

FORUM REVIEW ARTICLE

Emerging Omics Approaches in Aging Research

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Abstract

Significance: Aging is a complex trait that is influenced by a combination of genetic and environmental factors. Although many cellular and physiological changes have been described to occur with aging, the precise molecular causes of aging remain unknown. Given the biological complexity and heterogeneity of the aging process, understanding the mechanisms that underlie aging requires integration of data about age-dependent changes that occur at the molecular, cellular, tissue, and organismal levels.

Recent Advances: The development of high-throughput technologies such as next-generation sequencing, proteomics, metabolomics, and automated imaging techniques provides researchers with new opportunities to understand the mechanisms of aging. Using these methods, millions of biological molecules can be simultaneously monitored during the aging process with high accuracy and specificity.

Critical Issues: Although the ability to produce big data has drastically increased over the years, integration and interpreting of high-throughput data to infer regulatory relationships between biological factors and identify causes of aging remain the major challenges. In this review, we describe recent advances and survey emerging omics approaches in aging research. We then discuss their limitations and emphasize the need for the further development of methods for the integration of different types of data.

Future Directions: Combining omics approaches and novel methods for single-cell analysis with systems biology tools would allow building interaction networks and investigate how these networks are perturbed with aging and disease states. Together, these studies are expected to provide a better understanding of the aging process and could provide insights into the pathophysiology of many age-associated human diseases. *Antioxid. Redox Signal.* 29, 985–1002.

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Introduction

AGING IS A COMPLEX BIOLOGICAL PROCESS that is characterized by a progressive decline in physiological functions at multiple levels, including changes at the molecular, organelle, cell, tissue, and, finally, the whole organism level (Fig. 1A) (116). Over the past few decades, we have also learned that almost every biological process in the cell can contribute to physiological decline and progressive damage accumulation that eventually result in aging and death (47). Genome instability, DNA mutations, errors in RNA transcription, and protein translation, among other factors, can all contribute to organelle and cell dysfunction with aging (116, 137, 175, 177), but different macromolecules also interact with each other.

A number of genes have been shown to change their expression with aging. Although transcriptional regulation plays an important role in the control of gene expression, multiple factors beyond transcription factors, including the epigenetic modifications and the rate of mRNA degradation, contribute to determining protein concentration in the cell (Fig. 1B) (131). Additional processes, such as alternative pre-mRNA splicing and post-translational modifications (PTMs), add further complexity to the organism's proteome. Moreover, protein degradation through the autophagy and ubiquitin-proteasome pathways affects protein turnover and degradation, whereas spatial location and PTMs may influence activity of proteins. Finally, changes in protein expression and their activity can influence the patterns of

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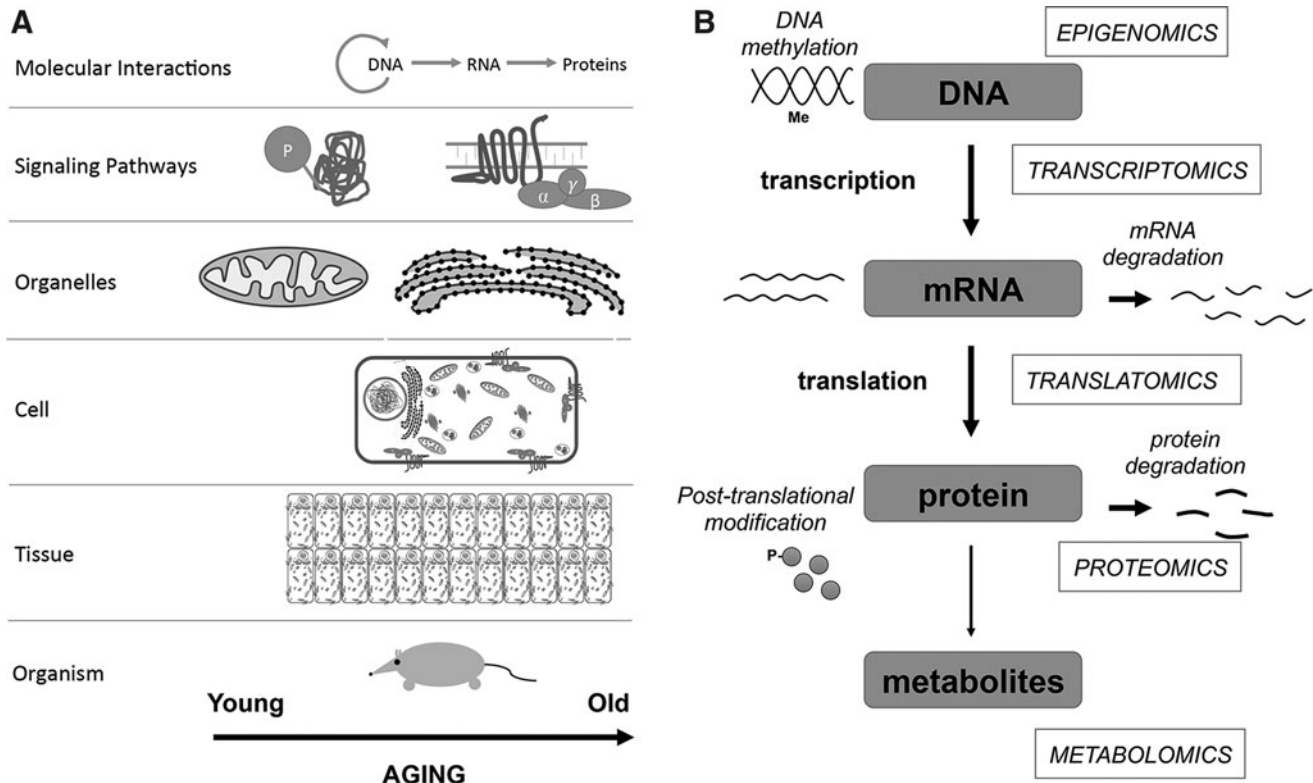


FIG. 1. Systems biology approaches in aging research. (A) Understanding the molecular mechanisms that underlie aging requires integration of multi-layered data about age-dependent changes that occur at the molecular, cellular, tissue, and organismal levels. (B) A scheme showing the Central Dogma of Molecular Biology and different “omics” approaches that are currently available for the high-throughput quantitative analysis of molecular changes associated with aging.

metabolites and change the metabolic state of the cell. Together, a gradual decline in all of the functions cited earlier may contribute to the aging process. Therefore, understanding of the mechanisms of aging is not possible without taking into account all components of the biological system, interactions between individual components, and the system’s hierarchy (18, 200).

Recent advances in the development of high-throughput sequencing and different “omics” technologies now allow quantitative analyses of biological molecules at multiple levels, and how they change with aging, with high specificity and sensitivity. Despite this progress, integration of high-throughput data remains a challenging problem. In this review, we describe recent advances and discuss emerging omics approaches in aging research. We then highlight current challenges in the field and point to the need for the further development of approaches for the integration of multiple omics data. Integration of multiple types of data would make it possible to investigate the cause-consequence relationships and understand the interactions between multiple aging factors, and understand how these factors can be manipulated to develop new therapeutic targets for age-associated diseases.

Genome-Wide Approaches in Aging Research

Transcriptomics

Aging is controlled by a combination of genetic, environmental, and stochastic factors. A number of diverse genes

have been shown to modulate aging. Studies in invertebrate organisms have led to the identification of hundreds of genes, whose deletion or knockdown can dramatically increase lifespan (27, 57, 129, 158). For example, a recent genome-wide analysis of yeast replicative lifespan in a non-essential gene deletion set revealed that more than 5% of the yeast gene deletions can significantly extend lifespan (129). Many of these genes were grouped into several functional categories (Fig. 2) that represent conserved biological processes, suggesting that they might be involved in the modulation of mammalian aging (40, 55). It has been also reported that the aging process can be delayed by environmental manipulations, including dietary restriction (DR) and various stress stimuli (*e.g.*, hypoxia) (107, 123). Given that there are multiple ways by which the lifespan of an organism can be extended, it is critical to understand how the various longevity-associated genetic and environmental manipulations are related to each other, and how the different aging genes and pathways are coordinately regulated.

Transcriptional regulation. Expression of many genes is altered with aging, and these changes may have causative effect on aging and the development of age-associated pathologies. A number of research groups used microarrays and RNA sequencing (RNA-Seq) to analyze age-dependent changes in gene expression in different model organisms (108, 113, 149, 189) as well as in humans (117, 152, 185). In addition, multiple transcriptome analyses have been performed to identify genome-

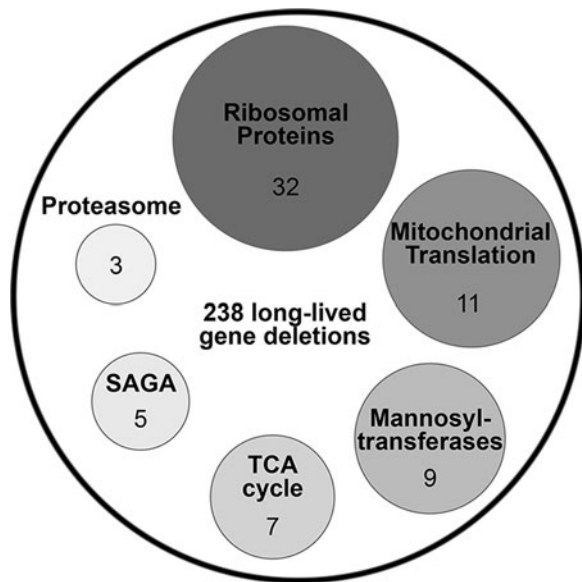


FIG. 2. Functional groups enriched among 238 long-lived gene deletions that were identified by the genome-wide analysis of replicative lifespan in 4698 yeast deletions strains. Number of genes is indicated for each functional category (129). SAGA, components of the Spt-Ada-Gcn5-acetyltransferase complex.

wide transcriptional changes associated with increased longevity (21, 30, 130, 136, 186). These studies revealed profound changes in the transcriptome with age, which reflect age-associated degenerative processes, but might also result from transcriptional response or adaptation to aging (33). Moreover, aging is accompanied by increased cell-to-cell and increased inter-individual variation in gene expression, which was attributed to stochastic variation or transcriptional noise (4).

To identify changes in mRNA transcript levels with aging, several bioinformatics tools and statistical methods have been developed for multiple comparison analysis and functional enrichment analysis of RNA-Seq data [reviewed in Hou *et al.* (72)]. In addition, gene expression clustering and dimension reduction approaches, for example, principal component analysis, are commonly used to identify genes with similar expression patterns, which could help identify potential regulators. However, it is hard to establish which of the differentially expressed genes are the regulatory factors and which are the consequences of regulatory changes based only on expression patterns.

To identify upstream regulatory factors, one of the commonly used methods is to search for enrichment of binding sites in the promoter regions of genes with a similar transcription pattern (37, 155). This approach relies on information about the promoter sequence binding sites identified by chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) analysis. A similar approach, named eDM_BN (for extended Deletion Mutant Bayesian Network inference algorithm), has been recently applied to identify clusters of genes and their upstream regulatory transcription factors and signaling pathways that mediate the effects of DR on lifespan in *Caenorhabditis elegans* (71). As opposed to other methods, this algorithm allows integration of transcriptome data with information about known regulatory re-

lationships between transcription factors and their targets from the modENCODE database (24) as well as the incorporation of real valued fold-change data for gene expression changes. However, care should be taken when interpreting results of these studies. Transcription factors often recognize overlapping motifs and may have overlapping sets of targets, which could present difficulty for establishing causative links.

In addition to changes in gene expression, aging is characterized by systemic decline in fidelity of transcriptional machinery, leading to transcription errors and loss of protein homeostasis (169, 175). Transcription is one of the fundamental biological processes in living organisms. Due to the inherent imperfectness of biological systems, numerous transcription errors are inevitably made during mRNA synthesis and processing. Although organisms evolved dedicated systems for degradation of aberrant RNA, increasing evidence suggests that some stable RNA intermediates can accumulate in cells, including fragments derived from intronic lariat RNA, tRNA, rRNA, as well as small noncoding RNA (43, 89, 198). Aberrant transcripts could also arise from genetic mutations, defects in transcription, premature polyadenylation, or errors in pre-mRNA splicing (174, 190).

Errors in pre-mRNA splicing. In a recent study, the link between RNA splicing machinery and longevity was established by using the *C. elegans* model system (62). Using transcriptomics and analysis of splicing in young and old worms *in vivo* with fluorescent alternative splicing reporters, researchers observed aberrant patterns of pre-mRNA splicing in old animals. Old worms were characterized by significantly increased levels of exon skipping, intron retention, and unannotated splice junctions compared with young animals, suggesting a dysregulation of splicing with aging (Fig. 3). Interestingly, DR and inhibition of mechanistic target of rapamycin (mTOR) signaling pathway, which are known to extend lifespan, shifted signatures of splicing to a more youthful state.

These findings also correlate with recent reports showing that the number of alternatively spliced genes is significantly increased with aging in mice and humans (104, 151), and suggest that manipulating RNA splicing could be used as a target for potential aging interventions in humans. Further analysis of the RNA splicing machinery in worms revealed that increased lifespan by DR and modulation of mTOR signaling is dependent on the functional SFA-1 splicing factor (62). The exact mechanisms linking DR- and mTOR-mediated longevity with SFA-1 remain unknown. Reversible protein phosphorylation has been shown to modulate the assembly and activity of the spliceosome (161). It is an attractive possibility that dysregulation of mTOR kinase activity or mTOR pathway downstream targets with aging may directly affect phosphorylation of spliceosome components and lead to altered patterns of pre-mRNA splicing. Additional genomics and proteomics studies are needed to characterize changes in spliceosome composition with aging. Integration of the results of these studies with the global analysis of PTMs (*i.e.*, phosphorylation) would shed light on how mTOR signaling modulates pre-mRNA splicing machinery.

Translatomics

Despite the importance of transcriptional regulation in the control of gene expression during aging, many of the genes

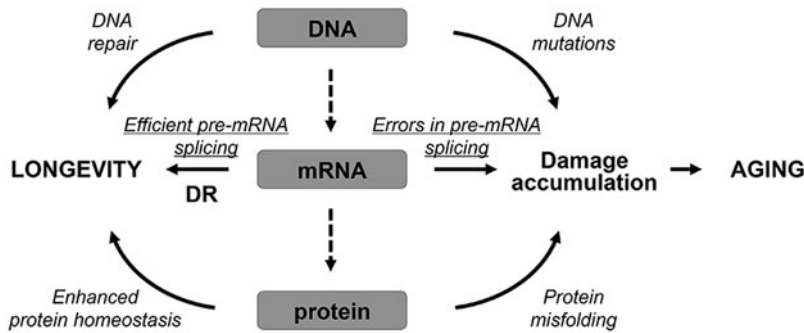


FIG. 3. A model for the role of pre-mRNA splicing in aging. Errors in pre-mRNA splicing lead to damage accumulation and aging. DR promotes pre-mRNA splicing efficiency, leading to lifespan extension. DR, dietary restriction.

are also regulated at the level of translation. However, the mechanistic details by which translational control affects the aging process remain poorly understood. Recent technological advances in proteomics and next-generation sequencing now enabled researchers to quantitatively measure the rates of protein translation at the genome-wide level that could facilitate investigation of translational control of gene expression during aging.

The rates of protein translation can be quantitatively measured by pulse labeling of proteins using Stable Isotope Labeling by Amino acids in Cell culture (SILAC) (139). Pulsed SILAC allows labeling of newly synthesized proteins with “heavy” ^{13}C - or ^{15}N -labeled amino acids, which can then be distinguished from pre-existing proteins containing “light” amino acids by using mass spectrometry (MS). Among the limitations of SILAC is sensitivity of detection, which is common for MS-based methods. Insufficient “heavy” isotope incorporation into proteins expressed at low levels may produce mass spectrometric signals below the detection limit. To overcome this limitation, an alternative method has been developed and is named puromycin-associated nascent chain proteomics (PUNCH-P) (2). This method involves separation of translating ribosomes by ultracentrifugation followed by enrichment of newly synthesized proteins using biotinylated translation inhibitor.

Recently, a novel approach based on next-generation sequencing, named ribosome profiling or Ribo-Seq, has emerged as a powerful technology to study mRNA translation and mechanisms of translational regulation at the genome-wide level. This technique uses deep sequencing to quantitatively analyze genome-wide ribosome occupancy and allows monitoring the rate of protein translation *in vivo* at codon resolution (78). Because ribosome occupancy is determined by overall mRNA abundance and translation efficiency, Ribo-Seq combined with RNA-Seq measurements can be used to identify genes that are specifically changed at the level of translation. Despite its advantages, Ribo-Seq has several technical limitations (77). For example, the choice of ribonuclease (44) and the use of translation inhibitors (76) in ribosome profiling experiments have been shown to affect the data interpretation. Furthermore, standardized bioinformatics protocols for analysis of the Ribo-Seq data are lacking. Although multiple reports have been published on utilizing Ribo-Seq in various model systems, which investigated translational regulation in different physiological and disease states, application of this technique to studying aging has been limited.

To elucidate the role of translational regulation in aging, our group has recently used ribosome profiling to investigate

genome-wide translational changes in several long-lived yeast mutants, previously identified in yeast replicative lifespan screens (10). By integrating transcriptional (RNA-Seq) and translational (Ribo-Seq) gene expression data, we identified common and unique patterns of protein synthesis associated with increased longevity, and we uncovered regulatory factors from gene and protein expression signatures. Our study revealed that the long-lived single gene deletions coordinately activate several common transcription factors and mRNA-binding proteins (RBPs), which specifically enhance the translation of proteins involved in cellular response to stress (Fig. 4) (10). At the same time, the long-lived mutants also contained adaptations that were unique for each of the strains, suggesting that there are multiple ways by which the lifespan of an organism can be extended. Consistent with the putative role of RBPs in translational regulation, we identified multiple targets of RBPs that were activated in the long-lived mutants at the level of translation.

Hundreds of putative RBPs that may play a role in translational regulation and can influence stability, transport, and

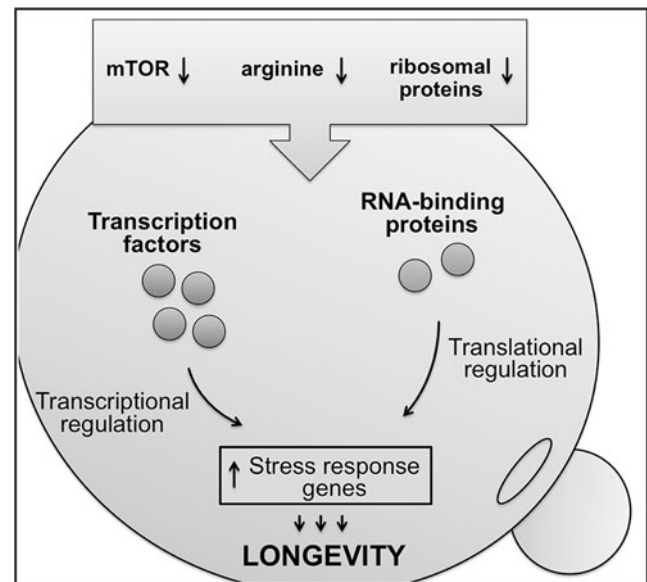


FIG. 4. A model for the role of translational regulation in yeast aging. Lifespan-extending manipulations, such as inhibition of mTOR signaling, impaired amino acid import, and ribosome deficiency, extend yeast lifespan by activating several transcription factors and RNA-binding proteins that coordinately regulate the expression of stress-response genes. mTOR, mechanistic target of rapamycin.

sub-cellular localization of specific mRNAs have been identified (5, 17). However, the functions of most of the RBPs in translational regulation during aging remain unknown. To characterize the role of these RBPs in translational control, there is a need to identify their downstream targets and mRNA-specific *cis*-regulatory sequences. Several methods have been developed to identify targets of RBPs that are based on immunoprecipitation, such as CLIP-Seq (cross-linking immunoprecipitation sequencing) (172) and RIP-Seq (RNA immunoprecipitation sequencing) (199). Integrating genome-wide translational profiling data obtained by using Ribo-Seq with information about RBP targets will allow researchers to identify genes that are translationally regulated during aging and characterize the role of RBPs in post-transcriptional gene regulation in aging.

In addition to RBPs, non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have been shown to play important roles in the post-transcriptional regulation of various biological processes (7, 8). Recent advances in omics techniques allowed identification of multiple regulatory non-coding RNAs and facilitated studies of their role in aging. In particular, miRNAs have been recently shown to modulate lifespan in *C. elegans* (13, 32) and *Drosophila* model organisms (114). Moreover, genome-wide profiling of non-coding RNAs revealed that many miRNAs and lncRNAs are up- or downregulated during mammalian aging (109, 122, 159) and may regulate cellular senescence (91, 97). However, the specific functions of many of the non-coding RNAs remain unknown. Similar to RBPs, miRNAs and lncRNAs may target multiple genes and have a potential to coordinate multiple pathways implicated in aging and longevity (79).

Proteomics

Although the rate of mRNA synthesis determines the bulk of the specific protein's concentration in the cell, and the regulation at the level of transcription for the most part governs protein expression, regulation at the level of protein translation and turnover appears to fine-tune protein concentration to the cellular needs (36). Current techniques for high-throughput analysis of protein concentration include liquid chromatography coupled with mass spectrometry (LC-MS/MS), antibody-based immunohistochemistry, protein arrays, and fluorescence-based imaging using fluorescent reporters (*e.g.*, GFP-tagged proteins). However, one of the common limitations for the proteomics measurements is sensitivity of detection. Currently, only a small fraction of the proteome can be quantitatively measured in a single experiment. Also, compared with transcriptomics that can detect different transcript isoforms produced as a result of alternative splicing, proteomics analysis is restricted to a limited number of protein variants.

Proteomics has been particularly useful for studying pathways regulating protein homeostasis and their role in aging. Loss of protein homeostasis, or proteostasis, has been linked to aging and multiple age-related diseases, but the impact of aging on different proteostasis mechanisms remains understudied. In a recent study, proteomic profiling of more than 5000 proteins at multiple time points during the lifespan of *C. elegans* revealed that the proteome of an animal undergoes extensive remodeling during aging (178). Although the concentration of many of the proteins declined

with aging, a large portion of the proteins increased their abundance, leading to protein imbalance and aggregation. Besides the loss of protein stoichiometry, increased protein aggregation can be induced by protein modifications and other covalent damage to proteins (*e.g.*, glycation, oxidation, nitration) accumulating with increasing age that may influence protein conformation and stability (92). Future proteomics studies in mammalian organisms are required to test whether similar changes in proteome are contributing to the loss of protein homeostasis during mammalian aging.

This study also revealed that proteostasis systems form a complex network containing multiple pathways that function in a coordinated manner. Although levels of ribosomal proteins declined with aging, the abundance of components of the proteasome system and small heat-shock proteins increased (178). These findings are in line with several reports demonstrating the crosstalk between proteasome and autophagy (87). For example, loss of proteostasis due to inhibition of the proteasome is compensated by an upregulation of macroautophagy (96). In addition, the inhibition of chaperone-mediated autophagy or macroautophagy leads to activation of a different form of autophagy (88, 127). This complexity allows cells to preserve protein homeostasis even if one of the systems fails by activating alternative mechanisms. The crosstalk between the protein degradation pathways should be taken into account when interpreting results from proteomic experiments. In future studies, it would be interesting to see how protein homeostasis network is changed with aging, and whether aging has a negative effect on the crosstalk between different protein degradation pathways.

Tissue-specific aging. One of the limitations of studies in invertebrate organisms, such as yeast and worms, is the extent to which these findings are relevant to the biology of aging in higher eukaryotes, including humans. Previous studies have shown that aging affects tissues and organs of the same organism in different ways, and different tissues age at different rates (63). For example, comparative transcriptome and proteome analyses between young and old rats revealed major organ-specific differences in ways that different organs (*i.e.*, brain and liver) age (142). Although changes in gene expression and protein translation signatures were subtle with aging (usually less than 5% of the genes were changed at the transcriptional and translational levels with aging within the same tissue), the changes in the transcriptome and proteome between different organs were more pronounced (142, 179).

This fact highlights the need to account for different physiological functions and proliferation rates between tissues when analyzing high-throughput transcriptomics and proteomics data. It is expected that tissues with low proliferation rates, such as brain, are more susceptible to damage accumulation compared with highly proliferative tissues that can regenerate, for example, liver. Consistent with a different capacity for cellular regeneration, brain and liver are characterized by different contribution of transcriptional and translational regulation to proteomic changes with aging (142). Interestingly, the majority of age-dependent changes in the brain proteome could be explained by changes at the level of translation, whereas changes in age-dependent proteome signatures in the liver were mostly regulated by changes in mRNA abundance (142).

Together, these findings imply that biological age of an organ and the way different organs age depend on its unique physiological function and specific cellular properties. Moreover, one tissue or organ can affect aging of other tissues and even the whole organism. It is important to emphasize the need to survey multiple organs and cell types and to integrate different omics data. Integration of multiple omics approaches such as transcriptomics, proteomics, metabolomics, as well as global analysis of pre-mRNA splicing, and methylation could reveal an in-depth picture of how different components deteriorate with aging, contribution of specific organs to aging, and a better understanding of aging at the level of the whole organism.

Post-translational modifications. Among other factors, the activity of the protein is determined by its expression, rate of protein synthesis and protein turnover, as well as protein localization within cells and regulatory PTMs. In turn, PTMs can affect the biochemical properties of the protein by changing its structure, enzyme active site environment, and protein-protein interactions. The most common PTMs of proteins include addition of small chemical groups (such as phosphorylation, acetylation, and oxidation), attachment of large sugar molecule oligosaccharides (*e.g.*, glycosylation) or lipid chains (*e.g.*, palmitoylation), and covalent attachment of ubiquitin protein moieties (ubiquitination), among others. However, it is important to distinguish between true functional PTMs and protein damage, for example, protein oxidation or dysfunctional adduct formation by sugars (glycation), methylglyoxal, nitric oxide *etc.*, that accumulates with aging (160). For example, oxidative modifications that are commonly considered as protein damage can be also used by cells in redox-regulated signaling pathways (28).

Similar to other reversible protein modifications, such as phosphorylation, oxidation of cysteine residues has been shown to play regulatory roles, and dysregulation of these processes has been linked to aging (99). Although detection of such modifications at the genome-wide scale is difficult, recently several large-scale proteomic approaches were developed to identify functional cysteines that can be oxidized *in vivo*, including OxiTMT (157), OxiCAT (106), and isoTOP-ABPP (182). More recently, targeted oxidation/reduction of methionine residues in proteins has been also implicated in signaling and redox regulation of diverse cellular functions (35, 124). Although the role of methionine sulfoxide-dependent signaling in aging awaits further investigation, future studies will benefit from newly developed proteomic methods (143) and fluorescent probes (168) for detection of methionine oxidation products.

One of the examples that illustrates multi-layered regulation of protein activity includes the 26S proteasome. Impaired 26S proteasome system has been implicated in the aging process (116). Consistent with the importance of the 26S proteasome in protein homeostasis and its multi-subunit composition, the activity of the 26S proteasome is regulated at multiple levels. In addition to transcriptional control, PTMs have been shown to regulate its activity (135, 150, 171). Previous large-scale studies of the phosphoproteome and other PTMs in yeast (93) and high eukaryotes (49) revealed that multiple proteasome subunits are subject to phosphorylation. In addition, proteasome activity might be modulated by other PTMs, such as acetylation, ubiquitination, addition of O-linked N-acetyl-glucosamine, S-glutathionylation, and oxidation (16,

154, 196). Notably, aging is associated with the decline in proteasome function that occurs at multiple levels, including reduced expression of genes encoding proteasome subunits (105), reduced proteasome activity due to oxidative damage (16, 181), and disassembly of the 26S proteasome complex (181).

Most of the prior studies that investigated age-dependent changes in PTMs have focused on one of the specific modifications that may regulate protein function (*e.g.*, phosphorylation or protein oxidative damage), but comprehensive and quantitative characterization of these complex factors at the genome-wide level and integration with transcriptional/protein translation data in the context of aging lagged behind. By combining genomics and proteomics approaches Ori *et al.* (142) have recently simultaneously analyzed changes in gene transcription, levels of protein translation, and protein phosphorylation status in the same tissue samples isolated from young and old rats. Although the majority of identified proteins that changed their protein abundance between young and old animals, specifically in the brain and liver, were primarily changed due to changes in the rates of protein synthesis, a fraction of the proteins also demonstrated changes in protein phosphorylation status. Moreover, several kinases have been found to be differentially expressed between young and old animals, which could potentially explain these differences in phosphorylation (142). At the same time, researchers identified a decreased level of phosphorylation in several proteins. Together, these findings highlight the complexity of the changes that occur with aging that could be missed by focusing on one aspect of age-associated gene expression studies.

Epigenomics

Recent studies revealed an important role of the chromatin-based epigenomic changes during aging (14). These epigenomic changes include DNA methylation and multiple histone modifications that can influence transcription of genes through inhibiting binding of transcription factors, heterochromatin formation, and affecting genome stability. The environmental and experimental perturbations of the epigenome and chromatin remodelers have been shown to directly affect lifespan in a variety of organisms. Further, epigenetic modifications have been recently proposed to be useful biomarkers of aging.

Methylation of cytosine bases in CpG dinucleotides (5-methylcytosine; 5mC) is a conserved epigenetic modification that is commonly associated with repression of transcription. Global hypomethylation of CpG sites with aging has been observed in humans in multiple tissues (56, 66). However, some CpG sites, which are preferentially located near promoters of transcription factors, tissue-specific genes, and genes involved in differentiation and development, become hypermethylated with the advanced age (11, 31, 121). Currently, the consequences of DNA methylation changes during aging on cellular function are not completely understood. One of the possibilities is that these methylation changes could influence binding of the transcription factors to the promoter regions and lead to altered expression of genes with aging (3, 195). Recently, several novel forms of DNA methylation have been identified in metazoa, including hydroxymethylation of cytosines (5-hydroxymethyl cytosine; 5hmC) (34, 145) and N⁶-methylation of adenines (6-methyl adenine; 6mA) (41, 51, 197). Interestingly, N⁶-methylation

of adenines has been implicated in regulation of transcription and activity of transposable elements as well as in transgenerational epigenetic inheritance (118).

Notably, genome-wide analyses of 5mC DNA methylation profiles have been used to predict chronological and biological age in human tissues. Recently, several methylation clocks have been developed that could predict the chronological age based on methylation changes at distinct sets of CpG sites in the human genome (56, 66, 183). These methylation sites are consistently changed with aging across different tissues and cell types (66) and can also predict age in a tissue-specific manner (56). Notably, epigenetic changes can be used to estimate not only chronological age but also biological age. The human DNA methylation clock is predictive of functional decline with aging (19, 22, 67, 70), increased mortality (125), and time to death (19). Moreover, accelerated epigenetic aging was associated with HIV infection (69) and was observed in patients with Down syndrome (68).

Beyond accelerated aging, an important question is whether epigenetic clock can be slowed and whether the organism's lifespan can be extended by reversing epigenetic modifications. Although answering these questions in humans is challenging, recently an epigenetic clock has been developed based on mouse blood DNA methylation profiles (147). Using reduced representation bisulfite sequencing of mouse blood samples, a set of CpG methylation sites has been identified in the mouse genome that could reliably predict mouse biological age. These CpG sites are scattered across the genome and are distinct from CpG sites observed in the human epigenetic clock. Importantly, the mouse DNA methylation clocks can robustly predict modulation of lifespan by different dietary and genetic interventions, for example, DR, rapamycin and gene knockouts known to extend lifespan (147, 180) as well as high fat diet and ovariectomy leading to shortened lifespan (166).

Because mice have a much shorter lifespan and are commonly used to study mammalian aging, the epigenetic clock in mice is an attractive biomarker of aging that could provide a valuable tool for researchers to test the effect of novel interventions and drugs to delay aging. However, precise assessment of biological age and comparison of data between different studies could be challenging. Among the limitations of early reports is a high prediction error due to the low number of samples available and the limited range of ages analyzed. Moreover, the lack of fully standardized procedures to quantitatively measure DNA methylation status and different genetic backgrounds could influence the estimation of the exact age. Recent technological advances in whole-genome bisulfate sequencing (WGBS) could further improve the resolution and predictive power of the DNA methylation clocks by surveying an increased number of CpG sites (100). In future studies, it would be also important to investigate to what extent different epigenetic clocks are able to predict biological age in different mouse tissues. Similar to human DNA methylation clocks (56, 66), the composition of the mouse epigenetic clock may change in a tissue-specific manner as a result of different developmental programs. These studies could help to explain differences between rates of aging among organs and may provide better understanding of molecular mechanisms that underlie aging in different organs and tissues (see the discussion on the Tissue-Specific Aging section).

In addition to changes in DNA methylation, other epigenomic changes such as histone modifications have been associated with aging. Histone methylation is dynamically regulated by histone methyltransferases and demethylases and can affect patterns of gene expression by regulating chromatin structure (54). Experimental perturbation of histone methylation has been found to modulate longevity in model organisms (52, 81, 128). Similarly, histone acetylation, which is regulated by histone acetyltransferases and histone deacetylases, has been shown to directly affect lifespan (29). More recently, studies in *C. elegans* revealed that histone methylation might have a long-lasting effect on lifespan that can be transferred through several generations (50, 53). Together, these studies suggest that multiple epigenomic changes are associated with the aging process, and that modulation of epigenetic modifiers can be used as a therapeutic strategy to slow and possibly reverse aging.

Metabolomics

Gradual accumulation of molecular damage, which is produced by numerous metabolic processes, has been proposed to be the principal driver of aging (48, 94, 193). Such cumulative damage is not limited to oxidative damage and may include byproducts of enzymatic reactions, alterations of gene expression, errors in RNA and protein synthesis, and accumulation of DNA mutations, among others (46). Moreover, the diversity of these damage forms and byproducts of various metabolic processes is expected to increase with advanced age (47). Therefore, analysis of metabolic signatures associated with the old age could provide a better understanding of the mechanisms of aging.

Two commonly used methods to quantitatively measure low-molecular-weight molecules, or metabolites, include the LC-MS/MS and nuclear magnetic resonance (NMR) spectroscopy. Currently, Human Metabolome Database (HMDB) contains more than 52,000 entries (187), including both water-soluble and lipid-soluble small molecule metabolites. However, out of this large number, only ~1500 can be identified by non-targeted metabolite profiling, and less than 500 by targeted MS-based metabolomics. Although fewer metabolites can be analyzed by using targeted metabolomics, this approach provides highly sensitive absolute quantification of metabolites with predefined structures. Despite lower sensitivity, the advantages of NMR spectroscopy include high reproducibility and ability to quantitatively measure metabolites over a wide range of concentrations. NMR can be also used to determine the structure of unknown compounds, and it offers advantages for compounds that are difficult to ionize or require derivatization for MS.

Recently, both targeted and non-targeted metabolome-wide profiling was used to characterize changes in levels of small-molecule metabolites associated with aging in several organisms, including nematodes (42), fruit flies (1, 64), mice (73, 170), and humans (83, 102, 194). These studies revealed significant changes in metabolic profiles with age across different species and organs. Most metabolites demonstrate organ-specific patterns, whereas some of the small-molecule metabolites correlate with aging across different organs. In addition to organ-specific changes, significant differences in metabolite levels were observed between men and women in humans (83). However, a set of small-molecule metabolites

consistently correlates with age independently of gender, suggesting that metabolite signatures could be used for the development of reliable biomarkers to predict biological age. Importantly, by using non-targeted metabolomics, researchers found that the diversity of metabolites (total number of distinct metabolites) was increased with aging (1). The appearance of new small-molecule metabolites with aging implies that some of them might represent byproducts of metabolism or other damage forms that accumulate in old organisms. Further advances in sensitivity of metabolomics techniques and structural identification of non-targeted hits that accumulate with aging may provide insights into causes of aging.

In addition to characterizing metabolic changes associated with aging, metabolomics has been applied to investigate patterns of small-molecule metabolites associated with increased longevity in model organisms (90, 146) as well as in humans (23, 84). Analysis of metabolic profiles in a group of centenarians demonstrated distinctive signatures in amino acid and lipid levels (23, 84). In particular, extreme longevity in centenarians was associated with significantly decreased levels of tryptophan, high sphingomyelin levels, a higher ratio of monounsaturated to polyunsaturated lipids, and decreased levels of lipid peroxidation markers in plasma. Decreased levels of polyunsaturated lipids may reflect enhanced resistance to peroxidation damage in centenarians that is consistent with the high sensitivity of polyunsaturated fatty acids to oxidative stress (23, 84).

Interestingly, lifespan-extending manipulations, such as DR, may reverse age-dependent metabolic signatures to a more “youthful” metabolic state (1, 64). Moreover, different metabolic processes are affected by DR in different tissues, suggesting that metabolic reprogramming by DR occurs in a tissue-specific manner (103). However, the exact mechanism by which DR induces the shift in metabolite concentrations is still not completely understood. More recently, targeted high-throughput analysis of metabolites in *Drosophila* revealed that among the most significantly changed metabolites with age are metabolites involved in methionine metabolism (144). Interestingly, deficiency of two S-adenosyl-homocysteine (SAH) hydrolase-like proteins dAhcyl1 and dAhcyl2, which are involved in regulation of methionine metabolism, leads to significant lifespan extension and is associated with suppressed H3K4 trimethylation (H3K4me3) resembling methionine restriction (144).

These findings highlight the importance to identify metabolites and/or factors that play a causative role in metabolic reprogramming with aging. Similar to metabolites involved in the methionine metabolism, other small molecules, for example, nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), acetyl coenzyme A (acetyl-CoA), and α -ketoglutarate, serve as cofactors or regulators of multiple enzymes that could affect aging through remodeling of chromatin, transcriptional regulation of gene expression, and other processes. Another level of complexity is based on the observations that increased levels of metabolites in one tissue can affect aging in a non-cell-autonomous manner and extend the lifespan of the whole organism (38).

However, many unresolved questions regarding the link between metabolome and aging remain. Because metabolome is influenced by both genetic and environmental factors at multiple levels, integration of transcriptomic, proteomic, and metabolomic data can provide important insights into causative

relationships between metabolome and longevity phenotypes. Further, it is not known how the patterns of metabolites change in species with diverse maximum lifespans. A detailed comparative analysis of metabolomes in different species could reveal the mechanisms by which the nature reprograms metabolomes to achieve a different range of lifespans (119, 120).

Automated Systems for Yeast *Saccharomyces cerevisiae* and worm *C. elegans* Lifespan Studies

Another important direction of research is how aging differs among individual organisms within the same species. For example, even in the population of genetically identical organisms, there is a huge variation in lifespans, which is determined by the stochastic nature of the aging process. To understand the mechanisms for phenotypic variation in aging and longevity, there is a need to quantitatively analyze age-associated phenotypes and molecular changes at the single-cell level. Yeast *S. cerevisiae* and worm *C. elegans* historically have been widely used for genome-wide aging studies. Recently, several microfluidic systems have been developed for these models that allow automation and performing aging experiments in a high-throughput manner. Here, we discuss advantages of microfluidic platforms for *S. cerevisiae* and *C. elegans*, current challenges and potential application of microfluidics for aging research.

Although manual microdissection of old yeast mother cells on agar plates is still the gold standard for analysis of replicative lifespan in yeast (162), it is a very labor- and time-intensive technique, which limits its application in high-throughput studies. Recently, several groups have developed microfluidic devices that allow monitoring of yeast cells with the single-cell resolution [reviewed in Chen *et al.* (20)]. Because microfluidic chambers allow trapping of individual yeast cells, when combined with time-lapse microscopy, these microfluidic systems can be used to observe multiple physiological parameters and monitor how they change with aging. For example, by using fluorescently labeled protein reporters, gene expression, protein expression, subcellular protein localization, organelle structure, and internal pH can be monitored throughout the aging process. Similarly, fluorescent dyes can be added to the media flow to track other physiological changes such as redox state and mitochondrial membrane potential (192). It is important to note that physiological changes measured by using microfluidic systems are obtained at the single-cell resolution, allowing to assess the penetrance of these changes within the overall population of mother cells. Moreover, they could be directly correlated with the life expectancy and the mother cell lifespan, which is monitored by scoring the periodic appearance and disappearance of daughter cells that are being constantly washed away by the fluid media (20).

To measure yeast replicative lifespan in real time, several designs of microfluidic systems have been used to trap mother cells. One of the major requirements to these devices is that they should trap and retain mother cells in the field of view through their lifespan, allowing automatic separation of the daughter cells from their mothers by media flow. Different designs have their strengths and weaknesses. Although none of the currently published designs are yet optimal, the devices containing a small set of polydimethylsiloxane columns/pillars (usually two or three) that form the trap (Fig. 5) are particularly attractive due to the ease of fabrication and the low manufacturing costs (26, 82, 115,

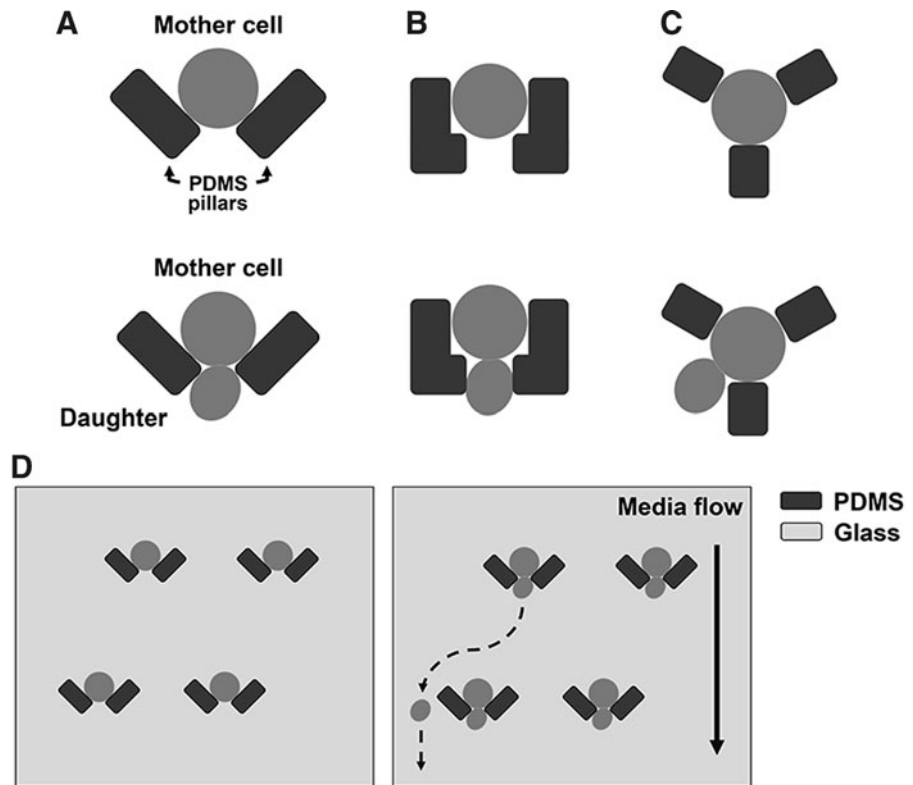


FIG. 5. Design of microfluidics systems for automated yeast replicative lifespan analysis. (A–C) At the beginning of the experiment, mother cells are trapped by using a two-column trap in the form of V shape (A) (26), two L-shaped PDMS pillars (B) (82), or a three-column jail (C) (115, 153). (D) Smaller daughter cells are constantly removed by media flow. Yeast lifespan is monitored by counting the number of daughter cells produced by each mother cell before it undergoes senescence.

153). Among the limitation of currently available microfluidic devices is the lack of data on how different designs and experimental conditions affect the robustness and reproducibility of lifespan measurements. In addition, care should be taken when interpreting the results of such microfluidic lifespan measurements in cases when genetic and environmental interventions under study can directly affect the yeast cell size (20). In future studies, it will be important to establish which microfluidic device design, and under what experimental conditions, most closely recapitulates the results obtained by using traditional replicative lifespan measurements.

As discussed earlier, the role of transcriptional and translational regulation in aging is not completely understood. Although several key regulatory factors, including transcription factors and regulatory RBPs, that coordinate transcriptional and translational responses have been identified, how their activity is modulated and how transcriptional and translational signatures are changed with aging remain unknown. Using fluorescence microscopy combined with automated microfluidic systems would allow researchers to monitor changes in gene expression and protein translation in yeast cells over their entire life. For example, quantitative single-cell tracking of the TEF2 translation elongation factor revealed a surprising increase in its expression in old mother cells near the end of their lifespan compared with young cells, suggesting a dysregulation of translational control with aging (192). In addition, microfluidic devices have been used to validate molecular markers, for example, Hsp104, and to assess their ability to predict lifespan in individual cells (192). Future studies using microfluidics systems will broaden our understanding of the aging process by quantitative analysis of the genetic and phenotypic factors that drive or accompany the aging process with the single-cell resolution.

Similar to yeast, the lifespan analysis in worms is a labor- and time-intensive procedure. In addition, worm longitudinal

lifespan studies suffer from low resolution and reproducibility, and can be influenced by human bias and multiple experimental variables. To overcome these limitations, several microfluidic devices have been developed to automate lifespan analysis in worms (75, 148, 191). However, currently such systems suffer from limited throughput and are not completely automated. Nevertheless, microfluidics systems are capable of monitoring physiological changes throughout the entire animal's lifespan, including body size, movement rates, age pigment accumulation, as well as fluorescent protein reporters. These microfluidic devices also allow for exposing the worms to different external stimuli by adding reagents directly to the media flow.

In addition to measuring lifespan, microfluidic systems could be applied to study reproductive aging. Recently, a new microfluidic platform combined with time-lapse imaging has been developed that allows quantitative real-time analysis of progeny from multiple animals and provides high-resolution data on reproductive age (110).

In addition to microfluidics systems, which analyze worm aging in liquid media, an alternative approach called the "Lifespan Machine" utilizes high-throughput imaging and tracking of movement in worms placed on plates with solid media (165). The Lifespan Machine allows monitoring a large number of individual worms by combining imaging using flatbed scanners with a novel image-analysis pipeline. This system was successfully used to compare the effects of different interventions, such as high temperature, oxidative stress, DR, and different genetic manipulations, to assess lifespan dynamics and high-precision mortality statistics in the large populations of worms (164).

Combining large-scale omics technologies with systems for automated lifespan analysis in *S. cerevisiae* and *C. elegans* could provide a better understanding of the mechanisms

that underlie aging and has the potential to transform the aging research field.

Data Integration

Although the development of high-throughput techniques led to production of a huge amount of data, integration of the omics data has been one of the biggest challenges in the field. Importantly, to perform direct correlation between parameters using multi-omics techniques, these analyses have to be performed and datasets have to be collected for the same sets of samples in a controlled laboratory environment. Due to variability of conditions, comparison and integration of omics data collected by different labs remains a challenge. Therefore, care should be taken when interpreting and integrating the data, taking into account experimental variables. Also, due to high technical variation, high-throughput approaches require proper data normalization and data quality control. Despite these challenges, the unbiased genome-wide analysis and monitoring how complex biological networks change with age are critical for understanding the mechanisms that underlie aging.

Specifically, the aging field will benefit from applying systems biology approaches to efficiently integrate the dynamic transcriptional, translational, and metabolic changes with aging and to reliably infer regulatory factors from multi-layered data (Fig. 6). Moreover, understanding of the aging process is not possible without taking into account molecular interactions (or “interactome”) occurring among different components of the biological system (proteins, lipids, nucleic acids, *etc.*) as well as how they affect physiological parameters associated with aging (*i.e.*, “physiome”). In fact, aging is indicated as one of the goals of the “Human Physiome project”, which is focused on precisely defining interactions that occur in the human body as a whole by using quantitative computer modeling (173).

Perspectives

Although significant progress has been made in studying changes associated with aging over the past decades, the

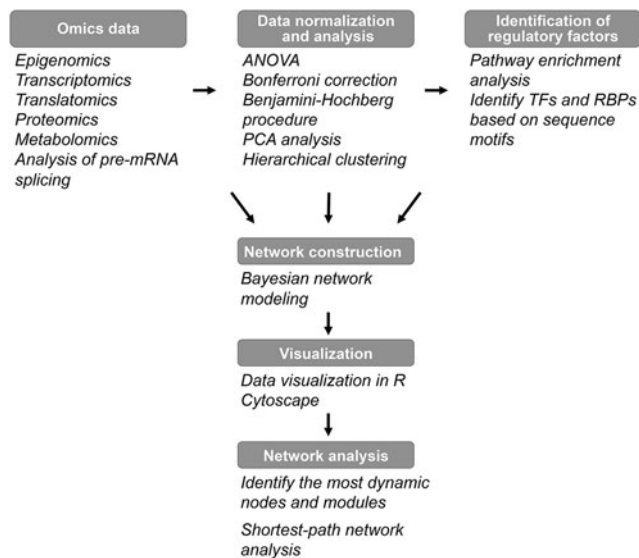


FIG. 6. Workflow for data integration. RBP, mRNA-binding protein.

mechanisms that underlie the aging process are not completely understood and many fundamental questions remain (Box 1). Answering these questions will allow researchers to build regulatory aging networks and help identify new targets for the development of new lifespan interventions to delay the aging process.

Recent advances in high-throughput “omics” methods have enabled detailed studies of physiological changes that occur with aging across different species as well as in different tissues and organs. The increasing number of available datasets that describe dynamic changes in transcription, translation, levels of metabolites, and PTMs with aging have prompted researchers to integrate these datasets. As the field progresses, additional datasets obtained from different organisms and multiple tissues, together with increased sensitivity of “omics” methods, will allow a more detailed investigation (*e.g.*, multiple time points, single-cell sequencing, and quantitative analysis of physiological changes *in vivo* using microfluidic platforms), and will be instrumental to identify causative relationships.

In addition to age-dependent changes, several studies analyzed changes in gene expression, protein translation, DNA methylation state, PTMs, and other factors in response to dietary interventions, genetic manipulations, and environmental conditions known to extend lifespan, which allowed to identify a number of regulatory factors and proteins (Table 1). In future studies, it would be important to examine the common and unique mechanisms of lifespan extension, and to investigate how different longevity-associated environmental and genetic manipulations are related to each other. These studies will enable the identification of risk factors and allow making predictions, which individuals will benefit the most from specific interventions based on their gene expression signatures or genetic background. For example, several bioinformatics and computational methods have been developed for identifying and predicting biologically active small molecules based on gene expression profiles (80, 101). However, these methods have not been extensively tested to predict drugs or small molecules with anti-aging properties and would be of interest in future studies (6).

Another important area of investigation is the mechanisms that regulate species lifespan. Mammals are characterized by more than 100-fold difference in lifespan. For example, the bowhead whale, the longest-lived mammal, is reported to live >200 years. Using comparative “omics” approaches to identify differences in genome sequences, gene expression profiles, and metabolic signatures among short- and long-lived organisms would allow researchers to identify key regulators of lifespan.

Scientists have been trying to understand aging for centuries, but what is aging is still not completely understood. Although many distinct, often contradicting each other, theories of aging have been proposed, at present no single unifying theory of aging exists that could explain all discordant observations (47, 94, 116, 176). Many of these theories assume that only one primary cause of aging exists or plays a major role, for example, oxidative damage (free radical theory of aging) (58), errors in protein synthesis (Orgel’s error catastrophe theory) (141), somatic mutations (Szilard’s model of somatic mutation accumulation) (167), or epigenomic changes (14). We argue that all biological processes contribute to aging, and none of these senescence factors individually is more important than others, rather the biological systems’ inherent susceptibility to errors lies in the center of the aging process (45). The advances

Box 1. Major Unsolved Questions

Which molecular changes associated with the aging process are causes of aging and which are consequences?
 Given that there are multiple ways by which the lifespan of an organism can be extended, how are the various longevity-associated genetic and environmental manipulations related to each other? How are different aging genes and pathways interconnected and coordinately regulated?
 How do aging regulatory networks dynamically change with age?
 Is aging programmed to lead to death at a species-specific age or is it a random process?
 Considering significant diversity (more than 100-fold difference) in lifespan among mammalian species, what are the genes and pathways that determine the differences among organisms' lifespan?
 What are the common gene expression and metabolite signatures associated with increased longevity?
 What are the mechanisms that underlie differences in lifespan among organisms within the same species?
 Can human aging be slowed or reversed therapeutically? Is there a therapeutic window in which age therapeutic interventions in humans will be the most beneficial?
 What is the role of epigenomic modifications and epigenome-modifying enzymes in the transgenerational effects on lifespan?

in next-generation sequencing, proteomics, and metabolomics may finally allow analyses of how different theories of aging relate to each other. Indeed, there have been considerable efforts in recent years to reconcile different theories and develop new theories based on the latest advances in “omics” research (45, 132).

For example, Orgel’s error catastrophe hypothesis, which implies a positive feedback between errors in proteins and the introduction of new errors in protein synthesis, has recently been re-examined and extended to somatic mutations to create

a new catastrophe theory of aging (132). In this theory, a primary role is played by somatic mutations with a contribution from other mechanisms. Because mutations in genes encoding enzymes involved in DNA repair are expected to introduce more somatic mutations, they would lead to an exponential increase in DNA damage, forming a feedback loop. This model is consistent with Szilard’s theory, but it also attempts to incorporate interactions between cellular processes (DNA repair, mRNA transcription, protein translation) and the system’s hierarchy. As opposed to errors in protein

TABLE 1. MAJOR INTERVENTIONS AND TARGETS OF GENETIC MANIPULATIONS THAT INCREASE LIFESPAN

<i>Intervention/signaling pathway</i>	<i>Molecular function/regulatory factors</i>	<i>Species</i>	<i>References</i>
17- α -estradiol	Estrogen agonist	<i>Mus musculus</i>	59
L-deprenyl	Monoamine oxidase inhibitor	<i>Rattus norvegicus</i>	95
Acarbose	Inhibitor of intestinal α -glucosidase	<i>M. musculus</i>	59
Aspirin	Anti-inflammatory agent	<i>M. musculus</i>	163
Ibuprofen	Anti-inflammatory agent	<i>Saccharomyces cerevisiae</i> <i>Caenorhabditis elegans</i> <i>Drosophila melanogaster</i>	61
Metformin	Antidiabetic drug, mTOR inhibitor, AMPK signaling pathway activator	<i>M. musculus</i> <i>C. elegans</i>	126, 140
Methylene blue	Mitochondrial complex IV activator	<i>M. musculus</i>	59
Nordihydroguaiaretic acid	Lipoxygenase inhibitor, anti-inflammatory agent	<i>M. musculus</i>	59, 163
Rapamycin	mTOR inhibitor	<i>M. musculus</i> <i>D. melanogaster</i>	12, 60, 133, 134
Resveratrol	SIRT1 activator	<i>M. musculus</i> <i>C. elegans</i> <i>D. melanogaster</i> <i>S. cerevisiae</i>	9, 74, 188
Dietary restriction	mTOR, AMPK, IGF-1	<i>M. musculus</i> <i>C. elegans</i> <i>D. melanogaster</i> <i>S. cerevisiae</i>	71, 112, 149, 184
GH signaling	GH, GHR	<i>M. musculus</i>	25, 39
Insulin/IGF-1 signaling	IGF-1, IGF-1R, PI3K, AKT, FOXO	<i>M. musculus</i> <i>C. elegans</i> <i>D. melanogaster</i>	15, 65, 138
mTOR signaling	mTOR, S6K	<i>M. musculus</i> <i>C. elegans</i> <i>D. melanogaster</i> <i>S. cerevisiae</i>	85, 86, 111, 156

GH, growth hormone; mTOR, mechanistic target of rapamycin.

synthesis, DNA mutations may not only lead to changes in protein sequences but also affect gene expression if they occur in regulatory regions or genes encoding transcription factors. Further experimental investigation is still needed to test this hypothesis, and it would require integration of genomic, transcriptomic, and proteomic data. Combining different omics approaches with systems biology tools and mathematical modeling has the potential to elucidate causality among multiple aging mechanisms and may also resolve the controversies between competing aging theories (*e.g.*, is aging a programmed or stochastic process?) (98).

Understanding a complex biological trait such as aging requires analysis of cause-consequence relationships between multiple, often influencing each other, factors in the biological system rather than studying individual components. Therefore, applying systems biology tools to integrate the data from different “omics” approaches obtained at multiple levels and at multiple time points throughout the organism’s lifespan is necessary to gain a global understanding of the aging process.

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Abbreviations Used

acetyl-CoA = acetyl coenzyme A
 DR = dietary restriction
 FAD = flavin adenine dinucleotide
 GH = growth hormone
 HMDB = Human Metabolome Database
 lncRNA = long non-coding RNA
 miRNA = microRNA
 MS = mass spectrometry
 mTOR = mechanistic target of rapamycin
 NAD = nicotinamide adenine dinucleotide
 PTM = post-translational modification
 RBP = mRNA-binding protein
 RNA-Seq = RNA sequencing
 SAGA = Spt-Ada-Gcn5-acetyltransferase
 SAH = S-adenosyl-homocysteine