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Proteomics of long-lived mammals

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Abstract

Mammalian species differ up to 100-fold in their aging rates and maximum lifespans. Long-lived mammals appear to possess traits that extend lifespan and healthspan. Genomic analyses have not revealed a single pro-longevity function that would account for all longevity effects. In contrast, it appears that pro-longevity mechanisms may be complex traits afforded by connections between metabolism and protein functions that are impossible to predict by genomic approaches alone. Thus, metabolomics and proteomics studies will be required to understand the mechanisms of longevity. We review several examples that demonstrate the naked mole rat (NMR) shows unique proteomic signatures that contribute to longevity by overcoming several hallmarks of aging. We also discuss SIRT6 as an example of a protein that evolved enhanced enzymatic function in long-lived species. Finally, we show that several longevity related proteins such as Cip1/p21, FOXO3, TOP2A, AKT1, RICTOR, INSR and SIRT6 harbor PTM sites that preferentially appear in either short- or long-lived species and provide examples of crosstalk between PTM sites. We discuss prospects of enhancing lifespan and healthspan of humans by altering metabolism and proteoforms with drugs that mimic changes observed in long-lived species.

Keywords

aging; proteomics; long-lived mammals; naked mole rat; SIRT6

Why study aging in the context of Comparative Biology?

Aging is a complex phenotype. Nine hallmarks of aging are generally recognized and include increased cellular senescence (and accompanying inflammation due to the senescence activated secretory phenotype or SASP), stem cell exhaustion, altered cellular communication, genomic instability, telomere loss, mitochondrial dysfunction, deregulated nutrient sensing, loss of proteostasis, and epigenetic alterations [1]. Deciphering root cause or effect relationships for the hallmarks is difficult. Perhaps a common underlying cause(s) manifests itself in many or all of the hallmarks. Alternatively, the cell may be designed to trigger other hallmarks if one is initiated. Several hereditary premature aging syndromes (such as Werner Syndrome or Hutchinson Gilford Progeria) implicate genome instability and/or loss of nuclear integrity as an underlying commonality that may trigger the other

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hallmarks [2]. Interestingly, calorie restriction (CR) or dietary restriction (DR) improves healthspan and increases lifespan in many species suggesting a common underlying connection between metabolism and avoidance of the hallmarks to increase longevity [3]. Combined “omics” (genomic, transcriptomic, proteomic, and metabolomic) approaches can provide insight into these connections and omics data obtained from long-lived species may yield new insights because their metabolism appears distinct from short-lived species.

Long-lived animals have evolved mechanisms to avoid, delay, or alleviate the hallmarks of aging [4]. One possibility is that they possess unique alleles of key aging proteins (that may be similarly optimized in human centenarians). Alternatively, long-lived species may employ entirely new strategies. Many aging-related pathways appear to be conserved from yeast to mammals [5]. The genome (and transcriptome) sequences of a number of long-lived species have been determined including the naked mole rat (NMR, *Heterocephalus glaber*) [6], the blind mole rat (BMR, *Nannospalax gallili*) [7], Damaraland mole rat (DMR, *Fukomys damarensis*), bowhead whale (BW, *Balaena mysticetus*) [8], and several bat species such as the insectivorous bat (*Myotis davidii*) or brandt’s bat (*Myotis brandtii*) [9, 10]. Several recent reviews highlight novel strategies that long-lived animals may employ based upon predictions from genome sequences [9, 11, 12]. Very few predictions have been verified at the functional level.

In this review we will discuss comparative proteomics as a tool for aging research. We will emphasize the emerging connection between proteomics and metabolomics studies. Next, we will provide several examples where proteomics has already revealed novel insights for comparative aging studies. We will also present an analysis of conservation of post-translational modification (PTM) sites in short- and long-lived species of mammals.

Why is Proteomics relevant for studying the comparative biology of aging?

Proteomics encompasses the studies of protein stability and degradation, interactions, cellular localization, and post-translational modifications (PTMs). Mass spectrometry (MS) has emerged as a powerful method due to instrumentation advancements, development of computational pipelines for data analysis, methods for protein preparation and enrichment, and strategies for quantitation [13]. In many cases MS is the only approach that can yield certain types of data such as characterizing a novel PTM or the co-occurrence of two PTMs on a contiguous peptide. Unambiguous assignments of raw MS data to matching predicted sequences requires reliable genome sequences. Thus, genome sequences of long-lived mammals have opened up the world of comparative biology of aging to proteomics.

Genomics enables proteomics; yet, gene sequences do not fully predict the proteome. Proteins are much more complex. The word “proteoform” was coined to signify an individual single contiguous peptide with all unique PTMs or splicing variants constituting a single form [14]. Single proteoforms may behave entirely differently [15]. Some proteins may exist as hundreds of unique proteoforms- significantly more variable than primary sequence predicts. As many as 400 different PTMs are known [16]. Often two or more different PTM chemistries compete for occupancy of the same site *and* multiple sites for PTMs often exist on any protein. In long-lived species, novel alleles add to proteoform diversity which may

facilitate a high level of protein specialization or diversification of functions. Comparisons of proteoforms of key aging-related proteins between species may reveal subtle differences in specialization that genomics cannot predict.

PTMs deserve special attention because they link protein regulation to metabolism. The chemical groups of many PTMs are derived from key metabolites [17]. Examples include ATP utilized for phosphorylation, acetyl-CoA (or other acyl-CoAs or acyl-phosphates) for acetyl/acylation, S-Adenosyl methionine (SAM) for methylation, UDP-sugars for glycosylation (such as UDP-GlcNAc for GlcNAcylation, NAD⁺ for ADP-ribosylation, and isoprenyl-pyrophosphate (IPP) for prenylation. The link between ATP, phosphorylation, and signaling is well-established and higher-order modeling of signaling networks is possible [18]. Similarly, clear ties exist between metabolites such as acetyl-coA or SAM and key histone PTMs such as acetylation and methylation (including mono-, di-, and tri-methylation) as are the role of key metabolites such as NAD⁺ (with sirtuins) or flavin adenine dinucleotide (FAD) or α -ketoglutarate (with lysine demethylases) in their removal [19]. Many diverse proteins are acetylated; yet, histones are the most acetylated protein in mammalian cells [20]. Interestingly, in the case of histones, acetylation and methylation compete for occupancy of the same site in cycles of addition and removal. Occupancy and cooperativity between sites can dramatically impact transcription.

Complicating matters, if fatty acid oxidation and ketogenesis occur, longer acyl-CoA chains may compete with acetyl-CoA leading to acylations [21, 22]. Sirtuins such as SIRT3, 4, or 5 (mitochondria or cytosol) and SIRT6, 7 (nucleus) employ NAD⁺ to deacetylate many previously unrecognized acylations [21, 23]. Other PTMs derived from metabolites such as ADP-ribosylation, and O-GlcNAcylation also impact a wide range of proteins including histones [24, 25]. The extent of cross-talk between all of these PTMs is unknown, but is also likely to depend upon metabolism.

Metabolic disorder (as obesity or in type II diabetes) and CR regimens confer opposing effects on aging and affect the levels of PTMs such as acylations of mitochondrial proteins or ubiquitination [21, 26]. Many of the changes that occur in metabolic disorder occur naturally during aging are accelerated by the disorder and alleviated by CR. It is not clear if similar changes occur during aging in long-lived species or if overfeeding would have negative effects on their PTMs. It is possible that long-lived species display altered metabolism, often appearing to mimic calorie restriction [9, 27–29] that will likely manifest as distinct changes in PTMs.

Proteomics and the Hallmarks of Aging

Proteostasis

Beyond human studies, some of the first proteomics studies of long-lived species was to examine proteostasis in the NMR. Loss of proteostasis occurs during aging in species ranging from yeast to mice [30, 31]. NMRs employ several mechanisms to achieve tight control over proteostasis compared to other short-lived species such as the mouse. A very early study found that NMR liver proteins appear more stable (with lower overall levels of ubiquitination) and did not show age-associated increases in cysteine oxidation, unlike

mouse proteins whose oxidation increased as animals aged [32]. NMR liver tissue extracts also showed more active proteosomal activity (and levels by Western blot) suggesting that damaged proteins are efficiently removed in the NMR [33]. Interestingly, NMR cells maintain an active proteasome due to interactions with a novel complex that contains heat-shock proteins from the HSP72 and HSP40/DNAJ families among other components [34]. Follow-up studies showed that autophagy as well as protein chaperone activities also appear enhanced in the NMR as well as in several other long-lived species [35]. Consistent with the idea that NMRs employ novel strategies to combat protein damage, using a novel MS-based approach, Heinze et al. found that the NMR liver contains very high levels of peroxiredoxin 1 (PRDX1) and thioredoxin reductase 1 (TXNRD1) proteins which reduce ROS and promote proteostasis [36].

Recently, Swovick et al. employed a quantitative MS with stable isotope labeling by amino acids in cells (SILAC) to examine global protein turnover rates in cultured fibroblasts derived from NMR and seven other rodents with diverse lifespans including mouse, rat, hamster, guinea pig, beaver, chinchilla, and blind mole rat [37]. They determined the turnover rate of a very broad spectrum of proteins from rapidly degraded to those whose half-lives are significantly longer than the rate of cellular proliferation. Interestingly, global protein turnover rates negatively correlated with the lifespan of species. Consistent with this result, a combined transcriptome/metabolome analyses of fibroblasts isolated from sixteen mammalian species found that animals with increased longevity displayed relatively lower expression levels of genes related to proteolysis, autophagy and apoptosis as well as higher levels of DNA repair/maintenance genes [28].

Metabolism may impact protein turnover differently in long-lived species. Heinze et al. argued that increased fatty acid utilization by the liver may contribute to NMR longevity since enhanced expression of fatty acid oxidation components was also observed in mice upon caloric restriction [3, 38]. Targeted metabolome profiling of four tissues (brain, heart, kidney, and liver) derived from 26 mammalian species revealed that long-lived mammals maintain lower overall levels of polyunsaturated triacylglycerides (TAGs) supporting the idea of increased fat utilization. Other proteomic analyses of the NMR brain motivated by the extreme insulin sensitivity of NMRs [39] found that several metabolic enzymes showed altered levels or phosphorylation status (using 2D gels and Pro Q Diamond staining) [40] supporting the idea that NMR metabolism is distinct from mice. Similarly, serum metabolome analysis indicated that NMR generally closely mimicked hibernating ground squirrels or rats subjected to dietary restriction [27]. Consistent with Swovick et al., a correlation between nutrient sensing/metabolism and slow global protein turnover in long-lived species is also supported by several mouse studies where global protein turnover was decreased during calorie/dietary restriction, rapamycin-treatment, overexpression of mitochondrial catalase (mCAT), as well as in long-lived Snell dwarf mice [30, 41].

Remarkably, another indication that the NMR proteostasis may be distinct came from an analysis of their unique ribosome structure. Azpurua et al. noted that NMR 28S ribosomal RNA (rRNA) is processed into two smaller fragments of unequal size and hypothesized that processing of the 28S rRNA may confer an enhanced ribosome function [42]. Using a luciferase-based assay, they found that NMR cells display approximately ten-fold higher

translational fidelity compared to mouse cells. Later, Ke et al. extended these findings using comparative approach and showed that translational fidelity appears to positively correlate with lifespan across 17 different rodent species [43]. It is difficult to prove that the cleavage of the 28S rRNA causes the increase in translational fidelity because reconstitution of a purified NMR ribosome has not yet been reported. However, these findings may echo the effects of lifespan extending molecules such as rapamycin (or ATP-competitive inhibitors) that inhibit the mTOR (mammalian target of rapamycin) kinase. Inhibition of TOR signaling using genetic or pharmaceutical interventions extends lifespan in multiple model organisms from yeast to mice (reviewed in [44]). mTOR responds to cellular cues such as nutrient availability to affect translation (among other processes) [45]. Among the key targets of mTOR are the S6 kinases (S6Ks), the inhibitory eIF4E-binding proteins (4E-BPs), and the eIF4G initiation factors which all play a role in translation in general as well as ribosome biogenesis by specifically affecting the translation initiation of mRNAs containing 5' terminal oligopyrimidine (TOP) motifs [46]. Yet, a clear relationship between translation rate and fidelity has not been determined. Given that translation efficiency (both rate and fidelity) declines in cells and tissues as they age (reviewed in [47]), the NMR may have evolved a high-fidelity translational apparatus as an additional mechanism to maintain overall proteostasis.

Epigenetic code

The epigenetic code consists of chemical modifications to DNA such as methylation and histone PTMs (or “histone code”) that tightly regulate the cell’s transcriptional program, chromatin organization, and correlate with cellular identity and differentiated state [48]. Robust MS-based methods permit quantitation of most known combinations of acetylated or methylated histone PTM sites using methods that rely on a set of synthetic peptide standards [49]. It is also possible to develop approaches to quantitate other known histone PTMs such as acylations [25, 50] [51].

During aging, the epigenetic code appears to deteriorate along with a concomitant loss of nuclear organization, transcriptional program (including gene silencing), and cellular identity [52]. Similar alterations in epigenetic state occur in cancer where a cell loses its identity and regains pluripotency after being terminally differentiated [53, 54].

Reprogramming of terminally differentiated cells into induced pluripotent stem cells (iPSCs) is a similar event- i.e. maintenance of the differentiated state must be overcome. iPSCs can be induced to overcome their differentiated state by the exogenous expression of transcription regulators/pluripotency factors OCT4, SOX2, KLF4, and c-MYC (OSKM) [55]. Similarities between reprogrammed iPSCs and tumor cells, including alterations in the epigenetic code, suggest that studying cell-fate/commitment to differentiation capacity may also reveal insights into tumorigenesis [54]. In fact, one of the assays that indicates pluripotency of the iPSCs is the ability to form a teratoma (a tumor-like mass) when the cells are transplanted into immunodeficient mice. Details regarding the connections between cancer or reprogramming and epigenetics are actively investigated in mouse and human, while very little is known about these relationships in other long-lived species.

Three separate groups attempted to test their limits of self-identity by generating iPSCs from NMR fibroblasts [56, 57]. Although the efficiency of generating NMR iPSCs was weak and varied among the reports, one result was consistent- the NMR derived iPSCs show a significantly reduced capacity to form teratomas in mice. These data suggest that the NMR cells maintain their differentiated state or self-identity more strictly compared to the mouse where reprogramming and teratoma formation is much more efficient. Since a strict maintenance of differentiated state may relate to epigenetic stability, Tan et al. hypothesized that the basal level of histone PTMs may be distinct in the NMR compared to the mouse [57]. Consistent with this idea, they found that the NMR fibroblast histones displayed lower levels of H3K27ac and higher levels of H3K27me1, H3K27me3, and H3K27me1K36me1 peptides. This trend of H3K27 methylation indicates a commitment to differentiated state and resistance to reprogramming/pluripotency thought to arise from less active gene expression (reviewed in [58]). In further support of this claim, Tan et al. found that NMR cells displayed lower levels of “open” chromatin by ATAC-Seq compared to the mouse, indicating that the chromatin was generally in a more repressive state. After reprogramming with Large T antigen, the NMR chromatin appeared more “open” by ATAC-Seq and values agreed more closely with the mouse. A second interesting finding by Tan et al. was that NMR histones displayed significantly less H3K9 and H3K56 methylation marks compared to mouse. These marks are normally associated with constitutive heterochromatin which also appeared as more densely immunostained foci in mouse compared to NMR cells. Lastly, H2A.J appeared more acetylated in NMR compared to mouse. While the impact of H2A.J acetylation is unknown, it may be connected to aging since H2A.J accumulates during senescence and promotes the expression of inflammatory senescent-associated secretory phenotype (SASP) factors [59]. Given the observed differences in the NMR histone code, the NMR epigenetic state may confer pro-longevity benefits compared to other shorter-lived species (Figure 1). An important caveat to consider when deciphering the histone code using the MS approach employed in Tan et al. is that previously unidentified PTMs such as recently-identified acylations (reviewed in [22]) or PTMs that cannot be easily synthesized on a peptide in vitro such as ADP-ribosylations (reviewed in [60]) may remain unaccounted for and may require novel methods for detection/quantitation. Thus, it will be interesting to see if the other PTMs that have not yet been explored are also distinct in the NMR and if the histone code of other long-lived species shows similar trends.

Genome and epigenome stability

Genome instability increases with aging, at least in part, due to decreased capacity to repair DNA damage. DNA double strand break (DSB) repair appears to be particularly compromised as a function of replicative age in both yeast and mammals as they approach senescence in culture [28, 61, 62, 63]. Moreover, there is significant data to indicate that DSB repair decreases during aging in mice and humans as indicated by increased numbers of phosphorylated H2A.X (γ -H2Ax) foci that appear in aged tissues [64]. This PTM has been so well characterized that it is generally accepted as a benchmark for DNA DSB repair. Yet, several other histone PTMs appear to be critical for DSB process such as H3K36me2, H4K20me2, or ADP-ribosylation at H3S10 and potentially others [65].

One of the best connections between PTMs, aging, and genome stability may be found in SIRT6 activity. SIRT6 is the only one of seven mammalian sirtuins found to increase lifespan when overexpressed as a transgene in mice [66]. Reciprocally, SIRT6 knockout mice die at a very young age (approximately 30 days old) [67]. Combined, these data indicate that SIRT6 shows a very strong connection to longevity. Mao et al. found that expression of exogenous SIRT6 rescued a reduction in DSB repair that occurs as human cells approach senescence in culture [62]. SIRT6 promotes genome stability via multiple pathways. It mediates gene silencing via their histone deacetylase activity [68]. In the case of DNA repair, SIRT6 recruits DNA-dependent protein kinase to DSBs [69] and also, in addition to its histone deacetylase activity, SIRT6 ADP-ribosylates and activates poly-ADP-ribose polymerase (PARP1) to enhance DSB repair [70]. Later Van Meter et al. showed that JNK phosphorylates and activates SIRT6 in response to oxidative stress which helps SIRT6 to recruit PARP1 to DSBs [71]. Recently, Tian et al. carried the idea one step further. They found that overexpression of SIRT6 from 18 different rodent species confers enhancement of DSB repair that correlates with the species lifespan [72]. Connections between SIRT6, PARP1, DSB repair, and longevity also agree with earlier data showing that PARP1 activity in leukocytes correlates with lifespan across 13 mammalian species [73]. Moreover, the SIRT6 biochemical activities deacetylase, ADP-ribosylase, and de-acylase also showed positive correlation with longevity. Tian et al. also found five amino acids that can be swapped between SIRT6 proteins from the species that showed the most extreme differences in their ability to stimulate DNA repair (beaver being the best and mouse being the worst) to swap their activities, making mouse SIRT6 as active as beaver SIRT6 and vice versa. These data suggest that SIRT6 deacetylase and ADP-ribosylase activities may play a role in determining longevity in diverse species and that more efficient enzymatic function may evolve with longer lifespan.

Beyond its ability to enhance DSB repair, a major role of SIRT6 is to repress the expression of ancient retroviral elements (LINE1 or L1) that account for a significant fraction of mammalian genomes [74]. The importance of LINE1 elements in aging as well as in the context of SIRT6 as a promoter of overall genome integrity was bolstered by two recent reports showing that showed that L1 elements become transcriptionally derepressed during aging and that this derepression activates a type-I interferon (IFN-I) inflammatory response [75] [76]. The effect can be suppressed by reverse transcriptase (RT) inhibitors to block expansion/reintegration of L1s. Furthermore, loss of L1 suppression is a major contributor to aging in SIRT6 knockout mice which can be partially rescued by adding RT inhibitors [76]. Upon the loss of SIRT6, the copy cytoplasmic L1 species accumulate triggering type-I interferon response via cGAS/STING pathway.

From a proteomics perspective, it is notable that SIRT6 deacetylase/deacylase *and* ADP-ribosylase activities both appear to contribute to longevity-enhancing effects. SIRT6 histone deacetylase and ADP-ribosylase activities are very weak *in vitro* (the k_{cat} for H3K9ac deacetylase is approximately 10^{-4} s^{-1} , or less than one turnover per hour) [77]. Longer acyl-chains can stimulate the deacetylase activity (although no evidence for SIRT6 removal of long acylations at H3K9 *in vivo* has been reported) [78]. It is likely that conditions *in vivo* enhance the catalytic efficiency for both deacetylase/deacylase and ADP-ribosylation reactions. In fact, SIRT6 deacetylase may stimulate its ADP-ribosylation activity directly.

This may represent a very unique example of PTM crosstalk where the two different activities may indeed be coupled (and may be unique to sirtuins). Previously, Fahie et al. provided evidence that sirtuin-mediated ADP-ribosylation can proceed via two distinct transition states- with or without prior deacetylation^[79]. Reactions that proceeded from prior deacetylation contain an otherwise transient 1'-O-alkylamidate intermediate of ADP-ribose that can be attacked by a neighboring nucleophilic residue which may be derived from a closely-interacting partner protein (see Figure 8C of Fahie et al.). That is, the switch between deacetylation, ADP-ribosylation, or concerted activities may be dictated by the availability of a particular nucleophile in the appropriate context *in vivo*^[80]. Recently, Liszczak et al. showed that SIRT6 deacetylation of histone H3K9ac is a prerequisite for PARP1 ribosylation of H3S10 during DNA repair^[81]. Given the prior data from Mao et al, this may be an example of a concerted action of SIRT6 protein where deacetylation (of H3) may enhance ADP-ribosylation. That is, SIRT6 deacetylation of H3K9ac may enhance ribosylation of PARP1 which would subsequently increase H3S10 ribosylation by PARP1/HPF1. This would represent a very high level of crosstalk to allow for precise coordination of proteins during an *in vivo* response to DNA damage. In fact, the crosstalk between neighboring acetylated Lys may be more widespread beyond histone H3K9ac because other proteins that are ADP-ribosylated by PARP1/HPF1 on Ser residues appear to reside adjacent to acetylated Lys residues^[81, 82]. Recently, Rezazadeh et al. reported that SIRT6 also ADP-ribosylates BAF170 (SMRC2), a component of the SWI/SNF chromatin remodeling complex, in order to promote the expression of NRF-2 responsive genes in response to oxidative stress^[83]. This role of SIRT6 is particularly interesting because it *activates* (as opposed to represses) gene expression. This may be another case where SIRT6 activity depends on other factors or context *in vivo* that confers specificity. One might ask if it is possible for SIRT6 to act on so many diverse substrates. Perhaps SIRT6 may have evolved to be versatile (depending on its interaction partners) analogous to O-GlcNAc transferase (OGT) that catalyzes O-GlcNAcylation of hundreds of diverse substrates and displays crosstalk between O-GlcNAcylation and other PTMs such as phosphorylation to impact many diverse pathways^[84]. It would be interesting to identify other proteins that show altered enzymatic functions in long-lived species.

Longevity-specific PTM sites?

Given the strong connection between PTMs, metabolism, and protein functions, several groups found that disease causing mutations or single nucleotide polymorphisms (SNPs) in patients can coincide with PTM sites (see ActiveDriver and AWESOME databases^[85]). Similarly, SNPs at PTM sites of proteins in long-lived mammals may generate proteoforms that promote healthy aging. Alterations of histone PTMs displayed by the NMR may only be one example of a much wider phenomenon. As a proof-of-concept we checked the conservation of residues of key longevity-associated proteins that are known to have PTMs in humans within a small set of species that span longevity. For the long-lived species we focused on a set of diverse taxa whose genomes have been sequenced and, in some cases, where metabolism or DNA repair has been studied^[12, 27–29, 72, 86]. Although the list of short-lived species is not nearly as diverse, we reasoned that if divergence of PTM sites were not observed in these extreme cases, then the role of such PTMs may not be related to

longevity. In addition, some of the short-lived rodents in our analysis are more closely related to long-lived species than other short-lived species. For example, the short-lived guinea pig is more closely related to the naked mole rat than other short-lived species. Similarly, the rat, hamster, and gerbil are more closely related to the blind mole rat than even the naked mole rat. To check for conservation of PTM sites, we downloaded known human PTMs as annotated FASTA sequences from PhosphoSitePlus database (note that this set is not exhaustive- it shows acetylation, methylation, phosphorylation, GlcNAcylation, ubiquitination, and sumoylation, but lacks modifications such as ADP-ribosylation or other acylations) and performed alignments using KAlign (which preserves case sensitivity of the annotated sites) [87]. Some of the proteins we checked are highlighted in Figure 2 which is an adaptation from the KEGG Longevity Pathway [88]. Majority of residues that show PTMs in humans are highly conserved among all of the mammalian species. However, we found several cases where PTM residues lacked conservation among this set of mammals, some of which appeared stratified based upon longevity. Table 1 shows notable substitutions (or lack of) at PTM sites in long-lived species. In the cases where substitutions may be interesting, we also examined predicted connectivity or “crosstalk” between the altered sites and other PTMs on the same protein using the webserver PTMcode 2.0 (<https://ptmcode.embl.de/index.cgi>) [89].

A few of the substitutions may be expected to affect protein activity or stability. For example, CDKN1A (Cip1/p21) phosphorylation at S123 and FOXO3 phosphorylation of S289 are both expected to stabilize the proteins based upon data in human cells [90, 91]. These residues are highly conserved among long-lived species and not conserved in the short-lived species set.

Similarly, S1495 and S1449 of topoisomerase (TOP2A) are only present in the long-lived species. Although modification of this residue has not yet been studied, yeast TOP2 hypomorphs extend replicative lifespan [63] and several mutations or truncations in the C-terminal domain of human TOP2 behave as hypomorphs that confer resistance to cancer drugs such as doxorubicin [92]. The C-terminal domain is not part of the conserved core and is thought to modulate protein interactions as well as overall activity. Therefore, changes in the ability to phosphorylate the C-terminus of TOP2A in different species may affect TOP2A activity or interaction partners to promote longevity. In further support of their significance, analysis by PTMcode 2.0 predicts that each of the TOP2A residues in Table 1 participates in a high level of crosstalk with distal sites (T1324, S1449, and S1495 with 10, 31, and 18 interactions respectively).

Another potentially interesting PTM site is RICTOR T1695 which appears conserved in humans, the bowhead whale, and the African elephant. Given its ties to mTOR signaling, we checked the conservation of this residue in all mammals available in NCBI. We found that most mammals, including all marsupials, all primates, and most ungulates, have RICTOR T1695. Yet, rodents, lagomorphs, microbats, cats, and bears substituted this threonine with an alanine or a branched-chain residue. Most of the species with substitutions consist of small-bodied species. Unlike many of the modifications identified by our screen, RICTOR T1695p has a known *in vivo* function in humans. T1695 is a GSK3 phosphorylation site that, upon phosphorylation, causes FBXW7-mediated degradation of RICTOR [93]. Based on

results from our screen we find that many small-bodied mammals lack T1695, and consequently, may lack the capacity for FBXW7-mediated degradation. Perhaps animals without T1695 require more basal mTOR complex 2 activity for thermoregulation of their larger surface area per body mass which may also impact other aspects of metabolism that influence aging.

SIRT6 T294 phosphorylation site is particularly interesting given the role of SIRT6 in regulating lifespan. A more thorough phylogenetic analysis of T294 reveals that it is only present in a few long-lived groups such as old-world primates, cetaceans, beavers, tree squirrels, as well as David's myotis bat; however, the majority of mammals appear to lack this site. Consistent with its potentially important role, phosphorylation of T294 is the most highly observed modification of human SIRT6 (cataloged in the PhosphoSitePlus database) [94]; yet, several studies failed to reveal its role. T294 resides outside of the highly conserved sirtuin catalytic domain within a proline-rich region that bridges to a less conserved C-terminus that may facilitate protein-protein interactions [95]. Substitution of T294 with glutamic acid (T294E) which in many cases would mimic a constitutive phosphorylation did not affect SIRT6 nuclear (or nucleolar) localization [96]. Similarly, alanine substitution (T294A) did not reduce SIRT6's ability to stimulate DNA repair after oxidative stress [71]. The presence of this phosphorylation site predominantly in long-lived species, suggests that it confers an additional level of regulation that is missing in the short-lived species. It will be important to understand the biological function of this PTM as it may relate to metabolic or epigenetic functions of SIRT6.

These examples support the idea that long-lived species may possess altered PTMs and proteoforms that confer longevity by allowing the organism to bypass the hallmarks of aging (Figure 3). Since many key longevity proteins such as Cip1/p21, FOXO3, TOP2A, RICTOR and SIRT6 as well as all of the modifiers and readers of the histone code exert their effects via PTMs such as phosphorylation, ADP-ribosylation, acetylation, or methylation, studying the conservation and role of PTM sites will likely yield critical insights.

Future Directions

It is important to establish connections between proteoforms, metabolism, and signaling and identify how these signatures are altered in long-lived species. Proteomics approaches similar to those reviewed herein can be used to determine if different long-lived species such as whales, elephants, or bats employ similar strategies to maintain proteostasis or a stable histone/epigenome code.

Quantifying the relative levels of proteins (especially key aging-related ones) in long-lived versus short-lived organisms may provide valuable insight. Continued analyses of PTMs/proteoforms in long-lived species will require well-established and novel proteomics methods as well as the help of existing databases such as dbPTM [97].

Given the effects of SIRT6 and PARP1 on longevity, it will be important to study ADP-ribosylation in long-lived mammals. Less destructive MS fragmentation by electron transfer dissociation (ETD) will facilitate ADP-ribosylation studies and permit the identification of

other labile modifications (such as O-GlcNAcylation) because it allows them to remain intact [98]. Similarly, protein oxidation can also be measured using proteome-scale approaches [99]. The ultimate characterization of a proteoform would be to analyze the intact protein and all of its associated PTMs by top-down approaches. Given that a large amount of material in relatively pure form is required, analysis of proteins isolated from cells or tissues will likely be challenging. Yet, method optimization decision trees are available [100] and proteins such as histones can be purified in large quantities from cells or tissues. All of these approaches will reveal differences in proteoforms in ways that genomics cannot predict.

Beyond the initial characterization of the PTM/proteoform catalog of long-lived species, the next step will be to place the PTMs into a biological context. This will require connecting metabolome analyses to PTM chemistries as well as understanding the role of “writers”, “readers”, and “erasers” of all PTMs. Given that a catalog of writers, readers and erasers of histones PTMs is known (except for any novel PTMs that remain undiscovered) [101], and novel ones are continuously added, we can begin to look at their activities, regulation, and PTMs in other long-lived species. Expanding this concept to other proteins would be a long-term goal. One example of this is the novel role of sirtuins such as SIRT3, 4 and 5 in regulating mitochondrial protein acylations [21]. A second area that would permit an understanding of biological context is to reveal crosstalk between PTM sites. Crosstalk may manifest as cooperative, negative, or intermediate effects between two different PTMs at distal sites within a single protein or between two interacting proteins [102]. Essentially, crosstalk often provides a very high level of control over the desired effects/response. One example of PTM crosstalk relevant to longevity is histone bivalency where two histone PTMs that normally oppose each other (H3K4me3 and H3K27me3) act synergistically in combination to promote a unique intermediate state of chromatin that appears “poised” for gene activation (reviewed in [48]). Another example of crosstalk relevant for longevity is the previously discussed case where H3K9ac interferes with ADP-ribosylation of H3S10 by PARP1 [81]. In this case, SIRT6 deacetylates H3K9ac to allow PARP1 to ADP-ribosylate H3S10. Based upon our studies comparing the long-lived beaver and short-lived mouse SIRT6 proteins, we would predict that beaver H3S10 would show a more robust ADP-ribosylation by PARP1 through crosstalk with a more active SIRT6 protein. It will be interesting to compile knowledge of PTM levels from bottom-up and top-down studies to see if any additional correlations between PTM sites in proteins with ties to longevity exist.

Biological context will also be gained from a catalog of the comparative histone PTM code among short-lived versus long-lived species. These data will inform ChIP-seq experiments to determine the location/occupancy of histone PTM “readers”/transcription factors. In parallel, gene expression effects determined by RNA-Seq will be correlated with particular histone PTMs. Next, these results will be superimposed with data derived from other methods such as Atac-Seq or 3D chromatin mapping techniques and aligned with ENCODE data to determine relative chromatin organization changes over time (4D) in each species [103]. When combined, these data may begin to explain the epigenetic differences between short- and long-lived animals during aging.

Since PTMs are not directly genetically encoded, engineering these features *in vivo* was beyond reach until recently. Novel engineering strategies such as protein ligation/splicing,

non-natural amino acid mutagenesis, or inactive CRISPR/Cas9-fusion protein-based localization of epigenetic modifiers may permit the engineering of designer PTMs/proteoforms *in vivo* [104]. We expect that future proteomics studies of longevity will incorporate these approaches to correlate PTMs/proteoforms with lifespan.

The goal of aging research is to develop interventions to increase human lifespan, but more importantly, to simultaneously increase healthspan. Promising examples such as caloric restriction mimetics, senolytics, anti-inflammatory drugs, sirtuin activators, or molecules that increase NAD⁺ levels already provide the proof-of-concept [105]. Long-lived species have evolved long lifespan and remarkable healthspan, as in the wild animals must remain fit for survival. For example, bats have to maintain the ability to fly to catch insects and bowhead whales must swim to catch fish till their last day. Understanding the connections between metabolism and proteomics that long-lived species employ to alleviate the hallmarks of aging may permit the co-option of these traits in humans through pharmaceutical or dietary interventions.

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Figure 1. Long-lived species such as NMR show differences in their histone PTMs or “Histone Code” that likely confer a stable epigenome compared to short-lived species such as the mouse. A stable epigenome could block attempts to reprogram differentiated cells, genome instability, senescence, and other hallmarks of aging.

Figure 2. Key longevity-regulating pathway proteins whose functions may be altered or enhanced by differential PTMs/proteoforms in long-lived species.

Proteins shown in yellow were checked for alteration of known PTMs. These proteins influence longevity in response to dietary restriction (DR) or DR mimetic drugs such as rapamycin, resveratrol, or metformin. The figure was largely adapted from KEGG pathways-Longevity Regulating Pathway, hsa04211. SIRT6 and other sirtuin effects which should also respond to NAD⁺ levels were also added. For a full description (including complete protein names) of pathway hsa04211 in KEGG, see https://www.genome.jp/dbget-bin/www_bget?map04211. Solid and dashed arrows signify direct and indirect effects respectively. +P, deAc, and mADPr signify phosphorylation, deacetylation, and mono-ADP-ribosylation.

Figure 3. Diet, stress resistance mechanisms, and overall metabolism of long-lived species likely confer pro-longevity PTMs that generate unique proteoforms that alleviate or bypass the hallmarks of aging leading to increased lifespan.

In addition, it appears that short-lived species may obtain some pro-longevity PTMs and proteoforms through a calorie restricted diet, drugs such as rapamycin, or mutations that influence metabolism.

Table 1.
Examples of conservation and substitutions at PTM sites of key longevity pathway proteins.

Substitutions are indicated by darker boxes. Long-lived species are in red and short-lived species are in blue. Species are as follows: *Homo sapiens* (human), *Balenoptera mysticus* (bowhead whale), *Nannospalax galili* (blind mole rat), *Heterocephalus glaber* (naked mole rat), *Loxodonta Africana* (African elephant), *Castor canadensis* (Canadian beaver), *Myotis brandtii* (Brandt's bat), *Oryctolagus cuniculus* (European rabbit), *Cricetulus griseus* (Chinese hamster), *Cavia porcellus* (Guinea pig), *Mus musculus* (mouse), *Meriones unguiculatus* (Mongolian gerbil), *Rattus norvegicus* (rat), ND indicates not determined. Letters indicate the following references: a= [91]; b= [90], c= d= [106], e= [107], f= [108], g= [109], h= [93], i= [110].

						Long-Lived Species						Short-Lived Species					
Protein	Residue	Substitution	Modification	Effect of PTM	Human	Bowhead	BMR	NMR	Elephant	Beaver	Bat	Rabbit	Hamster	Guinea Pig	Mouse	Gerbil	Rat
AKT1S1	T73	T->A	Phos	ND													
	S123	S->A,P,G	Phos	stabilize (a)													
CDKN1A	S289	S->A	Phos	stabilize (b)													
	S85	S->A	Phos	ND													
HSP70	S312	S->G	Phos	ND													
	K451	K->R	Ub	regulate (c)													
INSR	S1354	S->N	Phos	ND													
	S666	S->N	Phos	ND													
LKB1	S334	T->G	Phos	regulate (d)													
	S657	S->N,K,A	Phos	disassemble (e)													
RPTOR	T699	T->A	Phos	ND													
	S795	S->A	Phos	regulate (f)													
	S886	S->C	Phos	ND													
	T908	T->A,FP	Phos	activate (g)													
RICTOR	T1695	T->V,I	Phos	degrade(h)													
	T352	T->P	O-GlcNAc	activate (i)													
RELA	S374	S->N	O-GlcNAc	ND													
	S377	S->P,V,L	O-GlcNAc	ND													
PI3Kc2a	S108	S->G,N	Phos	ND													
	S1551	S->P	Phos	ND													

Protein	Residue	Substitution	Modification	Effect of PTM	Long-Lived Species							Short-Lived Species					
					Human	Bowhead	BMR	NMR	Elephant	Beaver	Bat	Rabbit	Hamster	Guinea Pig	Mouse	Gerbil	Rat
SIRT6	T294	T->A	Phos	ND													
TSC1	S871	S->P,A	Phos	ND													
TOP2A	T1324	S->A	Phos	ND													
	S1449	S->A,P	Phos	ND													
	S1495	S->E,N,G	Phos	ND													
TOP2B	T1292	T->I,V,A	Phos	ND													