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

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ABSTRACT

Our previous studies have shown that the liver from Naked Mole Rats (NMRs), 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, marsupials, and bats. Our results indicate that in all cases, macroautophagy was significantly enhanced in the longer-lived species, both at basal level and after induction by serum starvation. Similarly, basal levels of most HSPs were elevated in all the longer-lived species. Proteasome activity was found to be increased in the long-lived rodent and marsupial but not in bats. 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.

1. Introduction

Disruptions in proteostasis, a set of cellular mechanisms that maintain the stability of the proteome [1], 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 [2].

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

Abbreviations: NMR, naked mole rat; LB, little brown bat; EB, evening bat; SG, sugar glider; Opo, laboratory opossum.

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advancing age [3]. Dietary restriction (DR), the best characterized manipulation that extends lifespan and healthspan in mice, has been shown to increase the heat shock response and autophagy, processes that would be predicted to reduce protein misfolding and the accumulation of protein oligomers/aggregates.

Using a comparative biology approach, previous studies including our own, have shown that proteins present in extracts from the liver of a long-lived rodent and bats have better resistance to urea-induced unfolding when compared to mice, a shorter-lived species with similar body weight [4,5]. 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. Therefore, in this work we are comparing 3 pairs of long- and short-lived species: rodents [naked mole rats (NMRs; maximum lifespan (mls) ~30 year) vs. mice (mls ~4y), marsupials (sugar glider, mls ~18y vs. laboratory opossum (mls ~5y), and bats (evening bat, mls ~6y vs. little brown bat, mls ~34y).

2. Materials and methods

2.1. Species

The species studied were chosen based on their 1) well established longevity, 2) similar body 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*, 35gr and 4y], vs. Naked mole rats (NMRs) [*Heterocephalus glaber,* 30 gr and ~30y]; **Bats**: evening bat (EB) [*Nycticeius humeralis,*11gr and 6y] vs. little brown bat (LBB) [*Myotis lucifugus,* 8gr and 34y]; and **Marsupials:** laboratory opossum (opo) [*Monodelphis domestica,* 150 gr and 4,75y] vs. sugar glider (SG) [*Petaurus brevicaudus,* 100gr and 18y] (Supplemental Fig. 1).

2.2. 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% O_2, 5% CO_2, with the exception of NMR and mouse fibroblasts, which were cultured at 35 °C [6]. In all experiments, cells were used between passage 4 and 8. For NMR fibroblast cultures, we used Biocoat collagen I-coated tissue culture dishes (Advance Biometrix, San Diego, CA).

2.3. Protein degradation flux and macroautophagy

Degradation of long lived proteins was measured by radioisotopic pulse-chase labeling as described by Massey et al., 2008 [7]. Briefly, cells were incubated for 48 h 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/leupeptin (AC/L) for lysosomal inhibition. Cells were then chased for 4, 8, 12, or 24 h. 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.

2.4. Western blot analysis

Briefly, 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 [8]. The intensity of the bands was quantified by densitometry using Imagelab software (Bio-rad, Hercules, CA).

2.5. Heat shock response

A heat shock response was induced in fibroblasts by incubation at 41 °C for 1hr in 5% CO_2/95% air [9]. Then cells were quickly transferred to a 35 °C (mouse and NMR 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 h. 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.

2.6. 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.

3. Results

3.1. Enhanced macroautophagy in fibroblasts from long-lived species

Because autophagy plays a central role in proteostasis [10], we measured autophagy under both basal conditions and induced by serum deprivation, by monitoring the degradation of radioactively-labeled long-lived proteins (autophagy flux; Fig. 1A), 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) [11]. 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

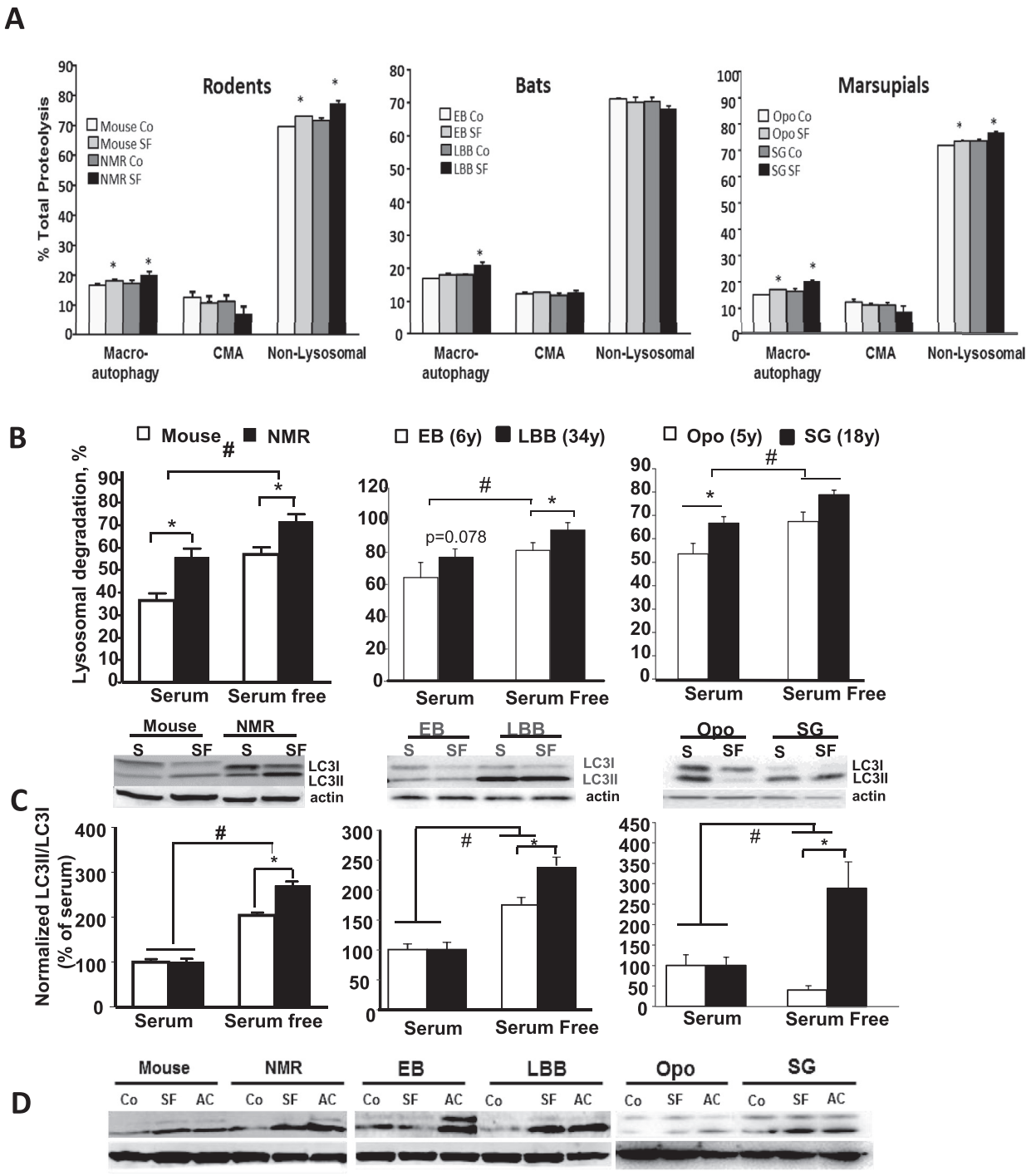

Fig. 1. Long-lived species have enhanced macroautophagy compared to short lived species. Protein degradation was determined by pulse and chase. Fig. 1A shows total rate of proteolysis in presence of 3-MA (macroautophagy measurement) and AC/L [Chaperone Mediated Autophagy (CMA) measurement]. Non-lysosomal 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 (Fig. 1B–D respectively). Representative gels for LC3II/LC3I per each especies 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 pound (#) denotes a statistically significant difference between serum and serum free conditions at $p < 0.01$.

both NMR and SG have a significantly higher rate of macroautophagy under both basal and serum-starved conditions, compared to the respective short-lived counterparts (Fig. 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 ($p = 0.078$; Fig. 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 the absence or in the presence and presence of AC/L [12]. In response to serum starvation, fibroblasts from each of the three long-lived species (NMRs, LBB, and SG) showed significantly increased LC3II/LC3I ratios compared to fibroblasts from short-lived species (induction of 50%, 40%, and 2-fold respectively; Fig. 1C).

LC3-II levels were most prominent after AC/L treatment, as indicated by the strong LC3-II band on the Western blot (Fig. 1D). The mTOR pathway is a major negative regulator of macroautophagy [13], therefore the 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 (NMR, LBB, SG), showing a further decline in S6 phosphorylation (~20, 20, and 25% respectively) under serum starvation, compared to short-lived species (Mouse, EB, Opp) (Supplemental Fig. S3).

3.2. The heat shock protein (HSP) response is elevated in fibroblasts from long-lived NMRs 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 [14], the levels of the major protein chaperones were measured by Western blot analysis. Under our experimental conditions, Fig. 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 (Fig. 2, middle panel). However under heat shock conditions, all chaperones measured were elevated in fibroblasts from NMRs and SG, compared to fibroblasts from their respective short-lived counterparts (mice and Opo), (Fig. 3A and C). However, in the longer-lived specie of bats, there were no statistical differences in the induction of any of the chaperones when comparing fibroblasts from short and long-lived bats (Fig. 3B).

3.3. Increased proteasome (20S) activity in fibroblasts from long-lived NMRs and marsupials but not bats

Increasing activity of the proteasome would be predicted to enhance protein homeostasis, and since we and others have previously shown that liver extracts from NMRs have increased activity of the proteasome compared to mice [4,6], 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 [8]. While the 20S protein content was similar between these species (Fig. 4 top panel), proteasome specific activity under basal conditions was greater in NMR and SG fibroblasts (40 and 30% respectively, Fig. 4A), compared to their shorter-lived counterparts. Furthermore, induction under serum free conditions was much more robust in longer-lived rodents and marsupials (Fig. 4B). For example, when compared to basal state (stripped bar; value = 1), NMRs had an 8-fold higher induction than mouse (~2-fold) fibroblasts, and SG had a 1.5-fold higher induction than Opo (~25%) fibroblasts (Fig. 4B, solid bars). Therefore using this 20S activity assay, NMRs 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 (Fig. 4A and B).

4. 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 *Caenorhabditis elegans* (20). Also, several studies suggest that cells from long-lived species are more resistant to a variety of stressors than cells from short-lived species [15,16]. 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 [17], 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 [18]. These data, coupled with evidence of sustained physiological function (healthspan) during aging in long-lived species [19], 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 NMRs 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 NMRs have been shown to have higher proteasome activity and a proteome that is more resistant to urea-

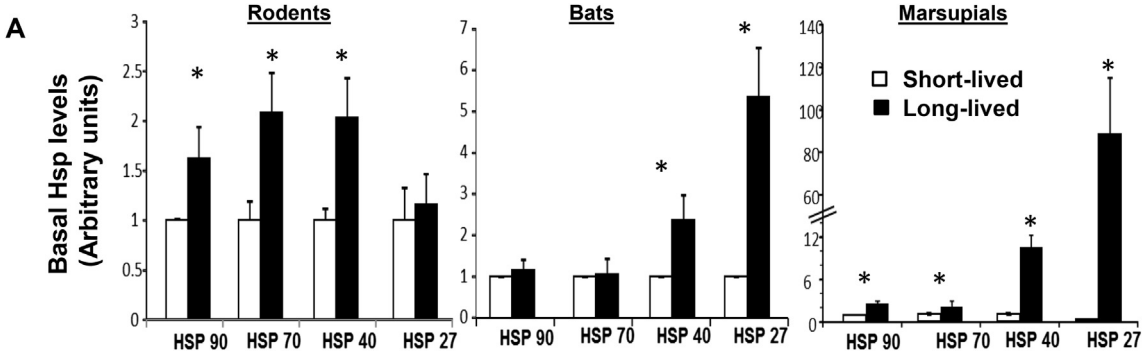

Fig. 2. Basal heat shock response is enhanced in long-lived species. The basal levels of chaperones (Hsp90, Hsp70, Hsp40, and Hsp27) were determined by western blot analysis in total homogenates from skin fibroblasts of short-lived species (open bars) and long-lived species (solid bars) of rodents, bats 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 $p < 0.05$.

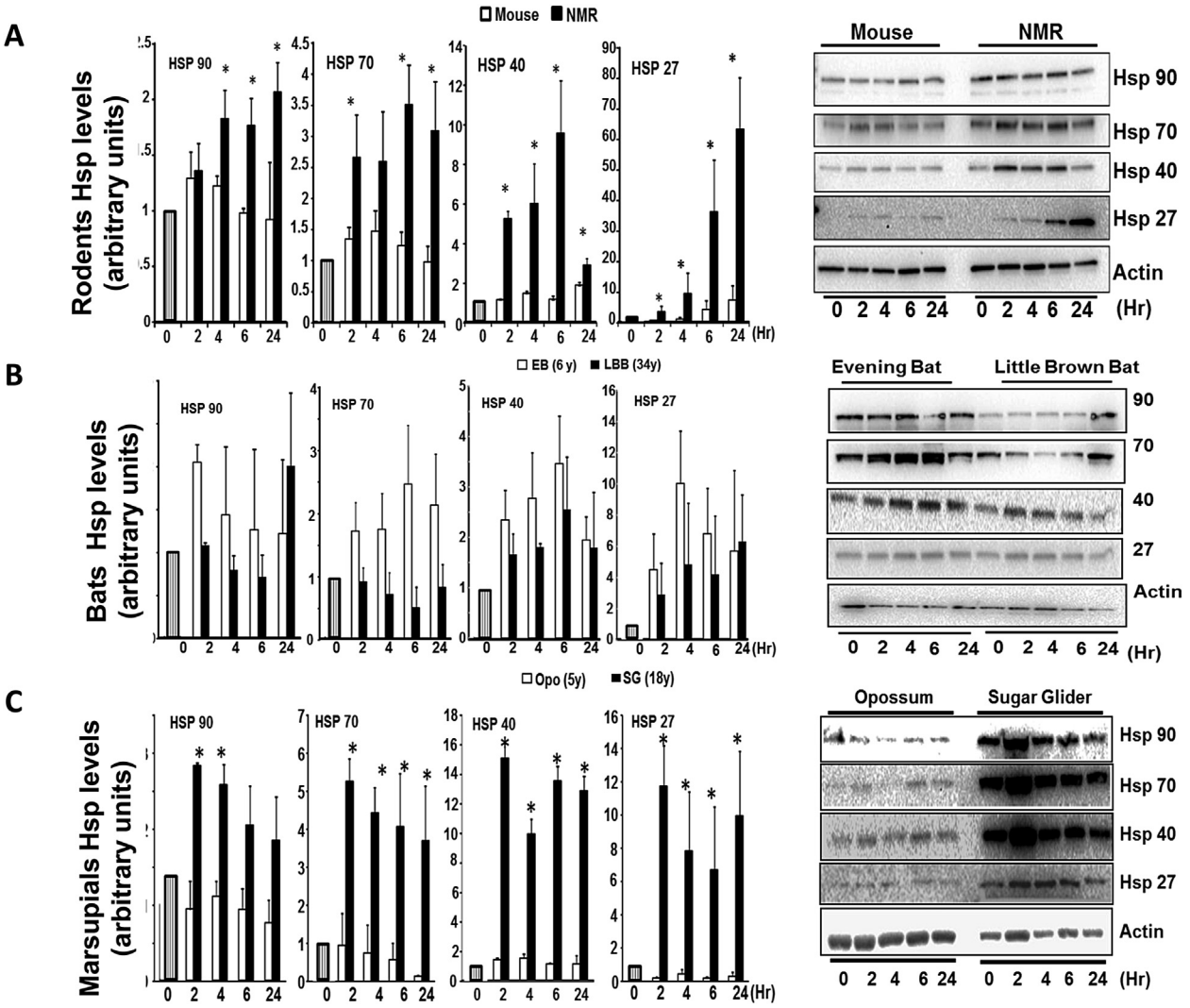

Fig. 3. The heat shock response is enhanced in long-lived rodents and marsupials, but not in long-lived bats. The heat shock response was determined by measuring protein chaperone levels (Hsp90, Hsp70, Hsp40, and Hsp27) over 24 h after heat shock treatment (41 °C for 1 h; panels A, B, and C) by western 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 $p < 0.05$.

induced unfolding [4], but also the recently published study where higher levels of chaperones have been observed in the liver of NMRs [20]. Considering that NMRs are extremely resilient to a variety of stressors and they are considered a model of successful aging [19], the available data strongly suggest that an improved proteostasis network may be necessary for the maintenance of longer health and life in NMRs.

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 [21,22]. 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 [11]. 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 Fig. S4). Our data agreed with recent data published by Zhao et al., 2014 [23], 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, NMRs and SG, but not in long-lived bats (Fig. 3). While HSPs are extremely well conserved across species [14], 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 and 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 (msl~ 12y)] showed better resistance to urea-induced unfolding, suggesting that these species may have a better chaperone response [5], this might be

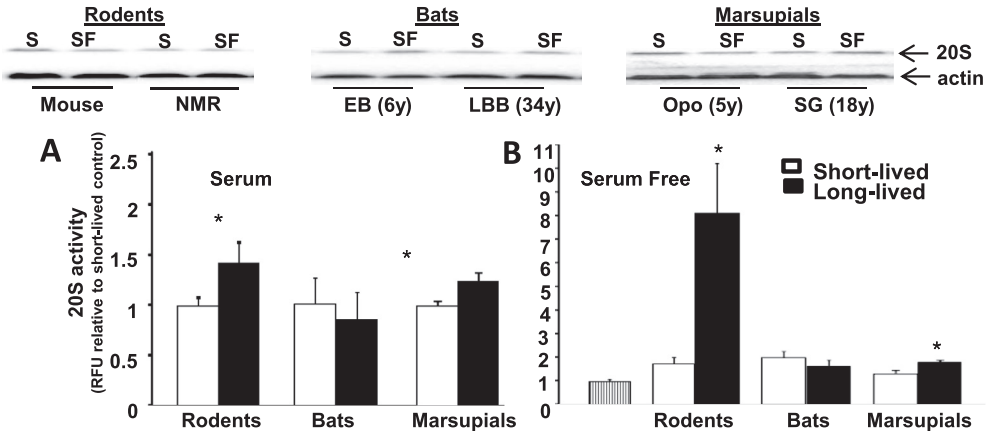

Fig. 4. Long-lived rodents and marsupials, but not bats, have enhanced proteasome activity compared to short-lived species. Proteasome activity was measured at both basal (serum; Panel A) and serum free conditions (Panel B) in total homogenates from skin fibroblasts of short-lived (open bars) and long-lived 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 $p < 0.05$.

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 a 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) [24]. 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 [5], 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 may be 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 activity might be needed in addition to the 20S proteasome.

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. It would be ideal to do these experiments with fresh tissue; however, at difference with common laboratory species such as mice, fresh tissue is hard to obtain when dealing with unusual species. Nevertheless, this data represents a first attempt to identify molecular mechanisms that seem important for species longevity.

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.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.046.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.046.

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