRs (*Heterocephalus glaber*) are mouse-sized (~35 grams) rodents that, in captivity, show exceptional longevity, living more than 28.3 years (Buffenstein, 2005). The relationship between life span and body size is usually explained in terms of *longevity quotient*. Longevity quotient of a species is the ratio of its maximum species lifespan to expected lifespan based on the body size. If the longevity quotient of a species is well above 1, it is considered as a long-lived species. So in this example, MRs have a longevity quotient around 5 (Buffenstein, 2005; Lindstedt and Calder, 1981). More importantly, besides the extraordinary longevity, MRs are able to maintain normal activity, with no obvious age-related increases in morbidity, around 80% of their lives (Edrey et al., 2011).

In my dissertation, I used a comparative biology approach, where I compared short- and long-lived species to investigate the role of proteostasis in protein aggregation in long-lived species. To do this we compared long- and short-

lived species belonging to same phylogenetic clade and similar body size to remove any confounding factors.

1.6 Dissertation hypothesis and Specific aims

The research on role on proteostasis in aging has primarily focused on working with short-lived models. Using comparative biology to study proteostasis in long-lived species is a relatively new approach and there is a lot of ground to cover. Research in this field so far has shown that fibroblasts from long-lived species are more resistant to different type of stressors than those of short-lived species (Elbourkadi et al., 2014; Harper et al., 2011), and the level of protein damage is less in long-lived species in the presence of different stressors (Pickering et al., 2014). In addition to this, previous work has shown that proteomes from MR liver homogenates are more resistant to urea-induced unfolding when compared to mouse (Pérez et al., 2009a). However, there is an enormous *gap in knowledge* in identifying components and mechanisms of proteostasis that play a key role in longevity of long-lived species. Therefore, the objective of my dissertation is to assess differences in PQCPs and protein aggregation between short- and long-lived species, and our general hypothesis of this work is that skin fibroblasts of long-lived species have superior PQCPs and have better resistance to form protein aggregation when compared to short-lived species.

We expected that the work done in this dissertation will provide a better understanding of the role of PQCPs and protein aggregation in aging and longevity. Moreover, the identification of these key longevity-contributing mechanisms is essential as they serve as a viable tool to enhance health span in humans.

To test my hypothesis, I pursued the following **specific aims**.

1.6.1 Specific aim1: To determine whether enhanced activity of protein quality control processes is common in long-lived species.

We hypothesized that fibroblasts of long-lived species have robust protein quality control processes (PQCPs) when compared to short-lived species. For this purpose we used skin fibroblasts from short- and long-lived species belonging to three different clades, which all belong to class mammalia. The objective was to identify if robust protein quality control is common to all long-lived species. Short-lived species and long-lived species belonged rodents, bats and marsupials. For rodents I used laboratory mice [*Mus musculus*, 35 g and 4 years], vs. naked Mole Rats (MRs) [*Heterocephalus glaber*, 30 g and ~30 years], for bats I used Evening Bat (EB) [*Nycticeius humeralis*, 11 g and 6 years] vs. Little Brown Bat (LBB) [*Myotis lucifugus*. 8 g and 34 years], and for marsupials, laboratory opossum (opo) [*Monodelphis domestica*, 150 g and 4.75 years] vs. sugar glider (SG) [*Petaurus brevicaudus*, 100 gand 18 years]. To test our hypothesis, we compared the activities of following three protein quality control processes.

i) Autophagy - LC3II/LCI ratio and pulse chase experiments were used to measure autophagy activity under both serum and serum free conditions.

ii) Proteasome – A fluorometric assay and western blot were used to measure the activity/levels of proteasome under both serum and serum-free conditions.

iii) Heat shock chaperones – Western blot was used to measure levels of HSP90, HSP70, HSP40 and HSP27 under both basal and heat shock conditions.

Specific aim2: To determine if there are any differences in protein aggregation between fibroblasts of short- and long- lived species.

We hypothesized that fibroblasts from long-lived species will have less protein aggregation and be more resistant to proteotoxicity induced by an aggregation prone protein model. For this purpose, I used fluorescent tagged aggregation prone polyQ model to study differences in aggregation in skin fibroblasts of long-lived and short-lived species. For this specific aim we explored following questions: Are fibroblasts from long-lived species more resistant to proteotoxicity induced by polyQ82-YFP? Do they have less protein aggregation when compared to short-lived species? High content imager (HCI) experiments on polyQ-YFP transfected fibroblasts were used to answer these two questions.

What is the role of each proteostatic mechanism (autophagy, Ub-proteasome, and chaperones) in providing resistance in polyQ82-YFP in long-lived fibroblasts? siRNA knock down was used to identify the importance of autophagy, proteasome, and HSP27 for survival after polyQ expression.

1.7 Figures

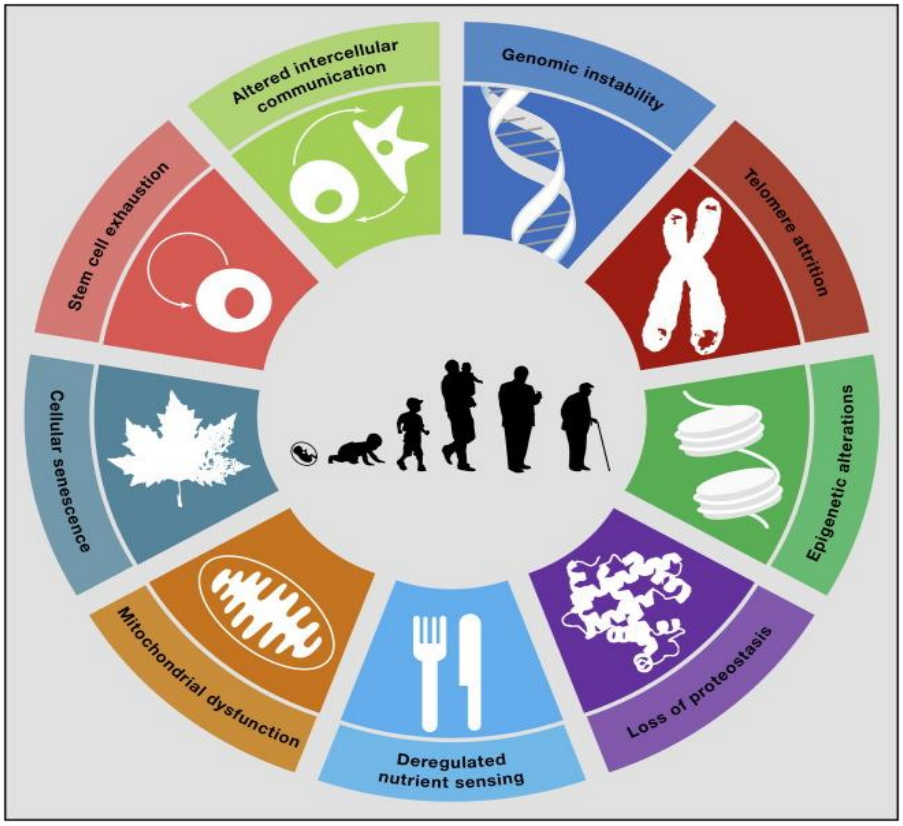

Figure 1.1. Hallmarks of aging. Cellular and molecular processes that are generally considered to contribute to the aging process. Proteostasis is one of the hallmarks of aging.

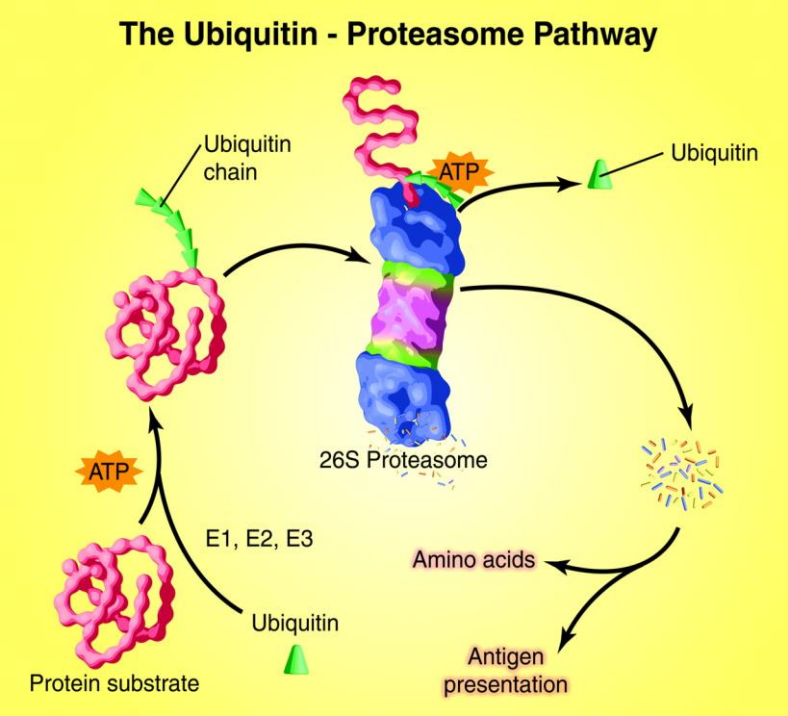

Figure 1.2. The ubiquitin (Ub)-proteasome pathway. Ub is conjugated to proteins that are targeted for degradation by an ATP-dependent process that involves three enzymes. A chain of five Ub molecules attached to the protein substrate is sufficient for the complex to be recognized by the 26S proteasome. In addition to ATP-dependent reactions, Ub is removed and the protein is linearized and injected into the central core of the proteasome, where it is digested to peptides. The peptides are degraded to amino acids by peptidases in the cytoplasm or used in antigen presentation.

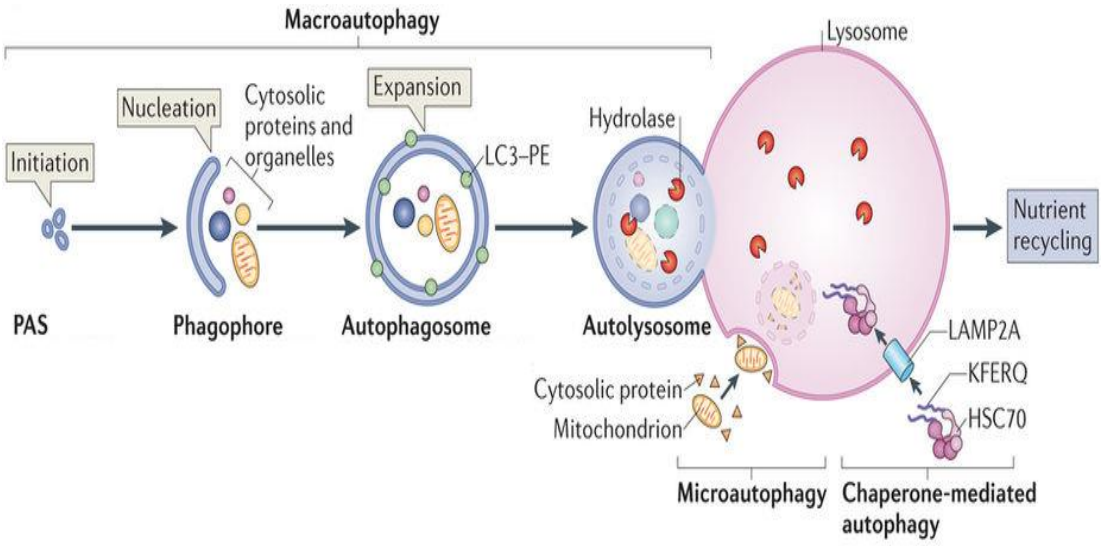

Figure 1.3. Types of autophagy. There are three types of autophagy, macroautophagy, microautophagy, chaperone-mediated autophagy. Macroautophagy starts with formation of a phagophore assembly site (PAS). This later expands to phagophore which ultimately forms autophagosome that surrounds a portion of cytoplasm. This autophagosome then fuses with lysosome to form autolysosome for degradation. Microautophagy involves invagination or exvagination of the lysosomal membrane to directly ingest the contents of cytoplasm for degradation. In chaperone-mediated autophagy (CMA), HSC70, a chaperone guides proteins with KFERQ sequence to the lysosome for degradation. LAMP2A is the lysosomal receptor which recognizes proteins targeted by CMA.

Chapter 2

Long-lived species have improved proteostasis compared to phylogenetically-related shorter-lived species

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2.1 Abstract

Our previous studies have shown that the liver from naked mole rats (MRs), a long-lived rodent, has increased proteasome activity and lower levels of protein ubiquitination compared to mice. This suggests that protein quality control might play a role in assuring species longevity. To determine whether enhanced proteostasis is a common mechanism in the evolution of other long-lived species, here we evaluated the major players in protein quality control including autophagy, proteasome activity, and heat shock proteins (HSPs), using skin fibroblasts from three phylogenetically-distinct pairs of short- and long-lived mammals: rodents (mice and naked mole rats); marsupials (opossum and sugar glider) and bats (evening and little brown bat). Our results indicate that in all cases, macroautophagy was significantly enhanced in the long-lived species, both at basal level and after induction by serum starvation. Similarly, basal levels of most HSPs were elevated in all the long-lived species. Proteasome activity increased in the long-lived rodent (MR) and marsupial (sugar glider) but not in bats (little brown). These observations suggest that long-lived species may have superior mechanisms to ensure protein quality and support the idea that protein homeostasis might play an important role in promoting longevity.

2.2 Introduction

Disruptions in proteostasis, a set of cellular mechanisms that maintain the stability of the proteome (Balch et al., 2008), can result in an increased burden of misfolded proteins, leading to toxic oligomers and the accumulation of insoluble protein aggregates, thought to play a role in many chronic diseases, including age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease, among others (Gidalevitz et al., 2010). The main players in proteostasis include the ubiquitin/proteasome system, autophagy, and heat shock chaperones. The ubiquitin/proteasome pathway is involved in the removal of short-lived proteins that have been damaged and/or misfolded, while autophagy is crucial for the degradation and recycling of long-lived proteins, macromolecular aggregates, and damaged intracellular organelles. Protein chaperones, in turn, promote protein quality control by covering hydrophobic regions of proteins that are exposed during the normal and dynamic process of unfolding/refolding of proteins, thus assuring that proteins acquire a stable folded conformational state and do not oligomerize and aggregate. These mechanisms are known to decline with age, and this might be at least partially responsible for the increased accumulation of oxidatively and otherwise damaged proteins and aggregates with advancing age(Morimoto and Cuervo, 2014). Dietary restriction (DR), the best characterized manipulation that extends lifespan and healthspan has been shown to increase the heat shock response and autophagy, processes that are known to reduce protein misfolding and the accumulation of protein

oligomers/aggregates(Hansen et al., 2008; Katewa and Kapahi, 2010; Ntsapi and Loos, 2016; Steinkraus et al.; Weindruch et al., 1986). Using a comparative biology approach, previous studies including our own, have shown that proteins present in extracts from the liver of long-lived rodent (MRs) and bats have better resistance to urea-induced unfolding when compared to mice, a short-lived species with similar body weight(Pérez et al., 2009; Salmon et al., 2009). While our previous work was done *in vitro* with liver extracts, in this report we used cultured skin fibroblasts to further evaluate whether an enhancement of several proteostatic mechanisms (macroautophagy, proteasome activity, and heat shock chaperones) is associated with longevity. In addition to comparing MRs [maximum lifespan (MLS) ~30 years (y)] and mice (MLS ~4 y), we extended our studies to two additional pairs of long- and short-lived species: marsupials (laboratory opossum, MLS ~5 y and sugar glider, MLS ~18 y) and bats (evening bat, MLS ~6 y and little brown bat, MLS ~34 y).

2.3 Materials and methods

2.3.1 Species

The species studied were chosen based on their 1) well established longevity, 2) similar body size, and 3) representation of a broad phylogenetic coverage within mammals, to ensure generality of our conclusions. The 3 clades chosen are: **Rodents**: laboratory mice [*Mus musculus*, 35 g and 4 y], vs. naked mole

rats (MRs) [*Heterocephalus glaber*, 30 g and ~30 y]; **Bats**: evening bat (EB) [*Nycticeius humeralis*,11 g and 6 y] vs. little brown bat (LBB) [*Myotis lucifugus*, 8 g and 34 y]; and **Marsupials:** laboratory opossum (opo) [*Monodelphis domestica*, 150 g and 4.75 y] vs. sugar glider (SG) [*Petaurus brevicaudus*, 100 g and 18 y] (Supplementary figure 1).

2.3.2 Cell culture

Skin fibroblasts from long- and short-lived species were obtained from the Comparative Biology of Aging Core in the San Antonio Nathan Shock Center. Briefly, cells were prepared by enzymatic digestion of skin from young animals, and cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) and 10% Cosmic Calf Serum (Hyclone Laboratories, Logan, UT, USA) in a 37°C incubator with a gas phase of 21% O2, 5% CO2, with the exception of NMR and mouse fibroblasts, which were cultured at 35°C(Liang et al., 2010). In all experiments, cells were used between passage 4 and 10. For MR fibroblast cultures, we used Biocoat collagen I-coated tissue culture dishes (Advance Biometrix, San Diego, CA).

2.3.3 Protein degradation flux

Degradation of long-lived proteins was measured by radioisotopic pulse-chase labeling as described by Massey et al., 2008 (Massey et al., 2008). Briefly, cells were incubated for 48 hours in DMEM containing 2 μCi/ml ^{3}H-valine (Perkin-Elmer, MA, USA). Two wells were harvested to determine total radioactivity

incorporated into protein at the zero time point and the remaining wells received the chase medium: DMEM containing 2.8 mM cold valine in the presence or absence of serum (serum free), 10 mM 3-methyladenine (3-MA) for inhibition of macroautophagy and 20 mM/100 μM ammonium chloride/leupeptine (AC/L) for lysosomal inhibition. Cells were then chased for 4, 8, 12, or 24 hours. Proteolysis was measured as the amount of acid precipitable radioactivity transformed into acid soluble radioactivity during the chase period. Macroautophagy was calculated as the percentage of lysosome-mediated protein degradation sensitive to 3-MA inhibition.

2.3.4 Western blot analysis

Total cell lysates were prepared in RIPA buffer supplemented with protease and protein phosphatase inhibitors (Calbiochem, La Jolla, CA) and subjected to SDS-PAGE followed by transferring to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated with antibodies specific for: S6, phospho S6 (P-S6), LC3, heat shock chaperones: 90, 70, 40, and 27 (Cell Signaling Technology, Inc Danvers, MA), Actin (MP Biomedicals, Solon, OH), 20S proteasome subunit(Bulteau et al., 2002). The intensity of the bands was quantified by densitometry using Imagelab software (Bio-rad, Hercules, CA).

2.3.5 Heat shock response

A heat shock response was induced in fibroblasts by incubation at 41°C for 1hr in 5% CO_2 / 95% air(Kim et al., 1995). Then cells were quickly transferred to a

35°C (mouse and MR fibroblasts) or 37°C (bats and marsupials) incubator (5% CO_2 / 95% air) and allowed experimental groups to recover for 2, 4, 6, or 24 hrs. The control group (0 time) was not exposed to heat shock. Fibroblasts were harvested using RIPA buffer supplemented with protease inhibitors (Calbiochem, Billerica, MA) and 30 μg of protein were subjected to western blot analysis. HSP90, HSP70, HSP40, and HSP27 were measured by Western blot analysis using specific antibodies for each of these proteins. The level of each protein was calculated by quantification of each band relative to the loading control actin, with attention to quantification of images where signals were not saturated.

2.3.6 20S proteasome activity assay

Fibroblasts were homogenized in homogenization buffer (50 mM Tris-CL, pH 8.0; 1 mM EDTA; 0.5 mM DTT) and protein concentrations were measured by BCA assay. For each sample, 100 μg total protein was assayed in triplicate in 96-well plates using a 20S proteasome fluorometric (AMC) assay kit as per instructions from the vendor (Calbiochem, Billerica, MA). In brief, the release of free AMC from the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC was measured over time at 37°C using a microplate fluorescence spectrophotometer. 20S activity was calculated by the slope of free AMC release over time after ~10 min period of normalization. 20S proteasome specific activity was calculated by normalizing 20S activity to the quantity of 20S proteasome as measured by Western blot; data were expressed as AMC

release per second per mg of protein. A proteasome inhibitor, Lactacystin, was used to verify proteasome-driven proteolysis.

2.4 Results

2.4.1 Enhanced macroautophagy in fibroblasts from long-lived species

Because autophagy plays a central role in proteostasis (Morimoto and Cuervo, 2009), we measured autophagy under both basal conditions and induced by serum deprivation, by monitoring the degradation of radioactively-labeled long-lived proteins (autophagy flux; Figure 2.1), as described by Massey et al., 2008. Macroautophagy was measured in the presence of 3-methyladenine (3-MA) as described by Wang et al., (2008)(Wang et al., 2008). Dose response curves reveal similar species sensitivity to this inhibitor (data not shown).

Our analysis showed that macroautophagy is enhanced in fibroblasts from all long-lived species. Specifically, fibroblasts from both MR and SG have a significantly higher rate of macroautophagy under both basal and serum-starved conditions, compared to the respective short-lived counterparts (Figure 2.1B, left and right panels). Fibroblasts from long-lived bats (LBB), showed a significant increase in macroautophagy only under serum starvation conditions (~15%), but no statistically significant increase at basal conditions ($p= 0.078$; Figure 2.1B, middle panel).

We confirmed these results by measuring the conversion of free LC3I to the lipidated membrane-bound LC3II form (ratio of LC3II/LC3I) in absent and presence of AC/L(Klionsky et al., 2012). In response to serum starvation, fibroblasts from each of the three long-lived species (MR, LBB, and SG) showed significantly increased LC3II/LC3I ratios compared to fibroblasts from short-lived species (induction of 50%, 40%, and 2-fold respectively; Figure 2.1C). LC3-II levels were most prominent after AC/L treatment, as indicated by the strong LC3-II band on the Western blot (Figure 2.1D). The mTOR pathway is a major negative regulator of macroautophagy(Tóth et al., 2008), therefore interspecies differences in the activation of mTOR signaling in response to serum starvation was determined by measuring the decrease in the phosphorylated form of ribosomal protein S6. Our data showed that mTOR inhibition was greater in long-lived species, as long-lived species (MR, LBB, SG) showed a further decline in S6 phosphorylation (~20, 20, and 25% respectively) under serum starvation, compared to short-lived species (Mouse, EB, Opp) (Supplemental Figure 2.3).

2.4.2 The heat shock protein (HSP) response was elevated in fibroblasts from long-lived rodents and marsupials but not bats

We measured the levels of some of the major heat shock proteins, to establish whether there is a difference in the level of chaperones in species with disparate longevity. Considering the highly conserved nature of heat shock proteins (Brignull et al., 2006), the levels of the major protein chaperones were measured by Western blot analysis. Under our experimental conditions,

Figure 2.2 shows that the basal levels of all chaperones measured were significantly elevated in fibroblasts from long-lived species compared to fibroblasts from their respective short-lived counterparts, although in the case of bats, the long-lived LBB only showed significantly higher levels of HSP40 and HSP27 (Figure 2.2, middle panel). However, under heat shock conditions, all chaperones measured were elevated in fibroblasts from MRs and SG, compared to fibroblasts from their respective short-lived counterparts (mice and Opo), (Figure 2.3A and 2.3C). However, in the long-lived species of bats, there were no statistical differences in the induction of any of the chaperones when comparing fibroblasts from short and long-lived bats (Figure 2.3B).

2.4.3 Increased proteasome (20S) activity in fibroblasts from long-lived rodents and marsupials but not bats

Increasing activity of the proteasome would be predicted to enhance protein homeostasis, and since we have previously shown that liver extracts from MRs have increased activity of the proteasome compared to mice (Pérez et al., 2009), we measured the 20S proteasome chymotrypsin-like activity in fibroblasts from the species under study by fluorescent-labeled substrate cleavage and normalized to protein levels of the 20S proteasome in serum and serum-free conditions (Bulteau et al., 2002). While the 20S protein content was similar between these species (Figure 2.4 top panel), proteasome specific activity under basal conditions was greater in MR and SG fibroblasts (40 and 30% respectively, Figure 2.4A), compared to their shorter-lived counterparts. Furthermore, induction under serum free conditions was much more robust in

long-lived rodents and marsupials (Figure 2.4B). For example, when compared to basal state (stripped bar; value=1), MRs had an 8-fold higher induction than mouse (~2-fold) fibroblasts, and SG had a 1.5-fold higher induction than Opo (~25%) fibroblasts (Figure 2.4B, solid bars). Therefore, using this 20S activity assay, MRs and SG had increased 20S activity under both basal and serum-free conditions. In contrast, we found no difference in 20S proteasome activity between short- and long-lived bat fibroblasts at either basal or induced conditions (Figure 2.4A and B).

2.5 Discussion

A direct correlation between a loss of proteostasis network and aging has been observed in diverse model systems. Moreover, either genetic or pharmacological enhancement of the proteostasis network reportedly extends lifespan and delays age-related disease in *C. elegans* (Morley and Morimoto, 2004; Raynes et al., 2012; Tóth et al., 2008; Walker and Lithgow, 2003). Also, several studies suggest that cells from long-lived species are more resistant to a variety of stressors than cells from short-lived species(Harper et al., 2011; Kapahi et al., 1999). However, there is little information on the cellular or molecular mechanisms that give rise to increased resistance to stress, or whether these two observations are mechanistically related. By removing damaged proteins and clearing damaged organelles such as mitochondria, endoplasmic reticulum and peroxisomes (Vellai et al., 2009), autophagy plays a major role in maintenance of the cellular proteome, and it has been shown to

be activated by DR. Recently Wang and Miller showed that fibroblasts from several long-lived mutant mouse strains have increased activity of the autophagy pathway (Wang and Miller, 2012). These data, coupled with evidence of sustained physiological function (healthspan) during aging in long-lived species (Buffenstein, 2008), suggest that proteostasis in long-lived vertebrates may be superior to that of short-lived species within the same phylogenetic group.

In this study we used skin fibroblasts in culture and comparative biology approaches to question whether enhanced proteostasis may be a common mechanism in the evolution of long-lived species. We focused upon species pairs from three evolutionarily distant clades that were approximately the same size. Overall, our results indicate that in all three clades (rodents, bats, and marsupials), the long-lived species had improved proteostasis. For example, we found that fibroblasts from MRs have higher activity of all three pathways studied (autophagy, chaperone levels and 20S proteasome activity), both under control and under stress induced conditions (i.e., serum starvation or heat shock). This data not only support previous results including our own, where proteins obtained from the liver of MRs have been shown to have higher proteasome activity and a proteome that is more resistant to urea-induced unfolding (Pérez et al., 2009; Rodriguez et al., 2014), but also the recently published study where higher levels of chaperones have been observed in the liver of MRs (Madeo et al., 2010). Considering that MRs are extremely resilient to a variety of stressors (Rubinsztein et al., 2011), and they are considered a

model of successful aging (Buffenstein, 2008), the available data strongly suggest that an improved proteostasis network may be necessary for the maintenance of longer health and life in MRs.

Autophagy (induced by serum deprivation) was a common mechanism that was enhanced in all three long-lived species, suggesting that autophagy may be linked to longevity, which is in accordance with previous studies (Feder and Hofmann, 1999; Zhao et al., 2014). Macroautophagy is the major pathway for the degradation of proteins and subcellular organelles; its activity has been shown to decrease with age in mouse liver, and this is correlated with an increase in the levels of damaged proteins (Wang et al., 2008). Our macroautophagy data obtained by flux measurement was corroborated by the measurements of the LC3II/LC3I ratio and inhibition of the mTOR pathway. However, we did not find significant differences in another marker for autophagy, p62 degradation, among any of the species studied (Supplemental Figure S4). Our data agreed with recent data published by Zhao et al, 2014 (Zhao et al., 2014), where hepatic stellate cells form NMR have higher autophagy compared to mouse cells.

We also found that the heat shock chaperones are increased in all long-lived species, although the response to heat shock (HSR) is enhanced in two of the three long-lived species studied, MRs and SG, but not in long-lived bats (Figure 3). While HSPs are extremely well conserved across species (Brignull et al., 2006), it is theoretically possible that some of the antibodies may recognize the proteins from some species better than the others. However, this

is unlikely to be true for all of them, because when we analyzed the sequence of the epitopes tested (antibodies) we observed between 99-100% homology over that region. The increased levels of heat shock chaperones in both basal and induced states for long-lived rodents and marsupials suggest that proteins from long-lived species are better protected from unfolding and thus they are likely to be less prone to aggregation. Although previous data have shown that compared to mouse, the proteome of two bat species [*Myotis velifer* (MLS~12 y) and *Tadarida brasiliensis* (MLS~ 12 y)] showed better resistance to urea-induced unfolding, suggesting that these species may have a better chaperone response(Salmon et al., 2009), this might be true only when comparing bats and mice, but not when comparing short- and long-lived bats. Of course, it is possible that differences might exist if additional species of bats are studied. However, this is unlikely since EBs are among the shortest-lived of bat species whereas LBBs are among the longest-lived. It is also possible that flying bats might need a different temperature to induce the HSR, or that heat shock might not the best insult to induce HSR in bats. In this sense, it may be possible that a better chaperone response can be induced using other insults, e.g. urea treatment, oxidative damage or others. In fact, it is known that HSPs have different degrees of inducibility, kinetics, and tissue expression depending on the species and each species' natural environment or ecology. For example, bats are naturally subjected to a wide range of temperatures (very high when they are flying, and very low when they are hibernating)

(Feder and Hofmann, 1999). This may be an important consideration in this study.

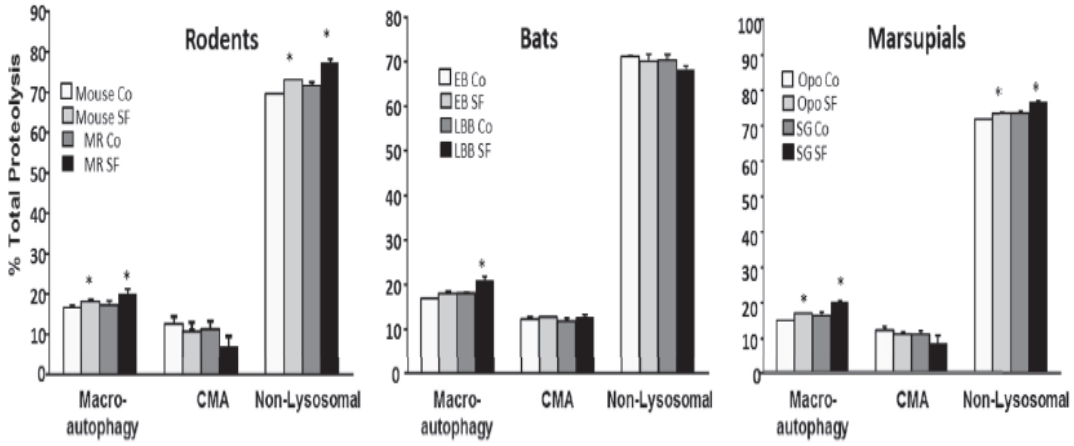
Similarly, the proteasome activity data also showed that this mechanism was enhanced in long-lived rodents and marsupials, but not in bats. Previous data have shown that bats did not have higher 20S proteasome activity when they were compared to mice (Salmon et al., 2009), and our data agrees with this notion. Currently we don't know why heat shock proteins and proteasome activity do not correlate with longevity in bats, but this may be related to a unique characteristic of flying species, or it might be an experimental difference because the animals used were wild caught, and therefore they may need different experimental conditions to asses these mechanisms. It is well-known that in mice, for example, wild animals have very different stress resistance, compared to laboratory-bred counterparts, and maybe even when dealing with cells in culture derived from these wild caught animals, so it is possible that with wild caught bats, different stressors (including different temperatures or times) might be needed to properly measure the HSR. Similarly, it is possible that measurement of the 26S proteasome might be needed, in addition to the 20S proteasome activity.

In summary, our data show that macroautophagy, both basal and induced by serum deprivation, correlates tightly with species longevity in three different clades. Collectively, enhanced proteostasis may be an important mechanism in the evolution of long-lived species, suggesting that they may adapt better to stressful environments by having a higher protein turnover and thereby

maintaining better cellular homeostasis. Future studies comparing a broader range of both long and short-lived species is needed to determine whether enhanced proteostasis is a mechanism common in the evolution of many long-lived species.

2 .6 Figures

A



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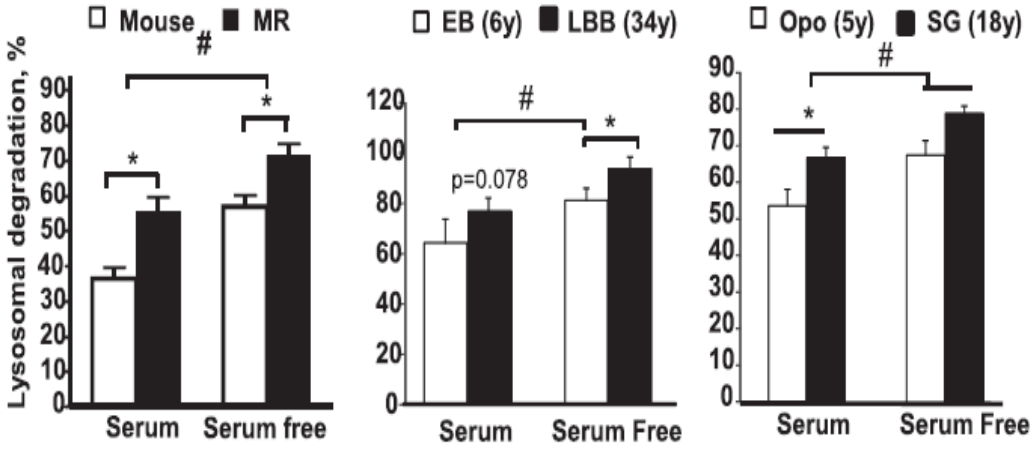

Figure 2.1. Long-lived species have enhanced macroautophagy compared to short-lived species.

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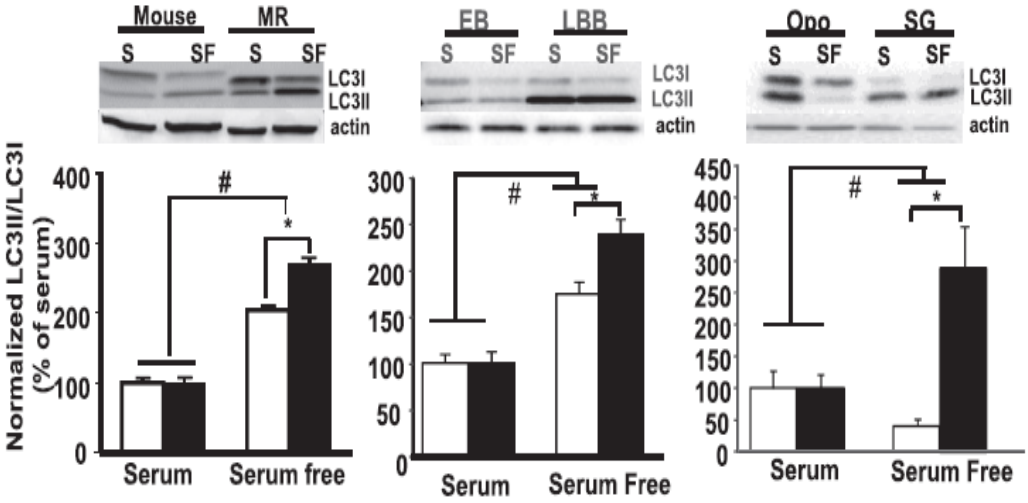
C



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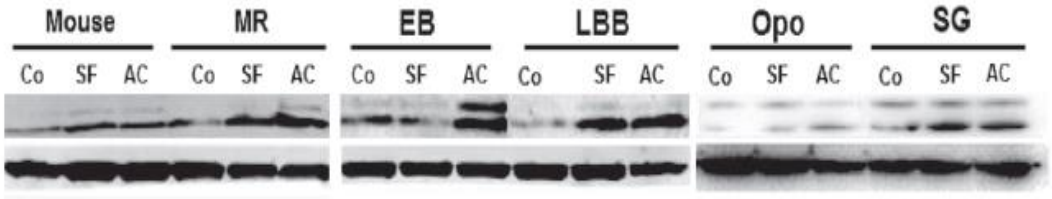

Figure 2.1. Long-lived species have enhanced macroautophagy compared to short-lived species.

(Continued)

Figure 2.1. Long-lived species have enhanced macroautophagy compared to short-lived species. Protein degradation was determined by pulse and chase. Figure 2.1A shows total rate of proteolysis in presence of 3-MA (macroautophagy measurement) and AC/L [Chaperone Mediated Autophagy (CMA) measurement]. Non-lysosomal degradation was measured as the residual value in presence of all inhibitors. Macroautophagy was determined by the percentage of lysosomal degradation and by the ratio of LC3II to LC3I conversion (LC3II/LC3I) in absence and presence of AC/L (Figures 2.1B, C and D respectively). Representative gels for LC3II/LC3I for each species are shown. The experiments were done both under basal (serum) and serum-free conditions in short-lived species (open bars) and long-lived species (solid bars). The data represent the mean ± SEM of triplicate measurements obtained from 3 different animal donors of each species and analyzed by the non-parametric test of ANOVA. The asterisk (*) denotes a statistically significant difference between species at the $p<0.05$, and the pound (#) denotes a statistically significant difference between serum and serum free conditions at $p<0.01$.

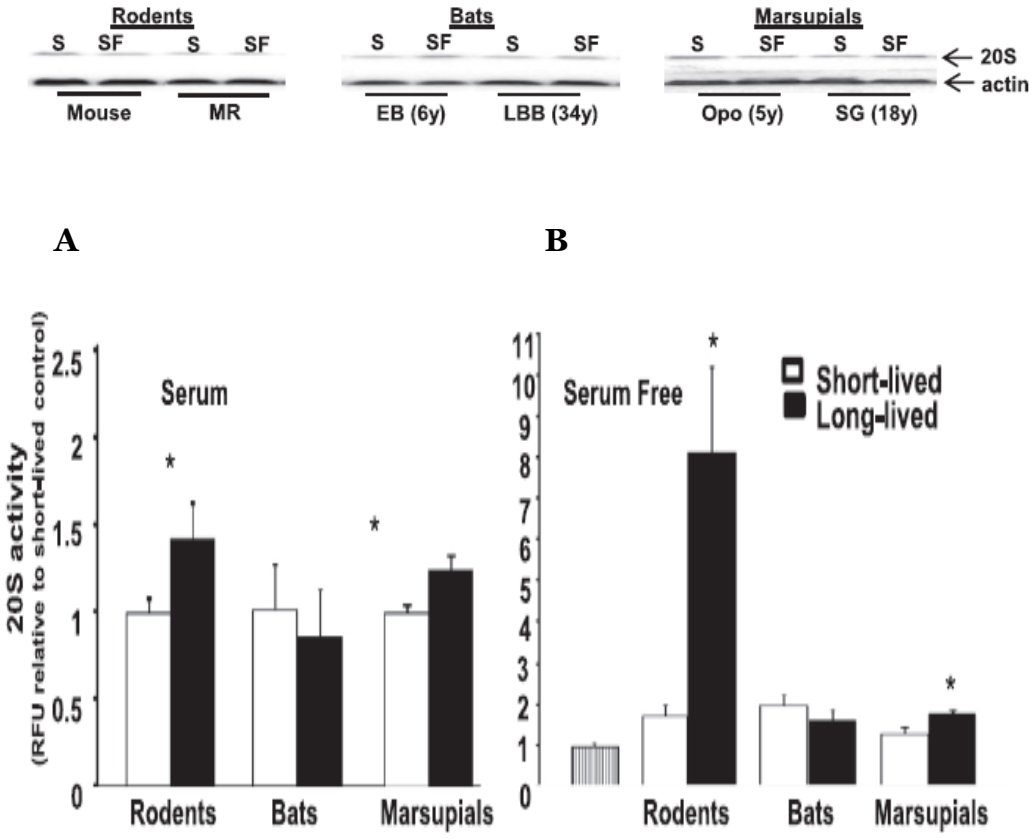

Figure 2.2. Long-lived rodents and marsupials, but not bats, have enhanced proteasome activity compared to short-lived species. Proteasome activity was measured at both basal (serum; Panel A) and serum free conditions (Panel B) in total homogenates from skin fibroblasts of short-lived (open bars) and long-lived species (solid bars) of rodents, bats and marsupials. The specific activity was calculated by normalizing 20S activity to the quantity of 20S (shown at the top of the figure) and expressed relative to the short-lived control (basal state) of each species (value of 1). The data are the mean ± SEM from quadruplicate assays from 3 different animal donors from each species and were analyzed by the non-parametric test of ANOVA. The asterisk (*) denotes a statistically significant difference between short and long-lived species at $p<0.05$.

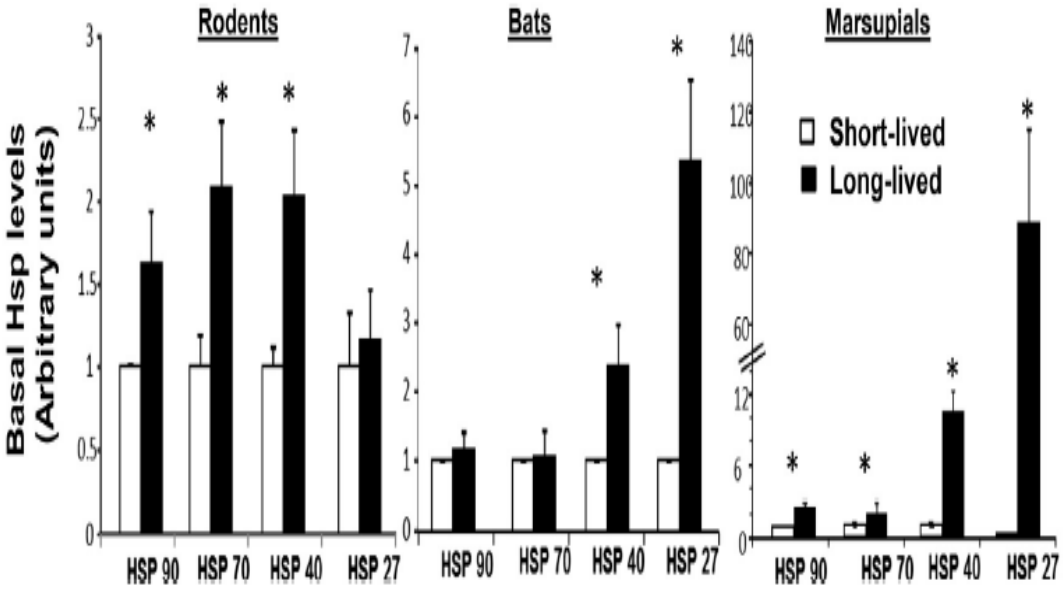

Figure 2.3. Basal heat shock response is enhanced in long-lived species. The basal levels of chaperones (HSP90, HSP70, HSP40, and HSP27) were determined by western blot analysis in total homogenates from skin fibroblasts of short-lived species (open bars) and long-lived species (solid bars) of rodents, bats and marsupials. The data is expressed relative to the short-lived species (open bars, value of 1) and they are the mean ± SEM from 3 different animal donors of each species and analyzed by the non-parametric test of ANOVA. The asterisk (*) denotes a statistically significant difference between short and long-lived species at $p<0.05$.

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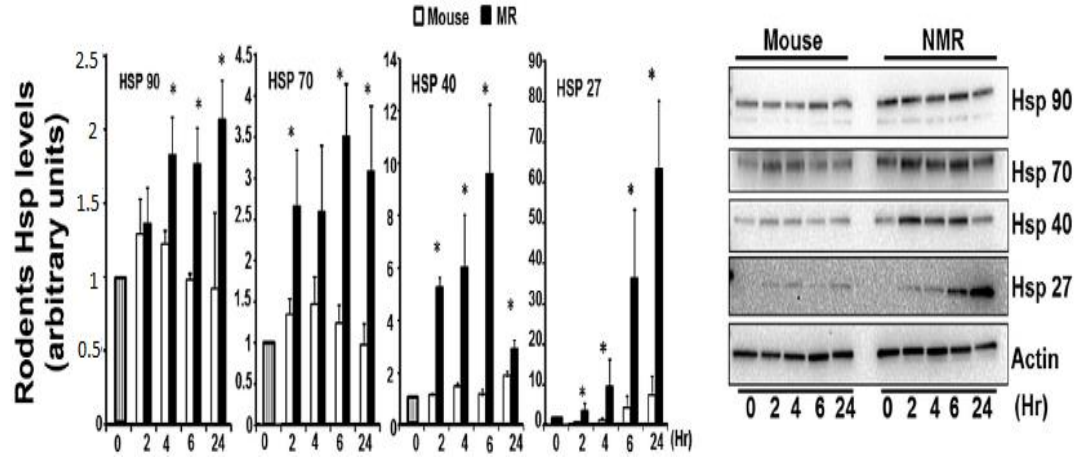

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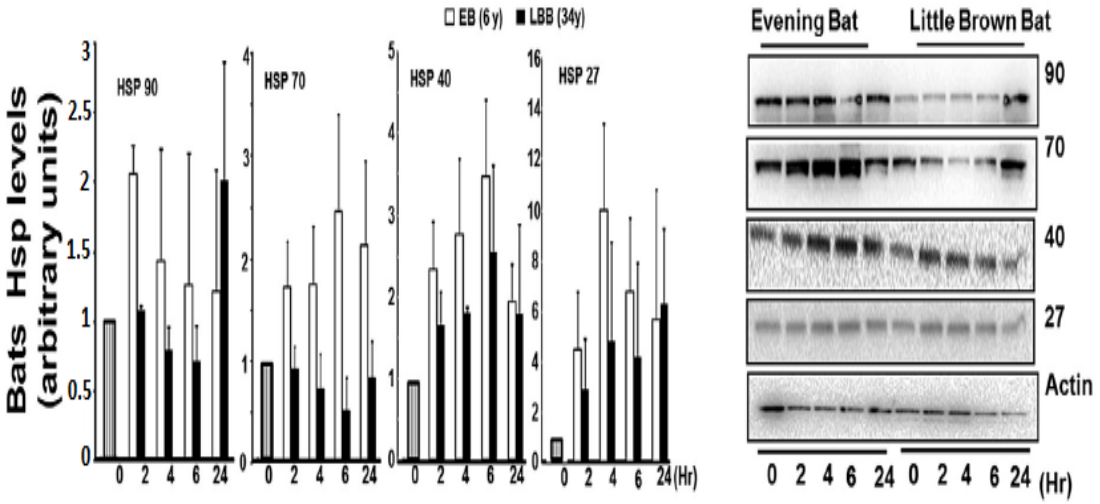

Figure 2.4. The heat shock response is enhanced in long-lived rodents and marsupials, but not in long-lived bats.

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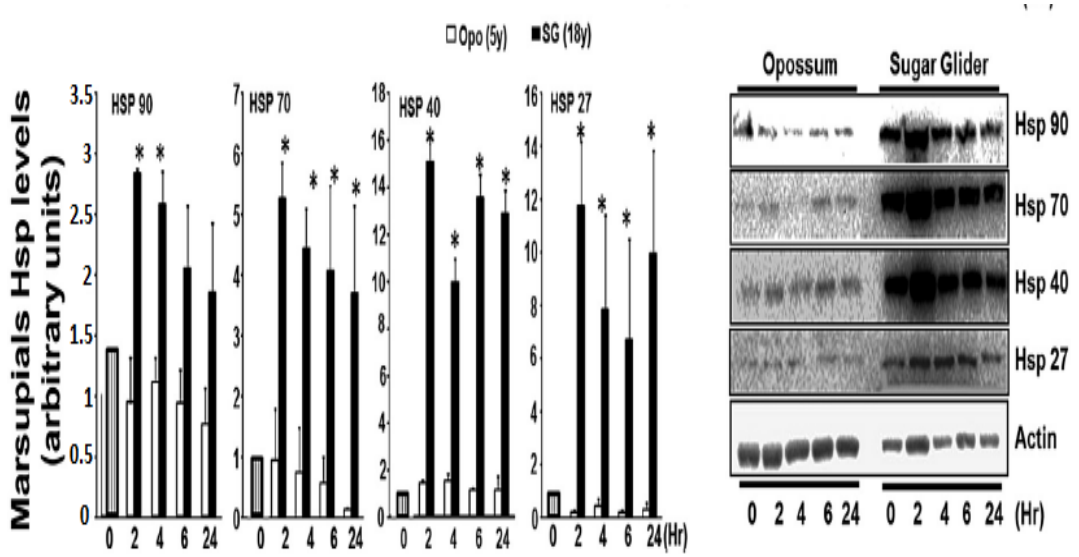
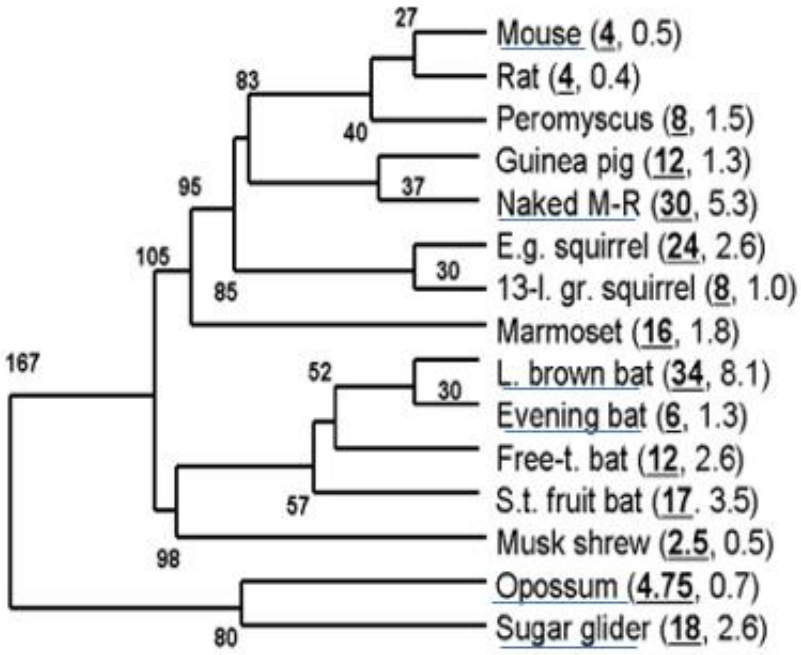
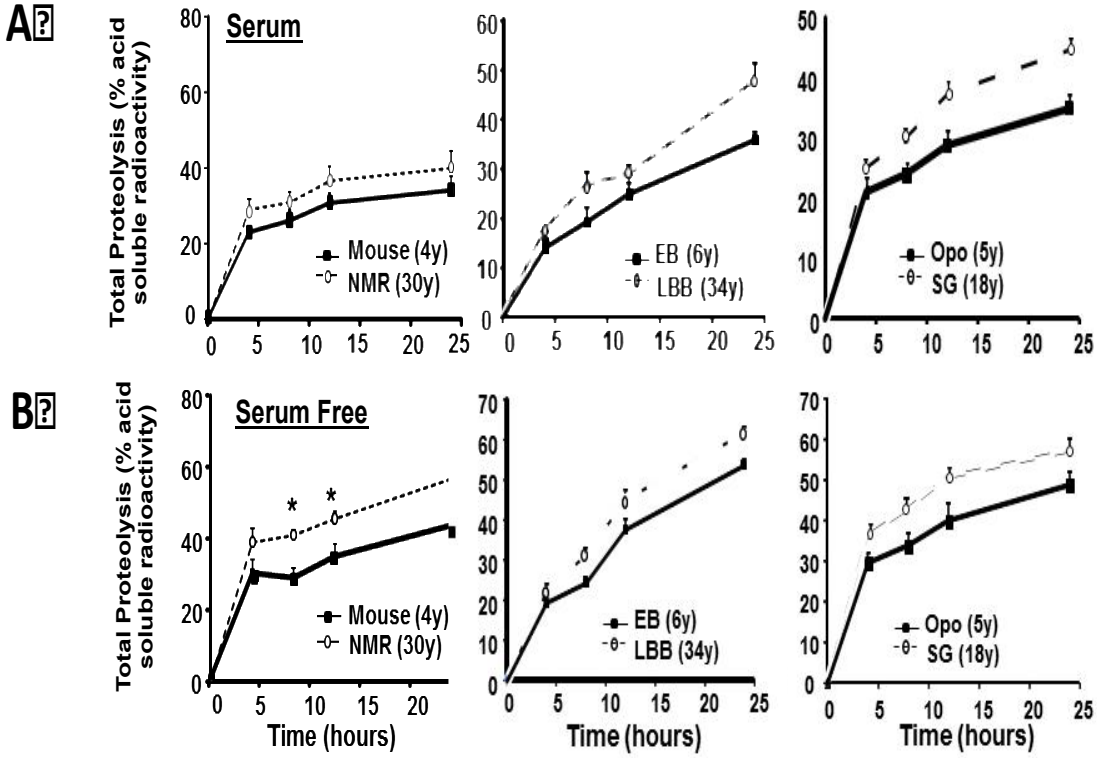


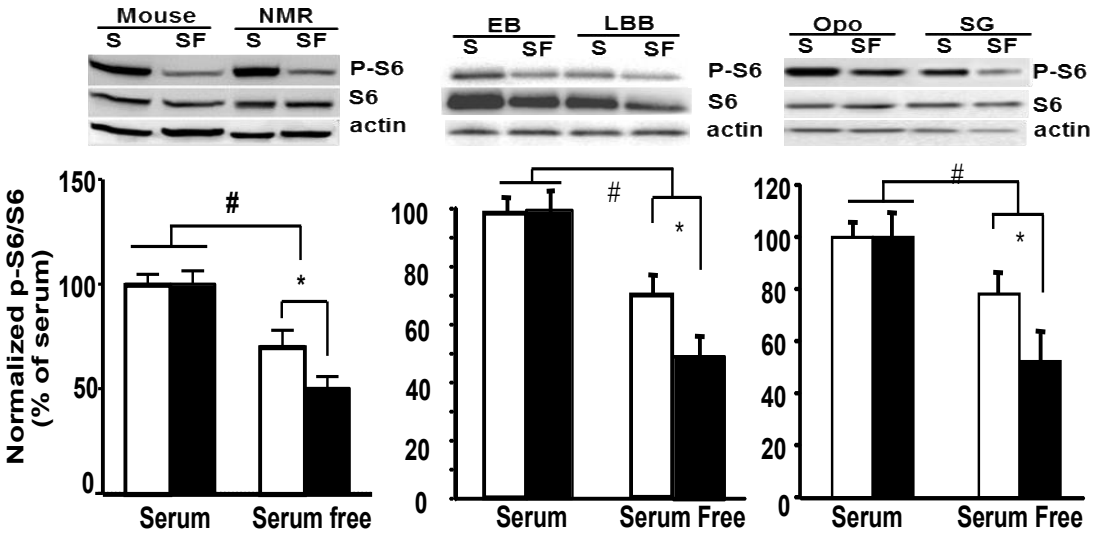
Figure 2.4. The heat shock response is enhanced in long-lived rodents and marsupials, but not in long-lived bats. The heat shock response was determined by measuring protein chaperone levels (HSP90, HSP70, HSP40, and HSP27) over 24 hours after heat shock treatment (41°C for 1 hr; panels A, B, and C) by western blot analysis in total homogenates from skin fibroblasts of short-lived species (open bars) and long-lived species (solid bars) of rodents, bats and marsupials (representative gels are shown). The data is expressed relative to the basal levels of the short-lived species (striped bars) and they are the mean ± SEM from 3 different animal donors of each species and analyzed by the non-parametric test of ANOVA. The asterisk (*) denotes a statistically significant difference between short and long-lived species at $p<0.05$.



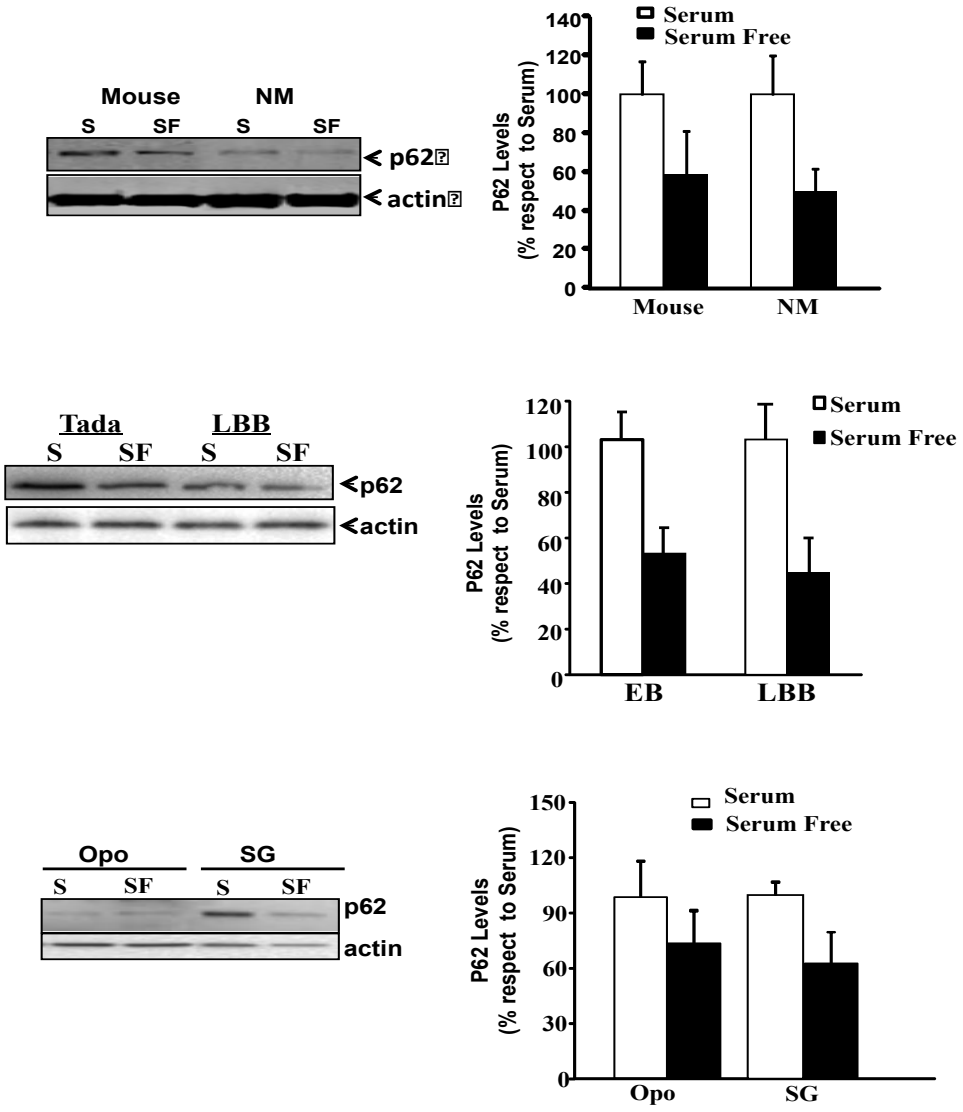
Supplementary figure 2.1. Phylogeny and species used. Numbers on the phylogeny itself represent estimated divergence times in millions of years and numbers after the species name are maximum recorded longevity in years (underlined) followed by longevity quotient (LQ: ratio of observed maximum lifespan to that predicted by body mass). Species from the 3 clades studied are underlined: rodents, bats, and marsupials.



Supplementary figure 2.2. Total proteolysis in short- and long-lived species. Protein degradation in skin fibroblasts from short-lived species (squares) and long-lived species (circles) was determined by a pulse-chase using [^{3}H]-valine (2 μCi/ml) for 24 hours, followed by a chase for the times indicated. Total rates of protein degradation were measured in medium supplemented with 10% FBS (Panel A) or in serum-free medium (Panel B). Left, middle, and right panels show data for rodents, bats and marsupials, respectively. The data represent the mean ± SEM of triplicate measurements obtained from 3 different cell lines of each species analyzed by the non-parametric test of ANOVA. The asterisk (*) denotes a statistically significant difference between species at the $p<0.05$.



Supplementary 2.3. mTOR signaling pathway: The mTOR signaling was measured as the ratio of PS6/S6. Representative gels for PS6, and S6 per each especie are shown. Western blot analysis of total cellular homogenates (30 ug each) from long-lived species (solid bars) vs. short-lived species (open bars) under both serum and serum-free conditions. The data represent the mean ± SEM of triplicate measurements obtained from 3 different cell lines of each species analyzed by the non-parametric test of ANOVA. The asterisk (*) denotes a statistically significant difference between short and long-lived species at $p<0.05$, and the pound (#) denotes $p<0.01$ between serum and serum-free conditions.



Supplementary figure 2.4. p62 protein levels. The levels of p62 were determined by Western blot analysis. Briefly, total cell lysates were prepared in RIPA buffer supplemented with protease and phosphatase inhibitors (Calbiochem, Billerica, MA) and subjected to SDS-PAGE gel followed by transferring to PVDF membranes (Millipore, Billerica, MA, USA). After blocking in 5% milk, the membranes were incubated with antibodies specific for p62 (Sigma-Aldrich, St. Louis, MO). Actin antibody (MP Biomedicals, Solon, OH) was used as a loading control. The intensities of the bands were quantified by densitometry using Imagelab software (Bio-rad, Hercules, CA).

Chapter 3

Long-lived species are more resistant to polyQ82 toxicity and form aggresome-like inclusions than short-lived species

Bharath Sunchu, Zhen Yu, Almog Ido, Andrew C. Drake, and Viviana I. Perez

3.1 Abstract

The capacity of cells to maintain proteostasis declines with age, causing rapid accumulation of damaged proteins and protein aggregates, and is considered to play an important role in age-related disease etiology. While our group and others have identified that proteostasis is enhanced in long-lived species, there is no data on whether this leads to better resistance to proteotoxicity. To evaluate differences in resistance to proteotoxicity, we measured cell survival in transfected fibroblasts from long-lived and short-lived species with polyQ82-YFP, a well-established polyglutamine model of protein aggregation. Additionally, we specifically down-regulated a key protein of each proteostatic mechanism (autophagy - ATG5; ubiquitin-proteasome – PSMD14; and chaperones – HSP27) in naked mole rat, the long-lived rodent, to evaluate their contributions to proteotoxicity resistance. Furthermore, we analyzed the sub-cellular localization of inclusion formation, and association with HSP27, which co-localizes with aggresome-like inclusions in a protective fashion. Here we show that fibroblasts from long-lived species were more resistant to proteotoxicity than their short-lived counterparts at least in part because they have an enhanced capacity to handle misfolded proteins, and form protective, aggresome-like, juxtanuclear inclusions. Taken together, our data elucidates some of the mechanisms through which long-lived species have enhanced proteotoxic resistance and identifies targets for remediating age-related loss of protein homeostasis.

3.2 Introduction

Proteostasis is a hallmark of aging (Kennedy et al., 2014; López-Otín et al., 2013) which involves multiple protein quality control processes that work together to ensure the health of the cellular proteome (Labbadia and Morimoto, 2014). A decline in proteostasis with age leads to the accumulation of misfolded proteins and protein aggregates, leading to proteotoxicity (Bulteau et al., 2002; Cuervo and Dice, 2000a, 2000b; Keller et al., 2000b; Taylor and Dillin, 2011; Viteri et al., 2004).

Using a comparative biology approach, we and others have shown that long-lived species have more robust proteostasis mechanisms, including chaperones, autophagy and the proteasome (Pickering et al., 2015; Pride et al., 2015; Rodriguez et al., 2012, 2014; Zhao et al., 2014). Similarly, studies using protein fractions from long-lived bats show low levels of protein ubiquitination, and resistance to urea-induced protein unfolding relative to the levels detected in similar fractions from mice (Salmon et al., 2009). Moreover, studies by Triplett et al, (2015) have shown that naked mole rats (MRs) maintain high levels of autophagy throughout most of their lifespan (Triplett et al., 2015). Finally, studies using primary skin fibroblasts from a larger set of long- and short-lived species, showed that there is a correlation between species longevity and the levels of insoluble proteins (Pickering et al., 2014), suggesting that long-lived species have better protection against misfolded protein and less protein aggregation.

Together, these data suggest that long-lived species might have very efficient mechanisms for maintenance of protein homeostasis. However, because such experiments tested endogenous proteins, a possible alternative explanation is that long-lived species have structurally more resistant proteins, leading to less aggregation.

In this study, we used a well-established model of exogenous protein aggregation, polyQ82 (Cohen et al., 2012; Morley et al., 2002), to evaluate the hypothesis that enhanced protein homeostasis might be the main contributor to the reduced level of protein aggregates observed in fibroblasts from long-lived species. Specifically, we transfected skin fibroblasts from two long-lived species [MRs and little brown bats (LBB)], and two short-lived species (mouse and evening bat) with polyQ82 and examined cell survival, cellular distribution, and levels of protein aggregate inclusions in relation to their resistance to proteotoxicity. We found that while resistance to proteotoxicity is superior in long-lived species, it was surprisingly not due to differences in the levels of protein aggregation. Instead, we observed that cells from long-lived species form aggresome-like inclusions, with a juxtanuclear cellular location, next to centrosomes. This was not observed in cells from short-lived species. Furthermore, our data indicate that inclusions of polyQ82 start to appear at lower concentrations in long-lived species, compared to short-lived species, suggesting that inclusion formation is a protective response to sequester toxic misfolded proteins and oligomers.

Overall our data suggest that the resistance to proteotoxicity observed in long-lived species is not due to a lower levels of protein aggregates but rather by enhanced handling of the protein aggregates through the formation of aggresome-like inclusions, a well-recognized protective mechanism against proteotoxicty (Kopito, 2000; Ouyang et al., 2012; Taylor et al., 2003; Zhou et al., 2014).

3.3 Materials and methods

3.3.1 Cell culture

Skin fibroblasts from short-lived species and long-lived species belonging to the same phylogenetic clade and having similar body size were used. Short-lived species and long-lived species studied were, rodents and bats. For rodents, we used laboratory mice [*Mus musculus*, 35 g and 4 y], vs. naked Mole Rats (MRs) [*Heterocephalus glaber*, 30 g and ~30 y] and the bats compared were Evening Bat (EB) [*Nycticeius humeralis*, 11 g and 6 y] vs. Little Brown Bat (LBB) [*Myotis lucifugus*. 8 g and 34 y]. Cells were prepared by enzymatic digestion of skin from young animals as described by Liang et al., 2010 (Liang et al., 2010). Mouse skin fibroblasts were isolated from C57BL/6 mice in the Linus Pauling Institute, Oregon State University. Fibroblasts from MR were obtained from the Comparative Biology of Aging Core in the San Antonio Nathan Shock Center, and bat cells were obtained from the Comparative Biology of Aging Core in the University of Birmingham Nathan

Shock Center. All cells were used at passages below 10 and all comparisons were made between similar passage cells. Cells were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM) and 10% Cosmic Calf Serum (Hyclone Laboratories, Logan, UT, USA) with 1% penicillin-streptomycin (Corning) in an incubator with a gas phase of 5% CO_2, 21% O_2. Mouse and MR skin fibroblasts were cultured at 35°C (Liang et al., 2010), while bat fibroblasts were cultured at 37°C. MR fibroblasts were cultured in collagen I coated tissue culture dishes (Advance Biomatrix, San Diego, CA).

3.3.2 Plasmids and cell transfection

pEYFP-N1 Q19-YFP, and pEYFP-N1 Q82-YFP plasmids were kindly donated by Richard Morimoto's lab. The plasmids pEYFP-N1-Q19 and pEYFP-N1-Q82 contain the polyglutamine-encoding sequences, cloned as a cassette, into the EcoRI site of the pEYFP-N1 (CLONTECH) resulting in the expression of yellow fluorescent protein (YFP) (Igarashi et al., 1998; Satyal et al., 2000). Thermo Fisher Scientific's Neon Electroporation System® was used for transfection of fibroblasts with polyQ-YFP plasmids. Fibroblasts were collected in media by trypsinization after reaching 70% confluency. The cells were centrifuged to remove the media and then washed with DPBS. The cell pellet was then suspended in the resuspension buffer (R buffer®). This cell suspension was mixed with the plasmid of interest and was electroporated. Cells were seeded in the respective cell culture dishes with pre-incubated media immediately after electroporation.

3.3.3 Immunofluorescence

Cells were seeded on collagen coated Zeiss no. 1.5 cover slips in 6 well plates. Cells were washed with DPBS (1X) twice and fixed with either methanol-free 4% formaldehyde or ice-cold methanol. Cells were incubated with primary antibody over night at 4° C or 1 hour at room temperature (for γ-tubulin). This was followed by incubation with the respective secondary antibodies for 1 hour at room temperature. The cover slips were mounted on microscope slides using a Prolong Gold Antifade Reagent®. A Zeiss LSM 780 confocal microscope was used for imaging. The primary antibodies used for immunofluorescence were γ-tubulin (c-20) rabbit pAb (Santa Cruz-7396-R), Vimentin(D21H3) XP rabbit mAb (Cell Signaling Technology-5741), Lamin A/C (4C11) mouse mAb (Cell Signaling Technology-4777), p62/SQSTM1(D6M5X) rabbit mAb (rodent specific, Cell Signaling Technology 23214), p62/SQSTM1 rabbit polyclonal antibody (Thermo Fisher Scientific, PA5-20839), HSP 27 (rodent preferred, Cell Signaling Technology 2442), and HDAC6 rabbit pAb (Abclonal A11429). Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) from Cell Signaling Technology (4414), and Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) from Cell Signaling Technology (4410) were used as secondary antibodies.

3.3.4 Location of inclusions

Inclusions were classified into two types based on the location with respect to nucleus: juxtanuclear – frequently forming dents in the nuclear membrane, and far- inclusions which are located far away from the nuclear membrane. Similarly inclusions were classified into two types based on the location with respect to centrosome: juxta-centrosomal – located within 2 μm of centrosome, and far – located greater than 2 μm from centrosome (Kopito, 2000).

3.3.5 High content imager experiments

The Image X press Micro wide field high content imaging (HCI) system®, a fully automated machine, was used to capture images of live cells. Live cells can be imaged over multiple days, as this imager comes with an incubator and an environmental control system to set the temperature, CO_2 supply and humidity. Transfected cells were seeded in Greiner® glass 96-well plates and time-lapse video microscopy was performed using the HCI system. The acquired images were analyzed using Fiji-Image J software. Mean cell fluorescence was calculated as in Bersuker et al., 2013 (Bersuker et al., 2013). Live cell images in which cells start to form polyQ82-YFP inclusions were selected. The outline of the cell was delineated using Fiji-Image J software and total cell fluorescence was calculated, which was divided by cell area to derive mean cell fluorescence at which inclusions started to form.

3.3.6 Cell survival

Using time-lapse cell imaging, we measured cell survival by following individual transfected cells. We considered a cell to be dead when it underwent rounding, shrinking and/or disintegration. We followed cultured fibroblasts for 72 hrs and the deaths were annotated over that time. The data was plotted as a survival curve using GraphPad Prism software and analyzed by log-rank test (Mantel-Cox test).

3.3.7 ATG5, PSMD14 and HSP27 SiRNA knock down

siRNA targeting HSP27, PSMD14, and ATG5 were purchased from ThermoFisher. Scrambled siRNA was used as control. Lipofectamine® RNAiMAX transfection reagent (Thermo Fisher®) was used to deliver siRNA (27.3 pmol siRNA with 6 µl RNA imax). 48 hr after siRNA transfection, cells were transfected overnight with polyQ19 or polyQ82. Cells were monitored by HCI as described previously. The silencing efficacy was determined by Western blotting using antibodies against HSP27 (Cell Signaling Technology 2442), PSMD14 (ThermoFisher 38-0200), and ATG5 (ThermoFisher PA5-35201), respectively.

3.3.8 Statistics

The survival curves were analyzed by GraphPad Prism software using log-rank (Mantel-Cox) test. Hazard ratios were calculated using GraphPad Prism software for two survival curves, which is a measure of how often a event, in this case death of the cell, happens in one group compared to how often it happens in another group over time. Here we report hazard ratios as significant if the lower limit of the 95% confidence interval of the hazard ratio is greater than 1. We also reported median cell survival, the time at which 50% of the cells are alive. For all other comparisons, a parametric Student-unpaired t-test was used in GraphPad Prism, and similar Standard Deviations were assumed for compared populations. All these tests were two-tailed, and a 95% confidence level was used to identify significant difference.

3.4 Results

3.4.1 MR fibroblasts were more resistant to polyQ-induced proteotoxicity than those of mouse

To determine if cells from long-lived species are more resistant to polyQ82 proteotoxicity, we measured cell survival by live cell imaging (Figure 3.1 A-B). While no significant differences in cell survival were observed between mouse and MR fibroblasts transfected with the control polyQ19, only mouse fibroblasts displayed a significant decrease in survival in the presence of polyQ82 ($p<0.0001$) (Figure 3.1 A). In contrast, we did not observe significant

differences between polyQ19 and polyQ82 in MR cells (p=0.1469) (Figure 3.1 B). The hazard ratio for mouse survival was 7.2, indicating that mouse cells transfected with polyQ82 were 7.2 times more likely to die than control (mouse cells transfected with polyQ19), whereas for MR the hazard ratio was 1.95. Furthermore, our data showed that median survival time of mouse cells transfected polyQ82-YFP was 22 h, while for MR it was 70 h. This data strongly suggests that MR fibroblasts are more resistant to proteotoxicity induced by polyQ82-YFP than mouse fibroblasts.

3.4.2 Knocking down autophagy, ubiquitin-proteasome, and HSP27 affected MR cell survival

Because previous data in our lab showed that MR fibroblast show enhancement in all three proteostasis mechanisms (Pride et al., 2015), we next wanted to determine the role of each of these proteostatic mechanisms in managing the proteotoxicity induced by polyQ82-YFP in MR cells. For this we used siRNA against key proteins for each mechanism: ATG5 (autophagy), PSMD14 (Ub-proteasome) and HSP27 chaperone. Then polyQ82YFP-induced proteotoxicity was analyzed using the live cell imaging system as described. As shown in Fig 3.2 A, there was no difference between survival of control and cells transfected with scrambled siRNA. However, we found that knocking down each of these mechanisms affected cell survival, even in the controls transfected with Q19 (Fig 3.2 B-D and supp. Fig 3.1 A). The most detrimental effect was with siRNA targeting autophagy (ATG5 gene, Fig 3.2 B), where both polyQ19 and polyQ82 showed 100 % cell death at 54 h and 27 h after

transfection, respectively (hazard ratios for polyQ82 compared to scrambled siRNA =3.6). Inhibition of the proteasome with siRNA against PSMD14 led to 63% and 100% cell death 72 h after transfection with polyQ19 or polyQ82 respectively (hazard ratios for polyQ82 compared to scrambled siRNA=2.3) (Fig. 3.2 C). siRNA against HSP27 showed a 46.6% and 87.3% cell death 72 h after transfection with polyQ19 or polyQ82 respectively (hazard ratios for polyQ82 compared to scrambled siRNA=1.8) (Fig. 3.2 D).

3.4.3 MR cells had a juxtanuclear cellular location of polyQ82 inclusions

During all our experiments using the live-cell high content imaging analysis, we observed a clear difference between mouse and MR fibroblasts in terms of cellular location of polyQ82-YFP inclusions. Specifically, we observed that inclusions in MR fibroblasts were located in the juxtanuclear region, whereas in mouse, inclusions were located more randomly and/or peripheral. To establish whether the inclusions are perinuclear or intranuclear, we used indirect immunofluorescence by targeting lamin A/C proteins to stain the nuclear membrane. These experiments clearly show that inclusions in mouse fibroblasts are randomly located, whereas in MR fibroblasts, inclusions were predominantly juxtanuclear or close to the nucleus (Fig. 3.3 A).

The juxtanuclear location suggests that these aggregates may be forming aggresomes, a protective type of aggregate that primarily localizes at the

juxtanuclear region, in close association with centrosomes (Kopito, 2000). Therefore, we explored whether polyQ82 aggregates in MR are near centrosomes as a marker of aggresomes. For this, we used immunofluorescence analysis using γ-tubulin antibody, a marker for centrosomes. Figure 3.3B showed that 48 h after transfection with polyQ82-YFP, MR cells showed a significantly higher number (over 65%) of inclusions close to the centrosome (red dots), when compared to mouse fibroblasts (~35%) (Figure. 3.3 B).

To find out whether there was any difference in concentration at which the polyQ82-YFP starts forming inclusions between mouse and MR fibroblasts, we used a method as described by Kopito's lab (Bersuker et al., 2013). The outline of the cell is marked using an imaging software (Fiji) at the frame where inclusions start to appear, and we calculated the mean cell fluorescence by dividing total cell fluorescence by total area of the cell. Our data showed that MR fibroblasts form inclusions at 65% less mean cell fluorescence than mouse fibroblasts (Fig. 3.3 C), indicating that MR cells start forming polyQ82 aggregates with less polyQ82 protein than mouse cells.

3.4.4 HSP27 co-localized with polyQ82 inclusions in MR fibroblasts but not in mouse fibroblasts

Using immunofluorescence and confocal imaging, we investigated if proteins that form part of the aggresome colocalize with polyQ82 aggregates. We found that p62 co-localized with polyQ82 inclusions in both mouse and MR

fibroblasts (Fig 3.4 A), while HDAC6 did not co-localize with polyQ82 inclusions in neither mouse nor MR fibroblasts (Fig 3.4 B). Interestingly, HSP27 co-localized with inclusions only in MR fibroblasts but not in mouse (Fig 3.4 C).

3.4.5 Improved survival to proteotoxic stress was apparent in other long-lived species

To test whether this was also observed in other sets of short and long-lived species, we used fibroblasts from the long-lived little brown bat (LBB; MLS=34 y) and compared them to fibroblasts from the short-lived evening bat (EB; MLS=6 y). Our previous data showed that, compared to EB fibroblasts, LBB cells have better autophagy and proteasome activity, but no difference in heat shock chaperones (Pride et al., 2015). Our results in Figure 3.5 showed that there was no difference in overall cell survival in response to polyQ82-YFP between long-lived and short-lived bat fibroblasts (LBB vs EB). The survival curves of EB and LBB have similar hazard ratios (2.24 and 2.03 respectively, Fig 3.5 A and 3.5 B). However, while fibroblasts from the short-lived EB have a median survival of 22.5 h, median survival for LBB fibroblasts is 37.5 h, indicating that LBB cells transfected with polyQ82 survive longer than EB. We also measured mean cell fluorescence at which inclusions start forming (as described above) and we observed that long-lived bat fibroblasts formed inclusions at 2 times less mean cell fluorescence than fibroblasts from short-lived bats (Figure 3.5C). We also analyzed the location of the polyQ82

inclusions with respect to the nucleus and centrosome (Figure 3.5D). Because the lamin A/C antibody did not work in EB cells, location of inclusions with respect to the nucleus was established using DAPI staining. Our data showed that 90.4% of LBB fibroblast inclusions were perinuclear, whereas in EB fibroblasts, ~60% of the inclusions were located randomly (Figure 3.5D). In addition, we also noticed differences in terms of proximity of inclusions with respect to the centrosome, where 64.1% of LBB fibroblasts had inclusions close to or co-localizing with the centrosome, whereas for EB fibroblasts it was only 35.5% (Fig 3.5E).

3.5 Discussion

Several studies, including our own, have shown that long-lived species have superior proteostasis compared to short-lived species from the same clade (Pérez et al., 2009; Rodriguez et al., 2012). Although lower levels of insoluble protein fractions in long-lived species (obtained from whole cellular extracts) have been used as an indicator of lower protein aggregation in long-lived species (Pickering et al., 2014), it is not known whether this is due to robust proteostasis processes or because of innate differences in the composition of their own cell's proteome. As a result, whether superior proteostasis in long-lived species leads to resistance to protein aggregation is not completely understood.

The objective of this work was to investigate whether enhanced proteostasis observed in long-lived species conferred a protection against protein

aggregation. We used a molecular approach where cells of both short- and long-lived species were transfected with the same exogenous aggregation-prone protein, polyQ82-YFP (Hsu et al., 2003; Kaganovich et al., 2008; Morley et al., 2002; Howarth et al., 2007). PolyQ proteins are broadly used as a protein aggregation model because their aggregation state is sensitive to genetic or pharmacological modifiers (Calamini et al., 2013; Morley et al., 2002), as well as different stressors and the age of the model organism (Mazzeo et al., 2012).

Our data showed that cells from long-lived species were indeed more resistant to proteotoxicity induced by polyQ82 and this effect was more robust in MR fibroblasts than in those from the long-lived bat (LLB) (Figure 3.1 and Figure 3.5). This is consistent with our previous data indicating that long- and short-lived bats show less differences in proteostasis mechanisms than observed between MR and mice (Pride et al., 2015). Also, even the short-lived bat is long-lived relative to the mouse, thus these differences may influence the resistance against proteotoxicity in the cells from these two species of bat. It may be relevant that these cells were obtained from wild-caught bats, thus it is also possible that differences in exposures, age and other factors may influence these preliminary findings, and more controlled experimental conditions may be needed to assess these mechanisms.

To identify the proteostasis pathways involved in resistance to proteotoxic challenges in MR cells, we used siRNA to block the various pathways. Our results_suggest that all three mechanisms are important for cell survival and

resistance to stress independent of proteotoxicity, because we observed a decline in cell survival even in control cells transfected with polyQ19. However, the toxic effect by polyQ82 was exacerbated because cell death was faster in the presence of all the siRNA tested. The most dramatic effect on cell survival was observed when macroautophagy was inhibited, indicating that this mechanism is crucial for cell survival and protection against general stress. For example, in ATG5 knockdown cells, expression of polyQ19 induced 100% cell death at 54 h after transfection and with polyQ82 100% of the cells are dead by 27 h after transfection (Figure 3.2B). The effect of knocking down the Ub-proteasome pathway or HSP27 was similar. In both cases the percentage of cell death by polyQ19 was increased by 50%, and for polyQ82 the increase in cell death at 72 h after transfection was 100% for Ub-proteasome pathway and 90% for HSP27 (Figure 3.2C and 3.2D). This data indicates that these proteostatic mechanisms are crucial for basal cell survival, not only to protect against the stress of protein aggregation, but also to protect against a general stress, i.e., the stress induced by the transfection and expression of an exogenous protein. Interestingly, our experiments using siRNA against HSF1, the transcription factor that regulates expression of heat shock chaperones, didn't show any difference in cell survival (data not shown), most likely because there were compensatory mechanisms that protected the cell against proteotoxicity.

Surprisingly, our data showed that, contrary to our expectations, MR skin fibroblasts did not have any less polyQ82YFP aggregation when compared to

mouse fibroblasts. Unexpectedly, we also observed that MR fibroblasts formed inclusions at a lower mean cell fluorescence (lower concentrations of polyQ) (Figure 3.3D), which may suggest that formation of inclusions could be a protective mechanism to prevent the toxic effects of misfolded monomers and smaller oligomers. Indeed, new evidence from multiple labs have shown that aggregates and inclusions are not toxic in all cases, and that in fact, they could be protective (Kuemmerle et al., 1999; Legleiter et al., 2010; Takahashi et al., 2008). It was also shown in the field of polyglutamine disease research, large inclusions are cytoprotective and smaller oligomers, misfolded monomers or microaggregates underlie pathogenesis (Todd and Lim, 2013).

These data correlate well with our finding that the perinuclear cellular location of polyQ82 aggregates in MR cells, whereas polyQ82YFP predominantly was located at the juxtanuclear region (sometimes forming indentations in the nuclear membrane). It has been shown that using artificial beta sheet proteins, as well as fragments of mutant huntington, the aggregation in the perinuclear region is more protective than in the cytoplasmic region because in this way the aggregates does not interfere with RNA transport (Woerner et al., 2016). Similarly, Kaganovich et al., showed that in yeast misfolded proteins can be sorted between two distinct quality control compartments: the juxtanuclear “soluble” quality control (JUNQ), where the aggregates can still be degraded; or the peripheral or “insoluble” protein deposits (IPOD), whereas terminally aggregated proteins are sequestered in the periphery (Kaganovich et al., 2008). Protective inclusions known as aggresomes are also found is

mammalian cells. Aggresome formation is a highly-regulated process that serves to organize aggregates of misfolded proteins into a single location in the cell (juxtanuclear) poised to be degraded later (Kopito, 2000). Based on this, we think that the difference in cellular location of protein aggregates in MR cells play an important role for cell survival. Our data showed that mouse fibroblasts do not form aggresomes, because polyQ82 inclusions were randomly located in the periphery of the cell and far from centrosomes and were not surrounded by the vimentin network. In the case of MR cells, although our evidence suggests that polyQ82 aggregates formed aggresomes, because they were juxtanuclear and juxta-centrosomal, we were unable to secure evidence for a presence of the vimentin cage due to incompatible antibodies.

Similarly, our data using immunofluorescence and confocal imaging for proteins that participate in aggresome pathway, i.e., p62, HDAC6 and HSP27 (Ouyang et al., 2012; Zaarur et al., 2008; Zhou et al., 2014); partially support our aggresome hypothesis, because we found a strong co-localization between HSP27 and polyQ82 in the perinuclear region of MR cells. Cox et al., also show that HSP27 inhibits cytotoxicity of alpha synuclein fibrils in a cell culture model (Cox et al., 2018). Also, several researchers have shown that polyQ-dependent toxicity correlates with a failure to upregulate or activate HSP27 (Evert et al., 2003; Zourlidou et al., 2007). Hence the HSP27 might be conferring protection against polyQ dependent toxicity in MR fibroblasts. In the case of p62, it is known to link the recognition of polyubiquitinated protein

aggregates to the autophagy machinery (Bjørkøy et al., 2006; Pankiv et al., 2007); Gal et al., 2007), and therefore is a common component of protein aggregates (Bjørkøy et al., 2005). Therefore, it was not entirely surprising when we found p62 co-localization with polyQ82 inclusions in both mouse and MR fibroblasts. Some reports using knockdown experiment with p62 showed that p62 does not affect formation of aggresomes, suggesting that p62 binds to aggresomes after their formation (Nagaoka et al., 2004). On the other hand, HDAC6 is one protein that plays a role in the formation of aggresomes, by recruiting and transporting polyubiquitinated misfolded proteins and aggregates to the aggresome via the microtubule network (Kawaguchi et al., 2003; Ouyang et al., 2012). Our data showed that polyQ82 inclusions do not co-localize with HDAC6 in either mouse or MR fibroblasts. However, the fluorescence for HDAC6 was very weak in both mouse and MR cells when compared to the strong signal of polyQ82, therefore it is not clear whether HDAC6 co-localized with polyQ82. Furthermore, there is another protein that participates in aggresome formation that is independent of HDAC6, BAG3, a chaperone which uses non-ubiquitinated substrates (Gamerdinger et al., 2011). The role of this protein in our model will be studied in more detail in future experiments.

Based on this data and previous research we think that HSP27 plays an important role in providing protection against polyQ82 proteotoxicity in MR fibroblasts. As was already proposed, we believe that when polyQ82 is overexpressed in MR cells, a chronic load of protein instability makes

functional recycling of unfolded aberrant proteins less feasible and thus it is better to simply sequester these inclusions into ordered aggregates (aggresomes) as the best option for cell survival (Vidyasagar et al., 2012) (Abisambra et al., 2010).

3.6 Figures

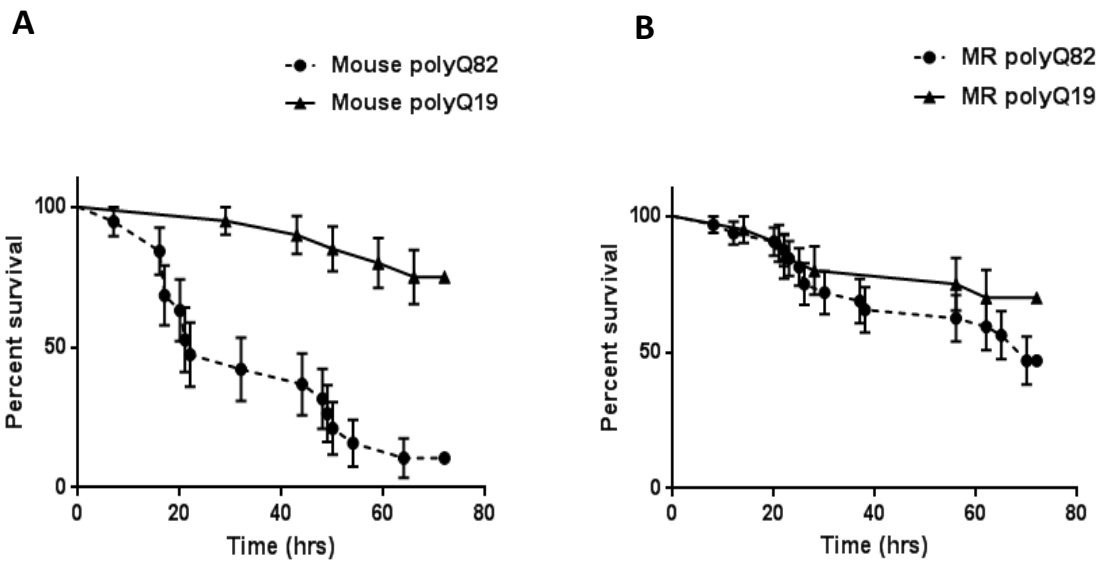


Figure 3.1. MR fibroblasts were more resistant to proteotoxicity induced by polyQ82 when compared to mouse fibroblasts. Fibroblasts of mouse and MR transfected with polyQ19-YFP (circle) and polyQ82-YFP (triangle) were followed using the live cell time-lapse imaging. Cell death was identified by collapsing of the cell and disintegration. Cell survival plotted as survival curve for mouse **(A)** and naked mole rat (MR) **(B)** fibroblasts (cells were analyzed for 72hrs after transfection). Dashed lines represent cells transfected with polyQ82 and solid lines represent with polyQ19. This data is representative of two independent experiments where two different cell lines for each animal were used, with an N of 19 to 32 cells, and analyzed by log-rank t test (mantel-cox test). PolyQ19 and polyQ82 survival curves in mouse cells were significant with a $p<0.0001$ and with a hazard ratio of 7.2. No significant difference in MR cells ($P=0.1469$) with a hazard ratio of 1.9.

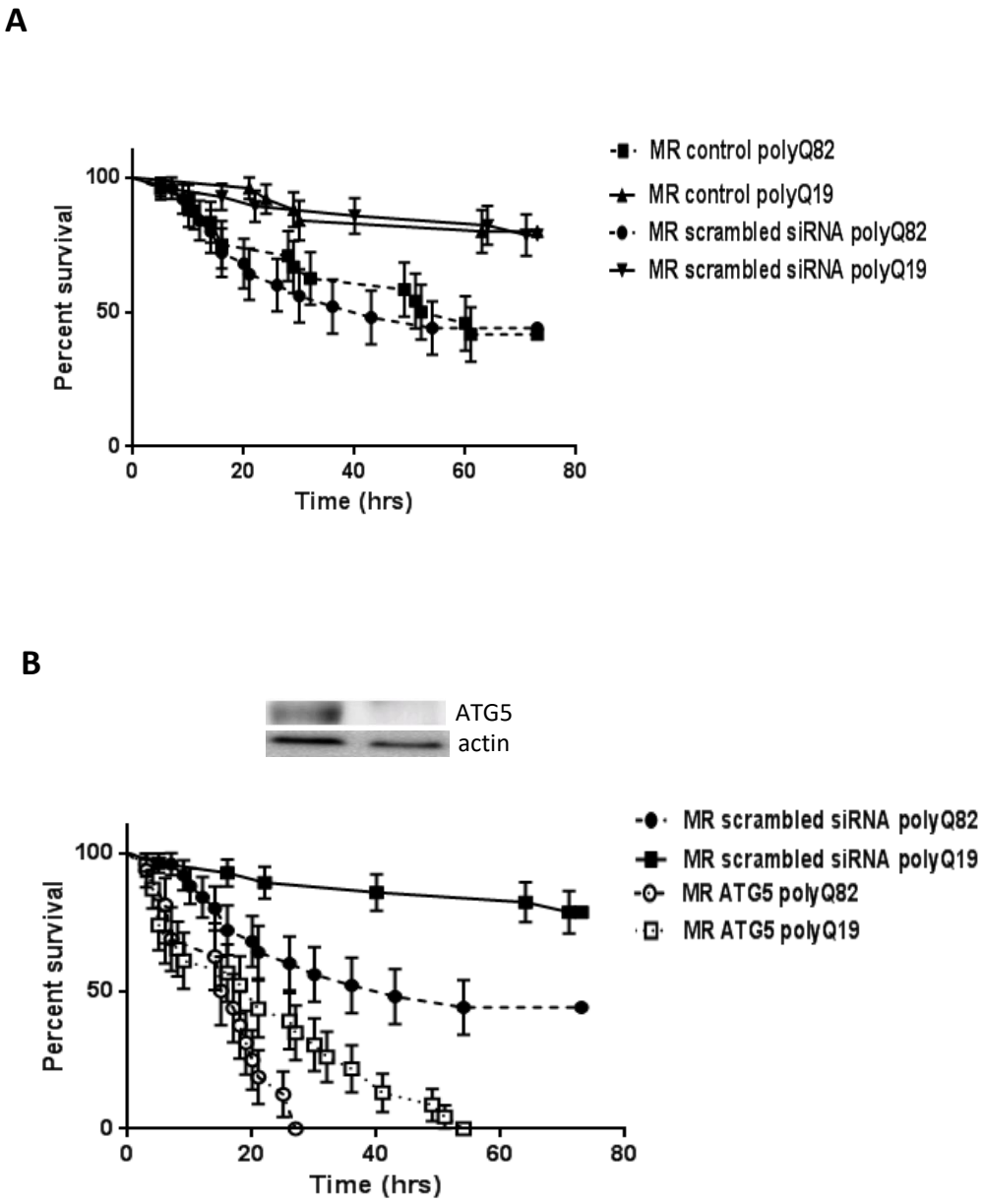


Figure 3.2. Knocking down of protein quality control processes affected MR cell survival.

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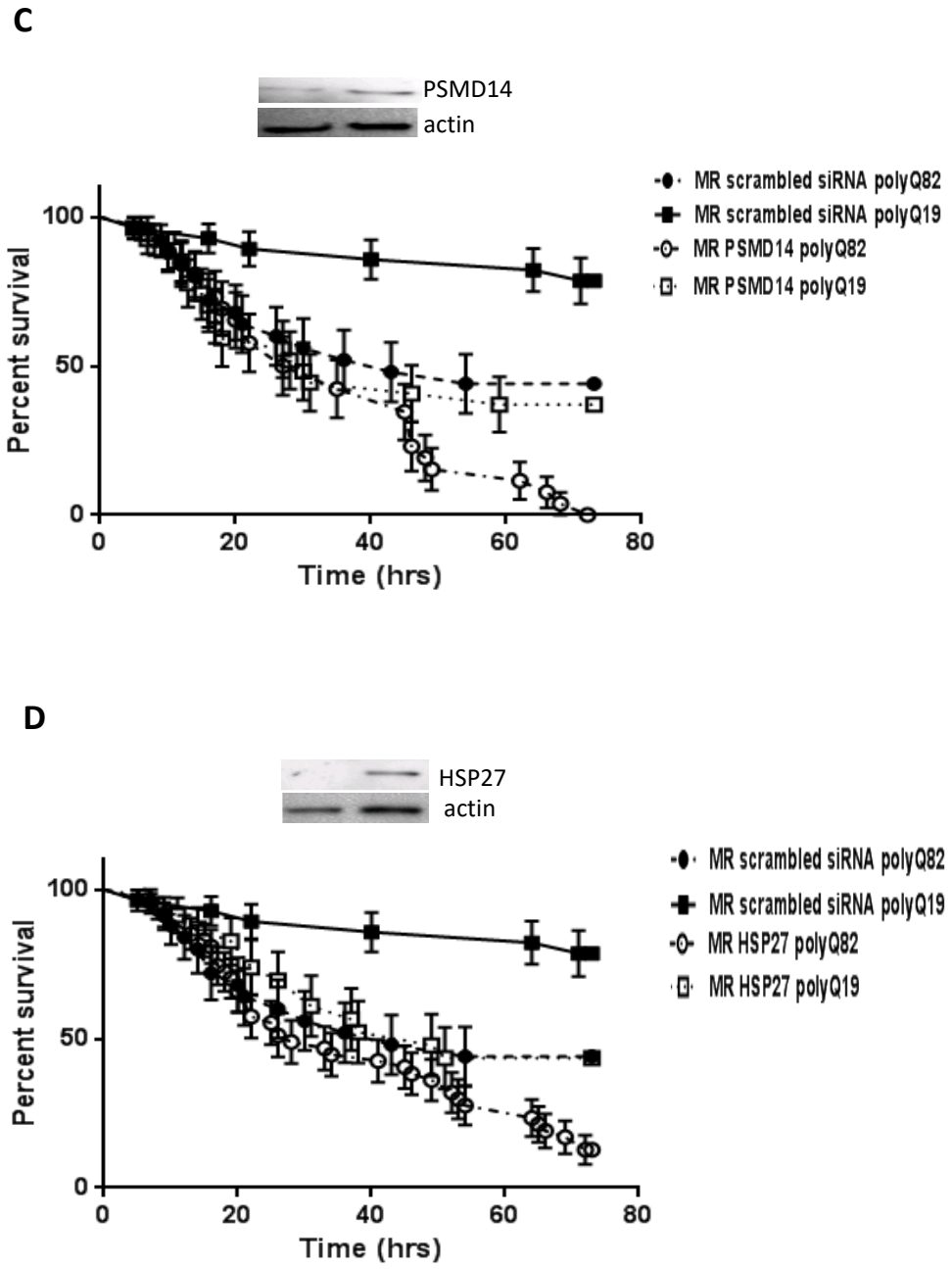

Figure 3.2. Knocking down protein quality control processes affected MR cell survival.

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Figure 3.2. **Knocking down of protein quality control processes affected MR cell survival.** Knockdown of autophagy, proteasome, and HSP27 was done using siRNA targeting ATG5, PSMD14, and HSP27 respectively. **A.** Survival curves of MR cells transfected with polyQ19 and polyQ82 and cells transfected with scrambled siRNA (polyQ19 and polyQ82). All survival curves were compared to the corresponding scrambled siRNA **B.** Effect of ATG5 knocking down in MR cell survival transfected with polyQ19 ($p \leq 0.0001$; hazard ratio of 10.09) and polyQ82 (($p \leq 0.0001$; hazard ratio of 3.60). A significance difference in ATG5 siRNA cells transfected with polyQ19 and polyQ82 ($p<0.05$; with a hazard ratio of 1.75) **C.** Effect of PSMD14 knocking down in MR cell survival transfected with polyQ19 ($p \leq 0.001$; hazard ratio of 4.18) and polyQ82 (($p \leq 0.01$; hazard ratio of 2.29). A significance difference in PSMD14 siRNA cells transfected with polyQ19 and polyQ82 was observed with a $P<0.05$; hazard ratio of 1.85) **D.** Effect of HSP27 knocking down in MR cell survival transfected with polyQ19 ($p \leq 0.01$; hazard ratio of 3.40) and polyQ82 (($p \leq 0.05$; hazard ratio of 1.82). A significance difference in HSP27 siRNA cells transfected with polyQ19 and polyQ82 ($P<0.05$; hazard ratio of 1.99). A western blot showing knock down of ATG5, HSP27 and PSMD14 is shown in the respective survival curve. Data represents one experiment with an N= 25 to 47 cells and analyzed by log-rank t test (Mantel-Cox test).

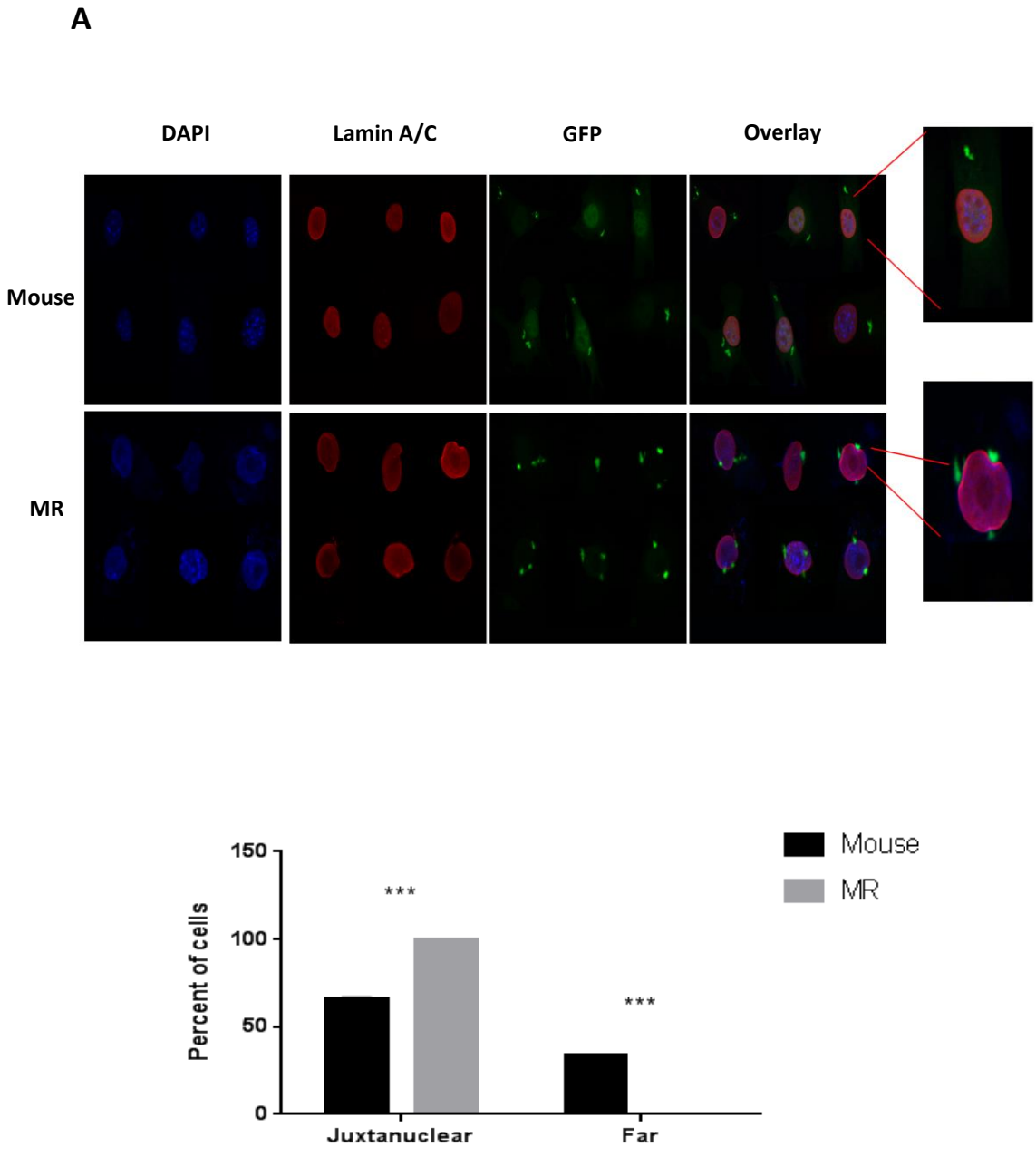

Figure 3.3. PolyQ82 inclusions in MR fibroblasts showed a juxtanuclear cellular location.

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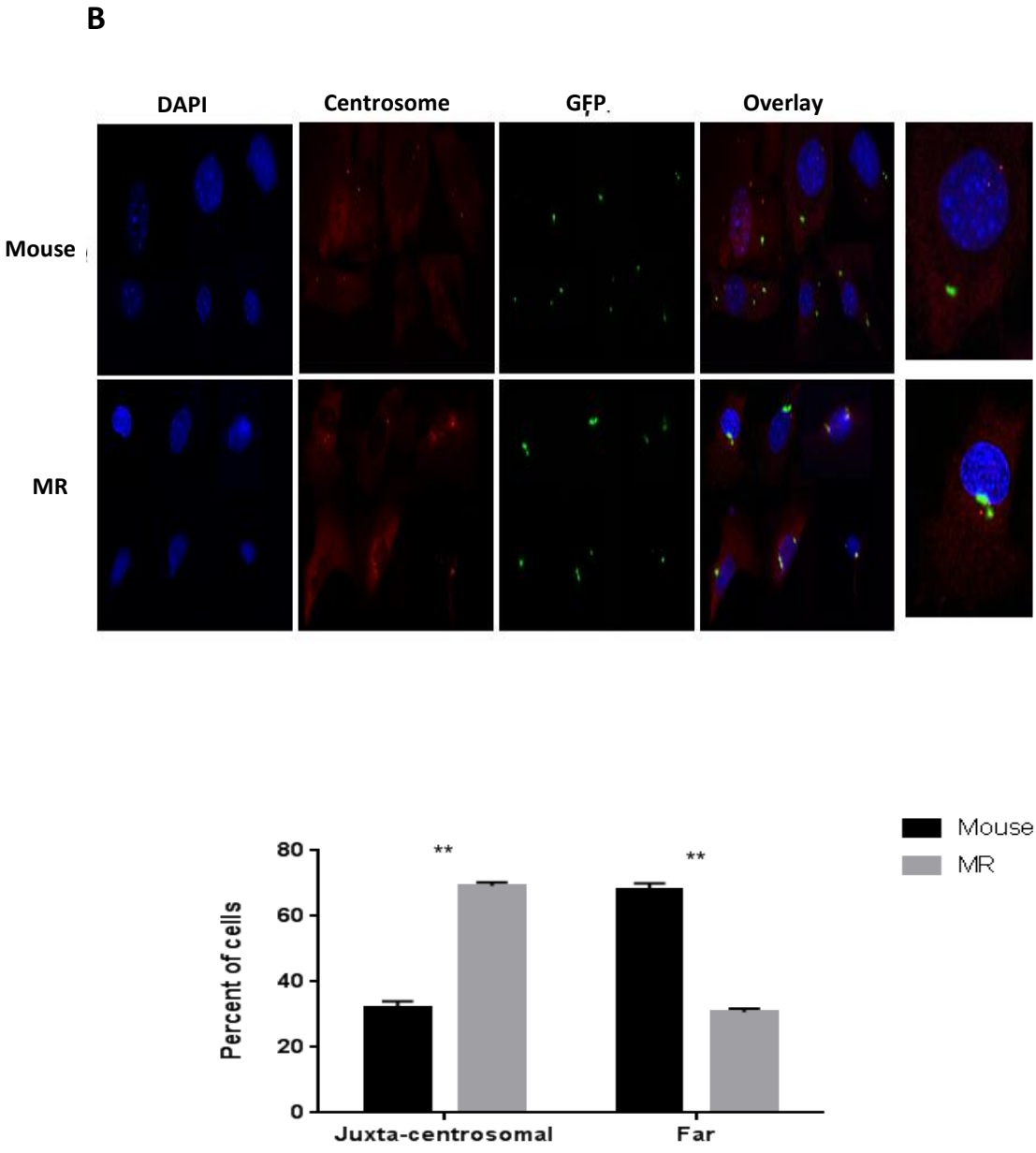

Figure 3.3. PolyQ82 inclusions in MR fibroblasts showed a juxtanuclear cellular location.

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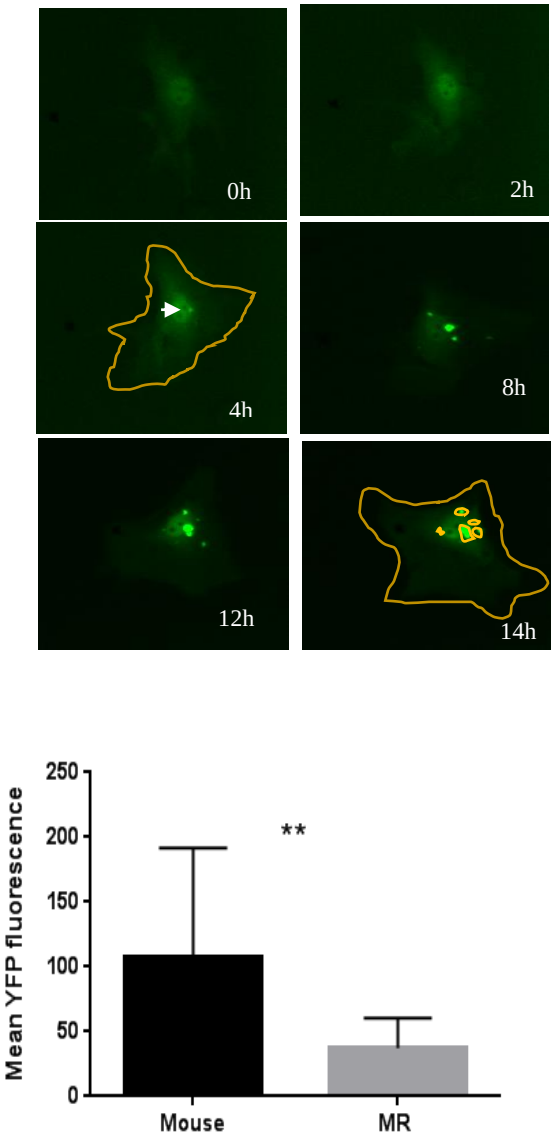

Figure 3.3. PolyQ82 inclusions in MR fibroblasts showed a juxtanuclear cellular location.

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Figure 3.3. PolyQ82 inclusions in MR fibroblasts showed a juxtanuclear cellular location. Indirect immunofluorescence was used to identify location of inclusions respective to nuclear membrane and centrosomes. **A.** Location of inclusions polyQ82-YFP inclusions (GFP) were identified with respect to nucleus (DAPI) and nuclear membrane [protein lamin A/C (alexa-647)], and overlay of three wavelengths in both top (mouse) and bottom (NMR) panels. Quantitation of inclusions with perinuclear location is shown in the bar graph to the right [x-axis: inclusion's cellular location based on proximity of inclusions to the nucleus: juxtanuclear (perinuclear), close and far; y-axis: percentage of cells]. **B.** Location of inclusions polyQ82-YFP inclusions (GFP) were identified with respect to nucleus (DAPI) and centrosomes (γ-tubulin) (Alexa 647), a marker for aggresomes. Overlay of three wavelengths in both top (mouse) and bottom (MR) panels. Quantitation of inclusions near to centrosomes is shown in the bar graph below [x-axis: inclusions location near to centrosomes: co-localized, close, and far; y-axis: percentage of cells]. **C.** Mean cell fluorescence was calculated in the frame where inclusions start to appear. The quantitation of the percent of mean cell fluorescence is shown in the graph below. The data represents mean ± SD of each cell line per species in an experiment. This is representative of two such experiments with different sets of cell lines. The data was analyzed by unpaired t-test. N= 19 cells were imaged in each experiment. The asterisks (**,***) denotes a statistically significant difference between mouse and NMR fibroblasts at $p \leq 0.01$ and $p \leq 0.001$ respectively.

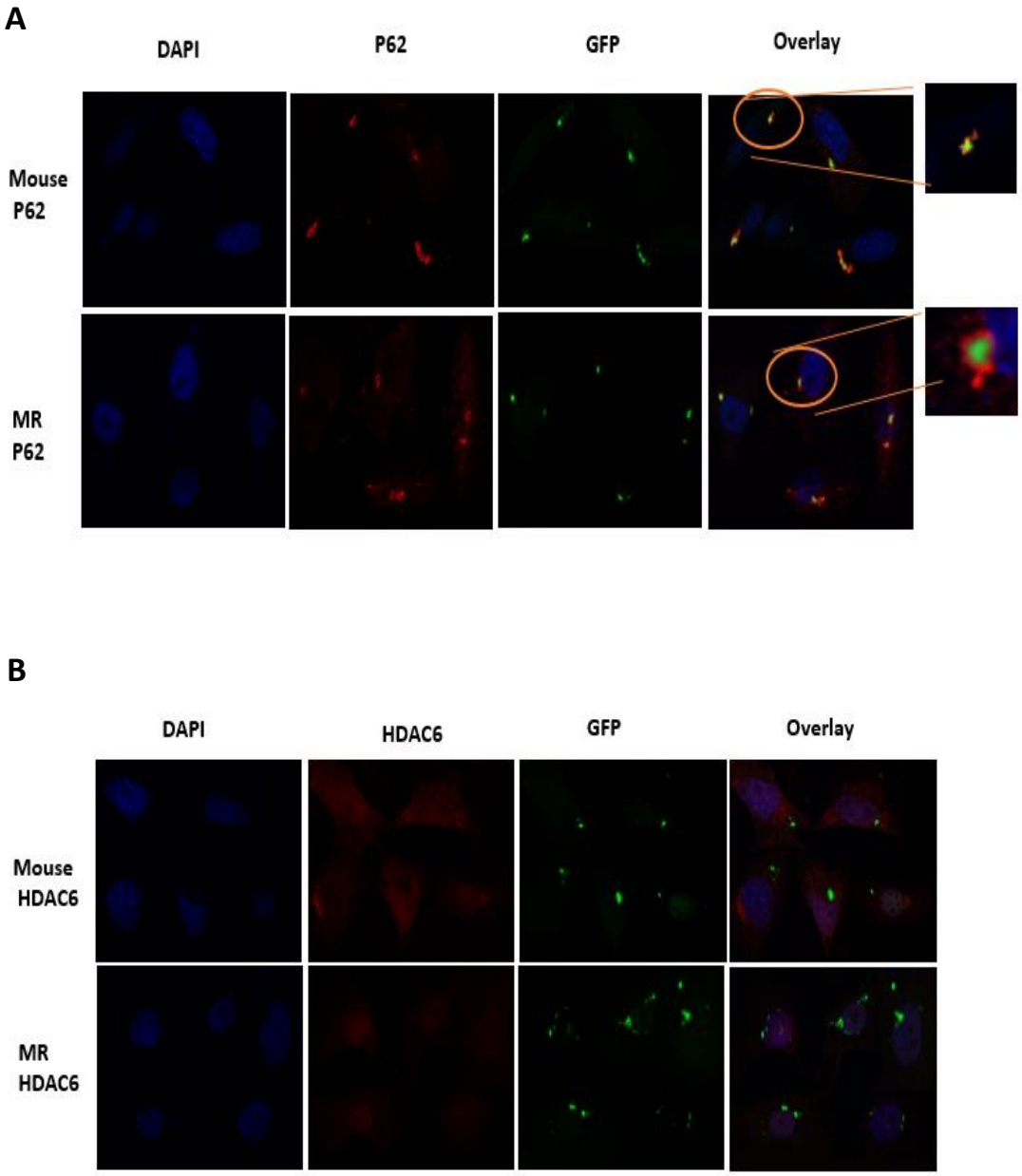

Figure 3.4. PolyQ82YFP inclusions co-localized with HSP27 in MR fibroblasts.

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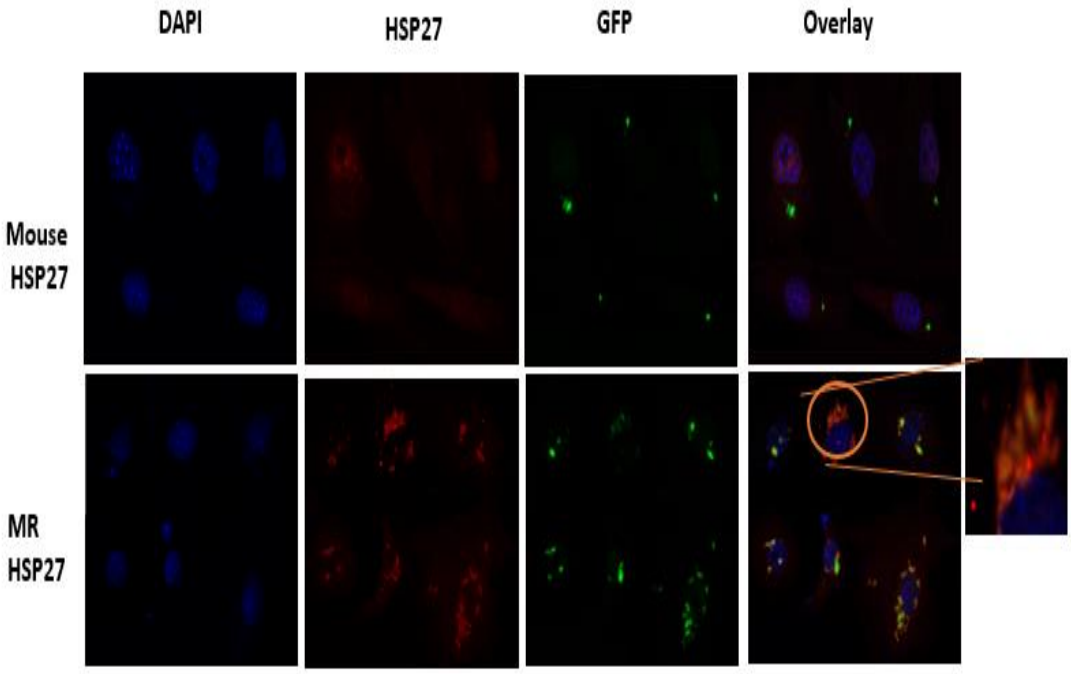

Figure 3.4. PolyQ82YFP inclusions co-localized with HSP27 in MR fibroblasts. Immunofluorescence using DAPI, polyQ82-YFP (GFP), p62 (alexa-647) **(A),** HDAC6 (alexa-647) (**B)**, and HSP27 (alexa-647) **(C)**, followed by the overlay of three wavelengths in both mouse (top) and NMR (bottom) panels. Data represent three independent experiments. Zeiss LSM 780 confocal microscope was used for imaging. 40X water objective, He Ne 633 laser line, and FL filter set 50 Cy5, EX BP 640/30, FT 660, Em BP 690/50 FL were used.

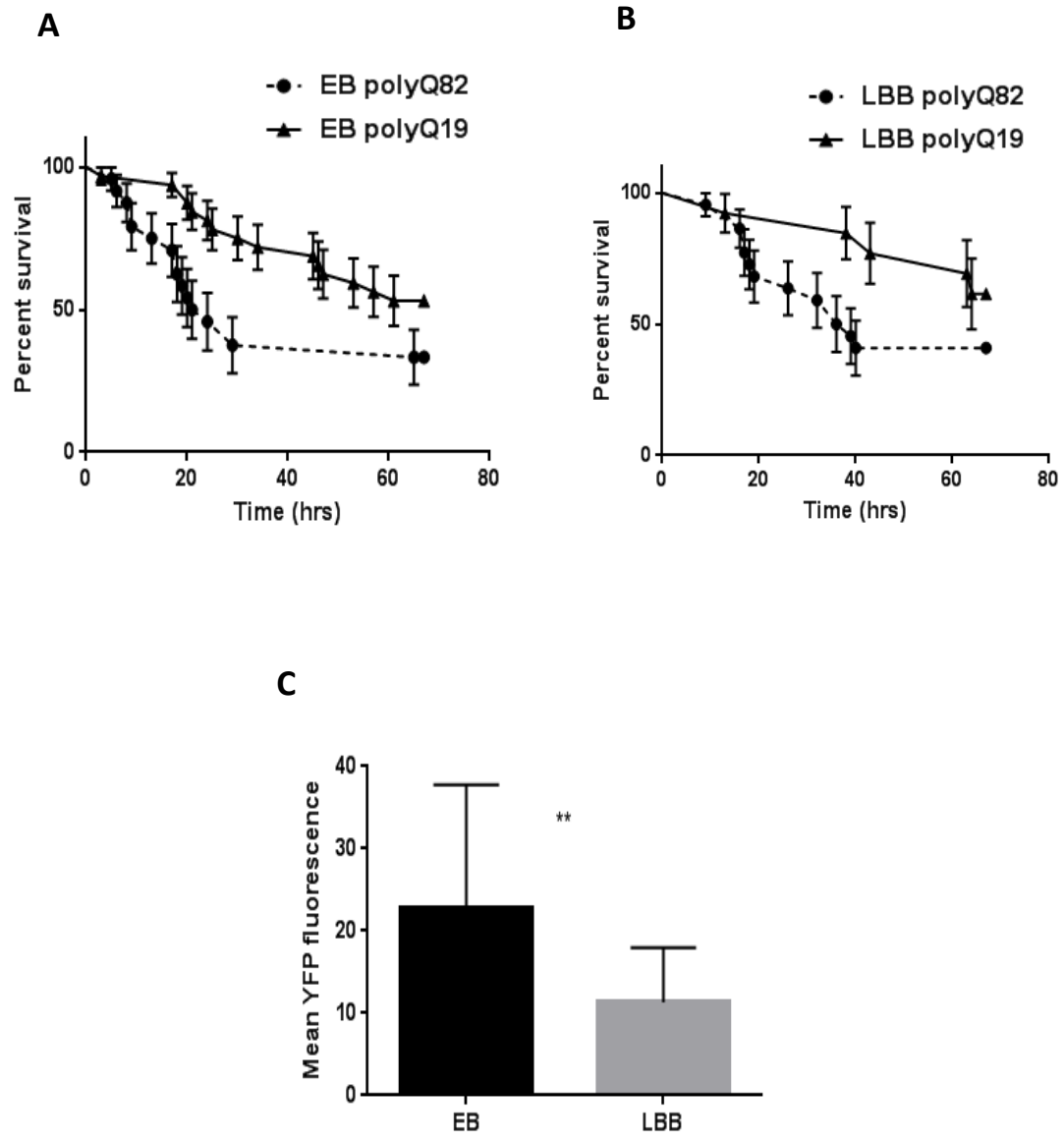

Figure 3.5. Improved survival to proteotoxic stress was apparent in other long-lived species

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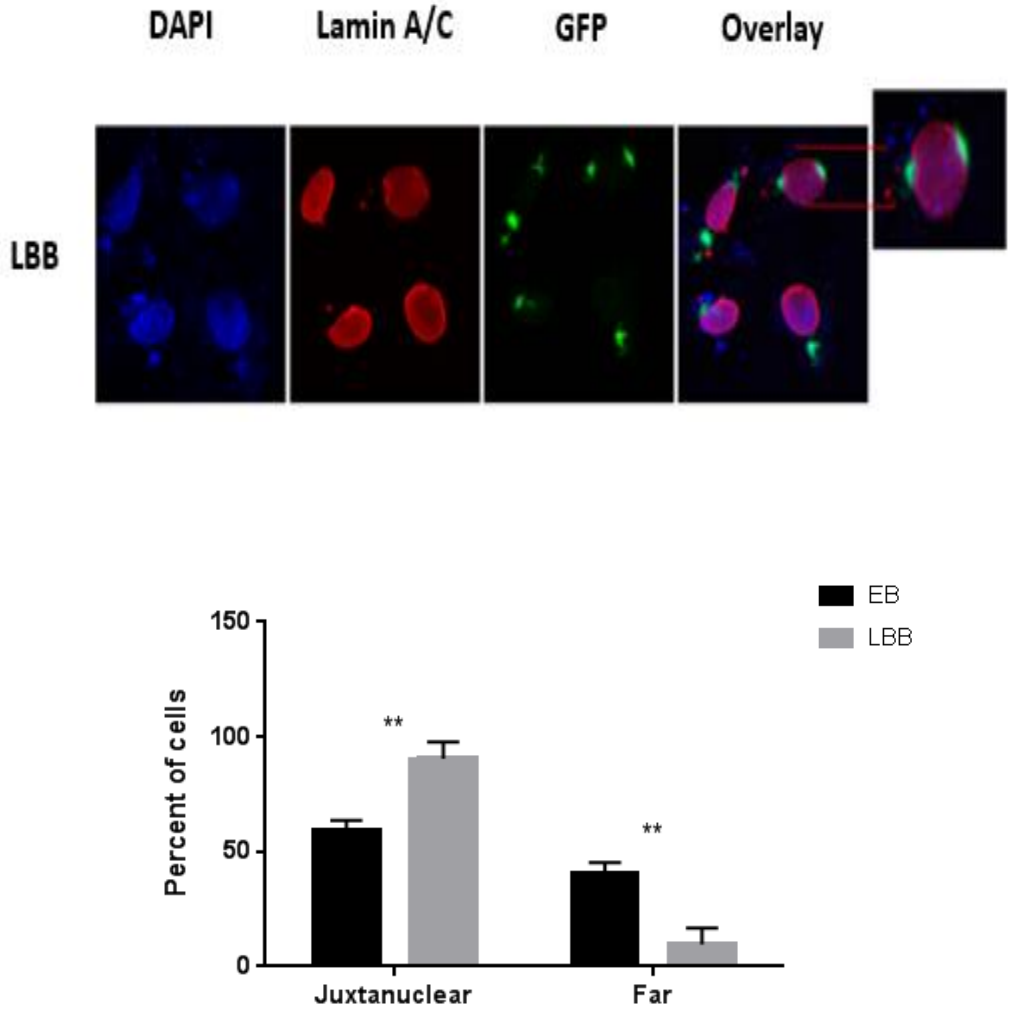

Figure 3.5. Improved survival to proteotoxic stress was apparent in other long-lived species

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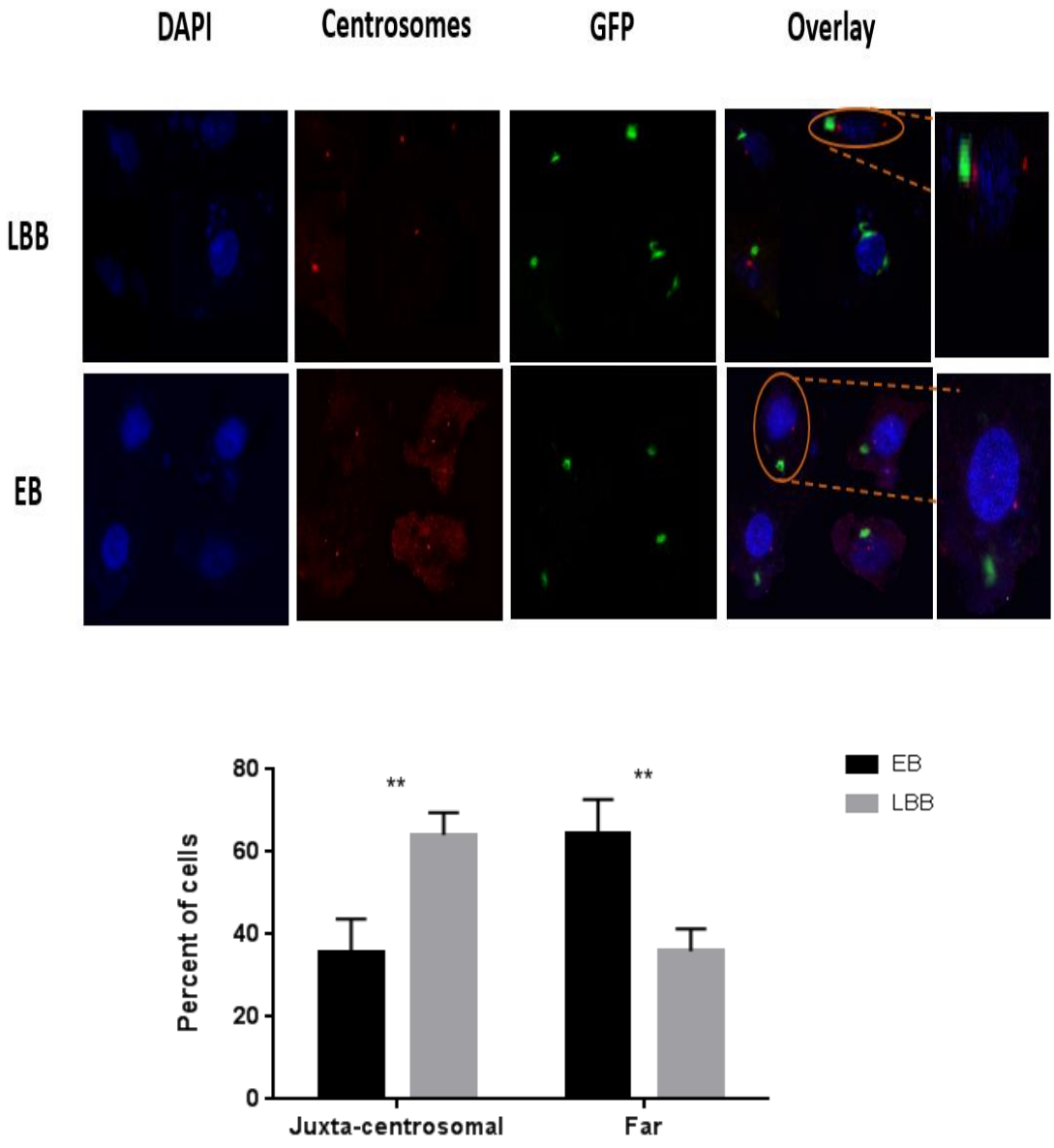

Figure 3.5. Improved survival to proteotoxic stress was apparent in other long-lived species

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Figure 3.5. **Improved survival to proteotoxic stress was apparent in other long-lived species.** Cell survival curves of skin fibroblasts from short-lived (EB) **(A)** and long-lived (LBB) **(B)** bats were transfected with polyQ19-YFP (solid lines) and polyQ82-YFP (dashed lines). **(C)** The quantitation of the percent of mean cell fluorescence is shown in the graph below. PolyQ-82 cellular localization in LBB cells using protein lamin A/C (alexa-647) **(D)** and γ-tubulin (alexa-647) **(E).** Quantitation of juxtanuclear and centrosomes localization are shown below each figure. Black bars represent EB cells and grey bars represent LBB cells. Survival curves were analyzed according to log-rank (mantel-cox) test. The hazard ratios for both EB and LBB cells is 2.10. Median survival for EB = 22.5 hrs, and for LBB=37.5 hrs . N=13 to 32 cells per cell line were analyzed. For mean YFP fluorescence, data shown is mean ± SD and analyzed by unpaired t-test. This data represents three independent experiments where different EB and LBB cell lines were used. N= 19 to 32 cells per cell line were analyzed. Data Asterisk (**,***) denotes a statistically significant difference between EB and LBB fibroblasts at $p \leq 0.01$ and $p \leq 0.001$ respectively.

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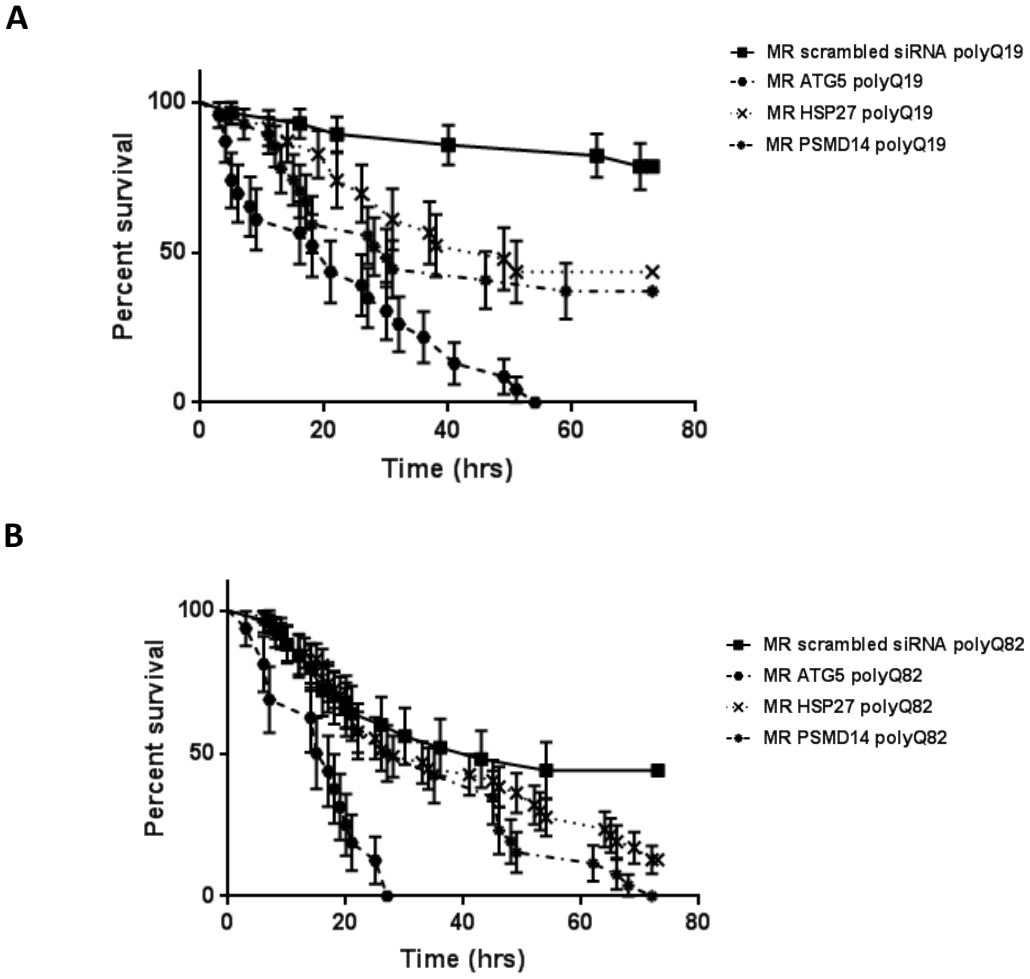

Supplemental figure 3.1. Knockdown of ATG5, PSMD14, HSP27 resulted in decreased survival of both polyQ19 and polyQ82 transfected naked mole rat (MR) fibroblasts. This figure is another way of depiction of data in figure 2. Survival curves of naked mole rat (MR) cells transfected with polyQ19 **(A)** and polyQ82 **(B)**. All survival curves were compared to the corresponding scrambled siRNA **A.** Effect of siRNA knocking down in MR cell survival transfected with polyQ19, ATG5 ($p \leq 0.0001$; hazard ratio of 10.09) , PSMD14 ($p \leq 0.001$; hazard ratio of 4.18) HSP27 ($p \leq 0.01$; hazard ratio of 3.40). **B.** Effect of siRNA knocking down in MR cell survival transfected with polyQ82, ATG5 (($p \leq 0.0001$; hazard ratio of 3.60), PSMD14 ($p \leq 0.01$; hazard ratio of 2.29), HSP27 ($p \leq 0.05$; hazard ratio of 1.82). Data represents one experiment with an N= 25 to 47 cells, and analyzed by log-rank t test (Mantel-Cox test).

Chapter 4

Conclusions

Bharath Sunchu

Most of the aging research has been performed using animal models that are classified as short-lived species, and not much is known about the importance of these mechanisms in determining longevity of the long-lived species. Long-lived species can be found in many phylogenetic clades, suggesting that longevity has evolved independently multiple times through evolution. Thus, studying proteostasis in long-lived species is a way to identify the mechanisms that evolution itself has used to extend life span (Austad, 2009). Hence in this dissertation we used a comparative biology approach to compare proteostasis in fibroblasts from short- and long-lived species of similar body size and belonging to the same phylogenetic clade.

Overall, we identified that fibroblasts from long-lived species have a more robust proteostasis compared to short-lived species. We found that autophagy (induced by serum deprivation) was the only mechanism enhanced in all three long-lived species. This data confirms that autophagy is an essential mechanism of defense in long-lived species as well, corroborating the relationship between autophagy and longevity previously identified in short-lived species (Madeo et al., 2010; Rubinsztein et al., 2011). We also found significant differences in the activities of proteasome and heat shock chaperones, with the sole exception of bats, where these mechanisms were found not to differ significantly between species of differing longevity. Our data confirmed previous results obtained using tissues from these long-lived species (Rodriguez et al., 2014; Perez et al 2009, Salmon et al, 2009).

From this data, we conclude that, in general, long-lived species have improved proteostasis, and this suggests that proteins from long-lived species might be better protected from unfolding, possibly allowing long-lived species to adapt better to stressful environments by having a higher protein turnover. In the case of bats, a possible explanation is that bat skin fibroblasts were obtained from wild-caught animals, which may have different physiological cellular conditions compared to cells from rodents and marsupials, that were obtained from animals in captivity (research laboratories). In addition, bats are adapted to a wide range of temperatures, i.e., they experience high temperatures during flight, and very low during hibernation (Feder and Hofmann, 1999), therefore, bat cells may need different experimental conditions to study protein quality control processes, including temperature, oxygen conditions, or other stressors. Also, because bats are unusually long-lived as a clade, it is possibly that even short-lived ones might have already elevated defense mechanisms, leading to a masking of the difference between long- and short-lived ones.

Our data from chapter 3 confirmed our hypothesis that enhanced proteostasis in long-lived species confers them to be more resistant to proteotoxicity induced by polyQ82-YFP. We also found that the differences in resistance to proteotoxicity were less robust between bat fibroblasts, which is in line with our results obtained in chapter 2, where the differences in protein quality control processes between short- and long-lived bat fibroblasts where not always enhanced in long-lived bats. Our data using siRNA against ATG5,

PSMD14, and HSP27 demonstrate that proteostasis mechanisms are indeed important for cell survival, and that autophagy is essential for cell survival.

Contrary to our initial expectations, we found that there was no less aggregation in MR fibroblasts when compared to mouse fibroblasts. Similar results were observed when comparing long- and short-lived bat fibroblasts. Furthermore, our data indicates that inclusions in short- and long-lived species are fundamentally different. Long-lived species formed polyQ82 inclusions at lower mean cell fluorescence than short-lived species and these inclusions were juxtanuclear and close to the centrosome, compared to a random location in short-lived species. These results support the idea the inclusion formation is not necessarily detrimental and may have a protective function depending on conditions in the cell. Many researchers have already proposed this idea, where in conditions of chronic protein instability, simply sequestering misfolded or damaged proteins into ordered aggregates is the best option for survival. This type of protective aggregation is generally known as aggresomes (Vidyasagar et al., 2012) (Abisambra et al., 2010). Indeed, when we further characterized the differences in inclusions between mouse and MR fibroblasts, we observed co-localization of inclusions with HSP27 in MR, but not in mouse fibroblasts. HSP27 is a chaperone that participates in aggresome formation, where it serves a "holdase" function, binding to hydrophobic patches of misfolded proteins. Previously it was shown that polyQ-dependent toxicity correlates with a failure to upregulate or activate HSP27 (Evert et al., 2003; Zourlidou et al., 2007). Based on this previous research and our current

work, we think that HSP27 plays an important role in providing protection against polyQ82 proteotoxicity in MR fibroblasts.

Although cultured fibroblasts are a good model to study proteostasis, in the future, our lab is planning to use induced pluripotent stem cells (iPS) to generate neurons and other cell types to expand our work in cell culture beyond skin fibroblasts. Current work in the lab is focused on generating stable cell lines where the polyQ expression can be induced at will. This will facilitate further studies by providing higher consistency in expression in and between cell lines. In fact, I attempted to generate stable cell lines with doxycycline inducible plasmids, but this effort was hampered by unanticipated technical difficulties such as doxycycline toxicity. In addition, some cells (marsupial fibroblasts) were refractive to transfection by any method attempted, including electroporation. This prevented me from obtaining results in marsupial cell lines for the polyQ aggregation project. However, since our results obtained in chapter 2 highly correlate with results obtained in chapter 3, we would expect that cells from long-lived marsupials would have shown improved resistance to proteotoxicity.

Future work in our lab will also expand this work to other protein aggregation models such as Aβ and tau aggregation. Also, by using lenti-viral shRNA plasmids, our lab is generating inducible knock out cell lines of autophagy, proteasome, and heat shock chaperones that will facilitate our study of the role of these mechanisms in protein aggregation.

I also started a collaboration with Dr. Dan Wustner of University of Southern Denmark who developed quantitative FLIP analysis (Wüstner et al., 2012). With this method we will be able to accurately detect and quantify any differences in the dynamics of polyQ82-YFP inclusions between different cell types.

Overall this dissertation shows that long-lived species have more robust protein quality control mechanisms than short-lived species, and that an active protein quality control machinery confers protection against proteotoxicity induced by misfolded proteins. I also identified some unexpected key differences in protein aggregation between short- and long-lived species, including a more efficient formation of aggregates at a lower threshold, and co-localization with HSP27, suggesting the possible involvement of protective aggresome-type mechanisms. In summary this data shows that enhanced proteostasis may be an important mechanism in the evolution of long- lived species.

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