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Review

Hallmarks of aging: An expanding universe

Carlos López-Otín,[1,2,3,*] Maria A. Blasco,[4] Linda Partridge,[5,6] Manuel Serrano,[7,8,9] and Guido Kroemer[10,11,12,*]
[1]Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología (IUOPA), Universidad de Oviedo, Oviedo, Spain
[2]Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), Oviedo, Spain
[3]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain
[4]Telomeres and Telomerase Group, Molecular Oncology Program, Spanish National Cancer Centre (CNIO), Madrid, Spain
[5]Department of Genetics, Evolution and Environment, Institute of Healthy Ageing, University College London, London, UK
[6]Max Planck Institute for Biology of Ageing, Cologne, Germany
[7]Institute for Research in Biomedicine (IRB Barcelona), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain
[8]Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain
[9]Altos Labs, Cambridge, UK
[10]Centre de Recherche des Cordeliers, Equipe labellisée par la Ligue contre le cancer, Université de Paris, Sorbonne Université, INSERM U1138, Institut Universitaire de France, Paris, France
[11]Metabolomics and Cell Biology Platforms, Gustave Roussy, Villejuif, France
[12]Institut du Cancer Paris CARPEM, Department of Biology, Hôpital Européen Georges Pompidou, AP-HP, Paris, France
*Correspondence: clo@uniovi.es (C.L.-O.), kroemer@orange.fr (G.K.)
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SUMMARY

Aging is driven by hallmarks fulfilling the following three premises: (1) their age-associated manifestation, (2) the acceleration of aging by experimentally accentuating them, and (3) the opportunity to decelerate, stop, or reverse aging by therapeutic interventions on them. We propose the following twelve hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. These hallmarks are interconnected among each other, as well as to the recently proposed hallmarks of health, which include organizational features of spatial compartmentalization, maintenance of homeostasis, and adequate responses to stress.

INTRODUCTION

Aging research explores the decline in function of organisms during adulthood. Since 2013, when the first edition of the hallmarks of aging was published in *Cell*,[1] close to 300,000 articles dealing with this subject have been published, which is as many as during the preceding century. Hence, time has become ripe for a new edition of the hallmarks of aging incorporating the main knowledge obtained a decade on.

The distinction among "hallmarks" is intrinsically diffuse, since they interact and are not independent of each other. Therefore, their classification is inevitably arbitrary, but we proposed three criteria that must apply for each hallmark of aging: (1) the time-dependent manifestation of alterations accompanying the aging process, (2) the possibility to accelerate aging by experimentally accentuating the hallmark, and—most decisively—(3) the opportunity to decelerate, halt, or reverse aging by therapeutic interventions on the hallmark. Rather than elaborating a compendium of age-associated alterations, we shall focus on the molecular, cellular, and systemic processes mechanistically accounting for their manifestation. That said, both in laboratory animals and in human medicine, objective quantification of morphological and functional decline affecting the aging organism is essential to measure biological aging. Indeed, disparity between biological and chronological age can reflect the efficacy of age-accelerating or -decelerating manipulations that evaluate the contribution of a given hallmark to the aging process. For this reason, standardized physiological measurements (e.g., respirometry to measure basal and maximal energy expenditure), functional tests (e.g., at the sensory, psychomotor, and cognitive levels), and ever more sophisticated "omics" technologies (e.g., genomics, epigenomics, transcriptomics, proteomics, and metabolomics), often applied at the single-cell level, are instrumental for evaluating the spatiotemporal patterns of health degradation and the (in)efficacy of anti-aging strategies.

In 2013, we suggested nine molecular, cellular, and systemic hallmarks of aging: DNA instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication.[1] Recent research has confirmed and extended the importance of all these hallmarks. They have withstood scrutiny by tens of thousands of aging researchers, but they require an update to deal with the discoveries of the last decade. For example, in 2013, much of the evidence on anti-aging interventions was limited to non-mammalian model organisms including yeast, nematodes, and fruit flies. Fortunately, experiments involving mice (and in some cases, non-human primates) have now

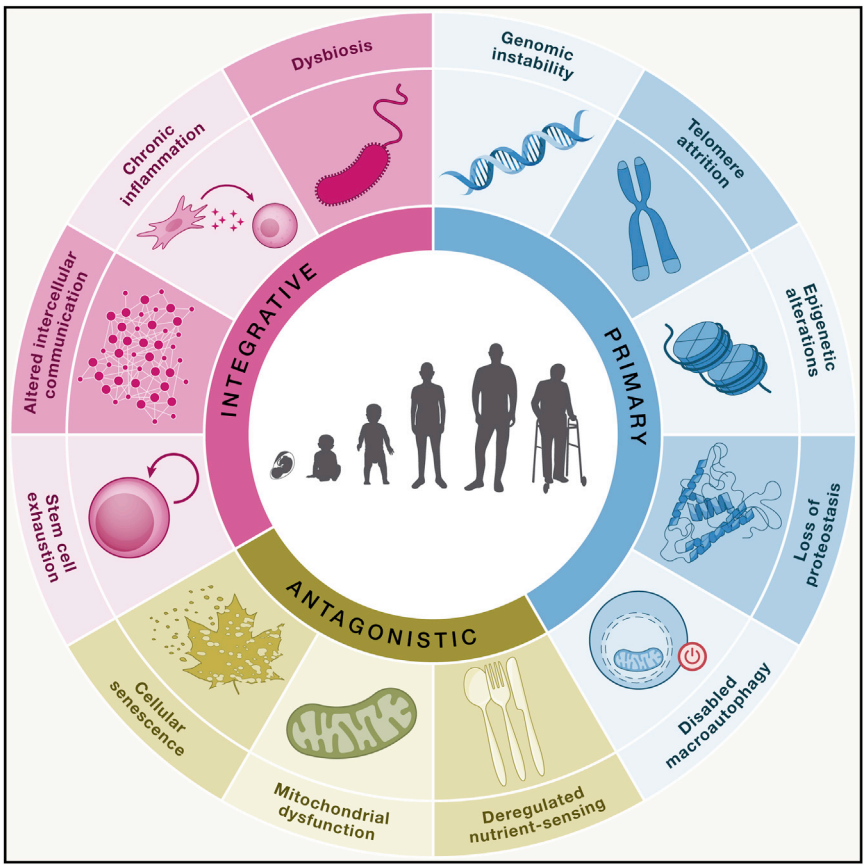

Figure 1. The hallmarks of aging

The scheme compiles the 12 hallmarks of aging proposed in this work: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. These hallmarks are grouped into three categories: primary, antagonistic, and integrative.

corroborated the validity of most of these hallmarks in mammals. Of note, human age-related diseases have statistically higher chances to co-occur and to share genomic characteristics when they are causally linked to the same hallmark rather than to different hallmarks,[2] clinically validating the approach that we have chosen.

Besides the necessary update of the previous hallmarks, we have also introduced some reorganizations and included the following three additional hallmarks of aging: disabled macroautophagy, chronic inflammation, and dysbiosis. Disabled macroautophagy was initially treated as a special case of loss of proteostasis. However, macroautophagy does not only affect proteins but can target entire organelles and non-proteinaceous macromolecules, justifying its discussion as a separate entity. Moreover, we considered that the final hallmark that we listed in 2013, altered intercellular communication, was too vast, requiring a separate discussion of chronic inflammation and age-associated dysbiosis (Figure 1).

The interdependence of aging hallmarks means that the experimental accentuation or attenuation of one specific hallmark usually affects other hallmarks as well. This underscores the fact that aging is a complex process that has to be conceived as a whole. Accordingly, each of the hallmarks should be considered as a point-of-entry for future exploration of the aging process, as well as for the development of new anti-aging medicines.

GENOMIC INSTABILITY

Genome integrity and stability are pervasively threatened by exogenous chemical, physical, and biological agents, as well as by endogenous challenges such as DNA replication errors, chromosome segregation defects, oxidative processes, and spontaneous hydrolytic reactions. The wide range of genetic lesions caused by these extrinsic or intrinsic sources of damage include point mutations, deletions, translocations, telomere shortening, single- and double-strand breaks, chromosomal rearrangements, defects in nuclear architecture, and gene disruption caused by the integration of viruses or transposons. All these molecular alterations and the resulting genomic mosaicism may contribute to both normal and pathological aging.[3] Accordingly, organisms have evolved a complex array of DNA repair and maintenance mechanisms to deal with the damage inflicted to nuclear and mitochondrial DNA (mtDNA) and to ensure the appropriate chromosomal architecture and stability. These DNA repair networks lose efficiency with age, which accentuates the accumulation of genomic damage and the ectopic accumulation of DNA in the cytosol[4] (Figure 2A).

Nuclear DNA

Cells from aged humans and model organisms accumulate somatic mutations at nuclear DNA.[5] Other forms of damage, such as chromosomal aneuploidy and copy-number variations, are also associated with aging. All these DNA alterations may affect essential genes and transcriptional pathways, resulting in dysfunctional cells that may finally compromise tissue and organismal homeostasis. This is especially relevant when DNA damage impacts on stem cells, hampering their role in tissue renewal or leading to their exhaustion, which in turn promotes aging and increases susceptibility to age-related pathologies.[6,7] The mutational burden in histologically normal human tissues is remarkable. For example, normal esophageal epithelium cells from young individuals already display hundreds of mutations and may carry more than 2,000 mutations per cell by middle age.[8] The accumulation of DNA mutations throughout life is likely tolerated because of the excessive energetic cost of the

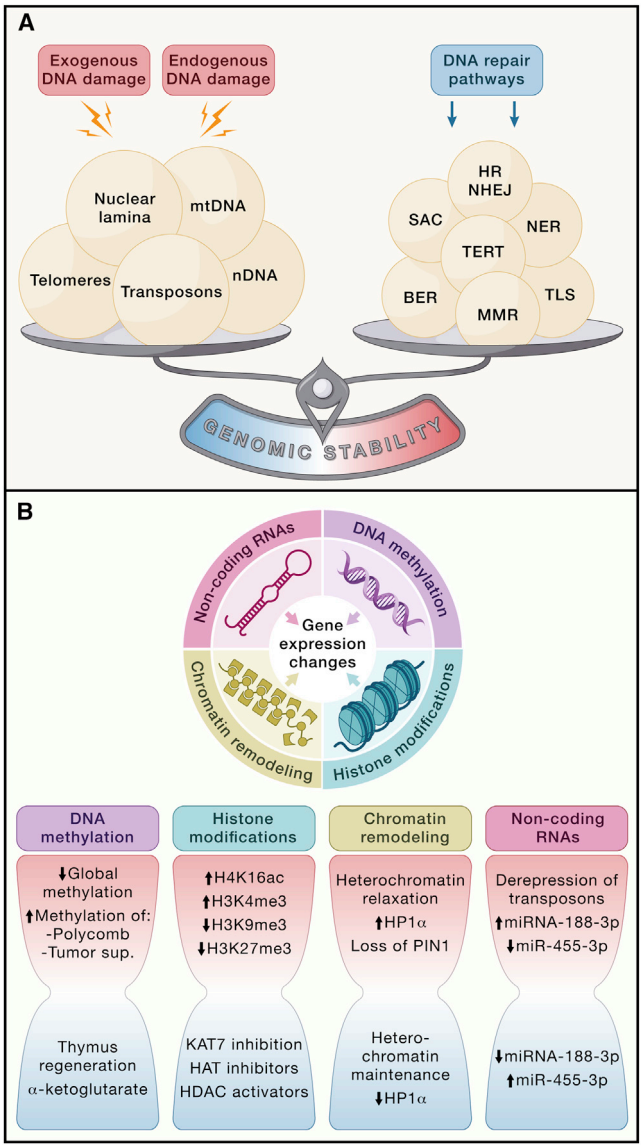

Figure 2. Loss of cellular integrity caused by genomic instability, telomere attrition, and epigenetic alterations

(A) Endogenous or exogenous agents cause a variety of DNA lesions that contribute to both normal and pathological aging. Such lesions can by repaired by a variety of mechanisms that lose efficiency with age. Excessive DNA damage, insufficient DNA repair, alterations in nuclear architecture, and telomere attrition favor the aging process. BER, base excision repair; HR, homologous recombination; NER, nucleotide excision repair; NHEJ, non-homologous end joining; MMR, mismatch repair; SAC, spindle assembly checkpoint; TERT, telomerase reverse transcriptase; TLS, *trans*-lesion synthesis.

(B) Changes in the acetylation and methylation of DNA or histones, as well as in levels or activity of chromatin-associated proteins or of non-coding RNAs (ncRNAs) induce epigenetic changes that contribute to the aging process. The red portions of the hourglasses indicate age-associated alterations and the blue portions strategies for avoiding them.

complete repair of all genomic damages caused by exogenous and endogenous challenges. Consequently, cells favor survival over genomic integrity.[9] These data also suggest that similar to carcinogenesis, driver mutations alone may not be sufficient to accelerate aging because they require a permissive microenvironment created by non-mutagenic promoting factors to become penetrant.[10]

Comparative analysis of the mutational landscape across mammalian species has shown that species-specific somatic mutation rate is inversely correlated with lifespan.[11] To date, there is no clear evidence that the normal rate of mutation fixation is responsible for aging, but numerous studies have shown that DNA repair deficiencies have the potential to cause aging. Thus, alterations in DNA repair mechanisms accelerate aging in mice and underlie several human progeroid syndromes.[12] Conversely, transgenic mice overexpressing the mitotic checkpoint kinase BubR1 exhibit an extended healthy lifespan[13] (Table 1). Moreover, studies in humans and other long-lived species have shown that enhanced DNA repair mechanisms coevolve with increased longevity.[14] Sirtuin-6 (SIRT6) may play a major role in this differential reparative efficiency across species. Overexpression of SIRT6 in mice reduces genomic instability, improves double-strand break repair, and extends lifespan[15] (Table 1), although other explanations, such as improved glucose metabolism and restoration of energy homeostasis, have been proposed to explain the prolongevity effects of SIRT6.[16] Notably, recent work has shown that small-molecule activation of 8-oxoguanine DNA glycosylase 1 increases oxidative DNA damage repair and may have therapeutic applications in the context of aging and other processes linked to excessive oxidative damage.[17] These findings suggest that interventions aimed at reducing the mutational load of nuclear DNA or at enhancing or rerouting its repair mechanisms may delay aging and the onset of age-related diseases, but further causal evidence in this regard is still missing.

Mitochondrial DNA

Genomic instability affecting mtDNA may contribute to aging and age-related pathologies.[96] mtDNA is strongly impacted by aging-associated mutations and deletions due to its high replicative index, the limited efficiency of its repair mechanisms, its oxidative microenvironment, and the lack of protective histones embracing this small DNA molecule. Somatic mtDNA alterations increase across human tissues during aging, but it remains unclear whether this increase truly impacts the aging process at the functional level. The causal implication of mtDNA mutations in driving aging has been difficult to assess because of "heteroplasmy," which implies the co-existence of mutated and wild-type genomes within the same cell. However, deep-sequencing of aged cells revealed that their mtDNA mutational load may substantially increase through clonal expansion events.[97] The accelerated expansion of mitochondrial mutations with age has also been observed in both primate oocytes and somatic tissues,[98] as well as in lymphoblasts from patients with neurodegenerative diseases.[99] Of note, ultra-sensitive sequencing indicates that most mtDNA mutations in aged cells arise from replication errors caused by mtDNA polymerase γ rather than from oxidative stress.[96]

Preliminary evidence that mtDNA mutations might be directly involved in aging and age-related pathologies was provided by human disorders that are caused by mtDNA damage and partially phenocopy aging.[100] Further causative evidence has arisen from studies on mice deficient in DNA polymerase γ that exhibit

Table 1. Examples of anti-aging effects of hallmark-targeted interventions in mammals

Hallmark	Species/model	Intervention	Outcome	Ref
Genomic instability	mouse	BubR1 overexpression	increased longevity	North et al.[13]
	mouse	SIRT6 overexpression	increased longevity	Tian et al.[15]
	HGPS mouse HGPS human	farnesyl transferase inhibitors	healthspan and lifespan extension	Gordon et al.[18]
Telomere attrition	mouse	telomere lengthening	lifespan extension	Tomás-Loba et al.[19]
	telomerase-null mouse	telomerase reactivation	lifespan extension	Jaskelioff et al.[20]
	mouse	pharmacological or genetic activation of telomerase	delayed aging	Bernardes de Jesus et al.[21]
	mouse	hyperlong telomeres	increased lifespan; metabolic health improvement	Muñoz-Lorente et al.[22]
	mouse	telomere maintenance in adult neurons	preservation of neuron survival and cognitive function	Shim et al.[23]
	mouse	telomerase activation by gene therapy strategy	improvement in models of pulmonary fibrosis and aplastic anemia	Povedano et al.[24] and Bär et al.[25]
Epigenetic alterations	human	α-ketoglutarate	delayed epigenetic clock	Demidenko et al.[26]
	human	thymus regeneration by GH +DHEA+ metformin	delayed epigenetic clock	Fahy et al.[27]
	mouse	Kat7 inactivation	lifespan extension	Wang et al.[28]
	human stem cells	KAT7 inactivation	decreased H3 acetylation, reduced cell senescence	Wang et al.[28]
	mouse	Sirt1 overexpression	improved genomic stability and metabolism	Bhatt and Tiwari[29]
	mouse	Sirt3 overexpression	reversed regenerative capacity of HSC	Bhatt and Tiwari[29]
	mouse	miR-188 depletion	alleviated age-related vascular problems	He et al.[30]
	mouse	miR-455-3p overexpression	improved mitochondrial and cognitive function; extended lifespan	Kumar et al.[31]
	aged or *Sirt6*$^{-/-}$ mouse	nucleoside reverse-transcriptase inhibitors	improved healthspan and lifespan	Simon et al.[32]
Loss of proteostasis	mouse	transgenic expression of LAMP2a in hepatocytes or HSC	improved hepatocyte viability in aged mice. Improved function and metabolic properties of HSC	Dong et al.[33]
	mouse	pharmacological induction of CMA	improved Alzheimer's pathology and arteriosclerosis in disease models	Madrigal-Matute et al.[34]

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Table 1. ***Continued***

Hallmark	Species/model	Intervention	Outcome	Ref
	mouse	intranasal administration of recombinant human HSP70	enhanced lifespan (m), improved cognitive functions, and proteasome activity; reduced brain lipofuscin	Bobkova et al.[35]
	mouse	4-phenylbutyrate administered to aged mice	reduced ER stress in cortex and hippocampus and improved cognition	Hafycz et al.[36]
	human	guanabenz in patients with ALS (FDA approved)	reduced progression to bulbar stage	Dalla Bella et al.[37]
Disabled macroautophagy	mouse	transgenic overexpression of *Atg5*	improved longevity, metabolic health and motor function	Pyo et al.[38]
	mouse	mutation of beclin 1 (*Becn1*$^{F121A/F121A}$) to reduce its inhibition by Bcl-2	extended longevity of C57BL/6 mice and progeroid klotho-ko mice. Prolonged neurogenesis	Fernández et al.[39] and Wang et al.[40]
	mouse	spermidine in drinking water	extended longevity, reduced cardiac aging and oxidative stress, sinusoidal dilation in liver	Eisenberg et al.[41]
	mouse	salicylates (salicylate, acetylsalicylate)	EP300 inhibition; autophagy-dependent hepatoprotection; improved cancer immunosurveillance	Castoldi et al.[42]
	mouse	nordihydroguairaretic acid	longevity extension (m) with EP300 inhibition	Tezil et al.[43]
	human	oral NMN in prediabetic women (phase III trial)	increased insulin sensitivity of skeleton muscle	Yoshino et al.[44]
	human	oral NR in Parkinson disease patients (phase 1 trial)	clinical improvement, reduced inflammatory cytokines in serum and cerebrospinal fluid	Brakedal et al.[45]
	human	NAM in patients with 2 non-melanoma skin cancers in the preceding 5 years (phase 3 trial)	reduced rates of new non-melanoma skin cancers and keratosis	Chen et al.[46]
	human	urolithin A to middle-aged adults (randomized phase 2)	improved aerobic endurance and physical performance; reduced plasma CRP	Singh et al.[47]
Deregulated nutrient-sensing	mouse	inducible GH receptor knockout at 6 months	longevity and enhanced insulin sensitivity; less neoplasms (m)	Duran-Ortiz et al.[48]
	mouse	caloric restriction by 30% of male C57BL/6J mice	lifespan extension of 10%–30%	Acosta-Rodríguez et al.[49]
	human	caloric restriction by 14% for 2 years (phase 2)	improved thymopoiesis and anti-inflammatory effects on adipose tissues. Reduced PLA2G7	Spadaro et al.[50]

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Table 1. ***Continued***

Hallmark	Species/model	Intervention	Outcome	Ref
			expression (gene knockout in mice ameliorates metabolic health)	
	mouse	ß-hydroxybutyrate in drinking water	higher energy expenditure, improved motor fitness and memory	Fan et al.[51]
Mitochondrial dysfunction	mouse	TPP-thiazole (inhibitor of respiratory chain complex IV)	improved mitochondrial metabolism, reduced visceral fat and higher glucose tolerance in old mice	Tavallaie et al.[52]
	mouse	CRMP that preferentially acts to uncouple hepatocyte mitochondria	administration to obese old mice reduces hepatosteatosis and liver insulin resistance	Goedeke et al.[53]
	mouse	elamipretide to inhibit mitochondrial permeability transition	improved mitochondrial function and avoidance of diastolic heart dysfunction, especially if combined with NMN	Zhang et al.[54]
	primates	spontaneous or diet-induced obesity in cynomolgus and rhesus macaques, treated with CRMP	enhanced hepatic mitochondrial fat oxidation, improved insulin tolerance, reduced hepatic and plasma triglycerides, reduced cholesterol	Goedeke et al.[55]
	human	clinical trial evaluating elamipretide on Barth syndrome (randomized phase 2/3 trial)	improved walking test, muscle and cardiac parameters, overall improvement	Reid Thompson et al.[56]
	human	clinical trial with L-carnitine supplementation to older man (phase 2 trial)	increased muscle carnitine content and fatty acid oxidation during exercise	Chee et al.[57]
Cellular senescence	mouse	genetic ablation of p16-expressing cells	increased health- and lifespan. Treatment starting at 1 year age	Baker et al.[58]
	mouse	senolytic treatment with dasatinib + quercetin	increased health- and lifespan. Treatment starting at 2 years age	Xu et al.[59]
	mouse	senolytic treatment with fisetin	increased health- and lifespan. Treatment starting at 1.6 years age	Yousefzadeh et al.[60]
	human	senolytic treatment with dasatinib + quercetin of patients with pulmonary fibrosis (phase 1 trial)	improved physical performance; reduction in pro-inflammatory and pro-fibrotic factors in serum; elevation of αKlotho in urine	Justice et al.[61]
	human	senolytic treatment with dasatinib + quercetin of patients with diabetic kidney disease (phase 1 trial)	reduction of senescent cells and macrophages in adipose tissue, and of pro-inflammatory factors in serum	Hickson et al.[62]

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Table 1. ***Continued***

Hallmark	Species/model	Intervention	Outcome	Ref
Stem cell exhaustion	mouse	transgenic expression of OSKM	longevity extension of HGPS progeroid mice. Preservation of hippocampal neuro- genesis and function in normal mice. Improved tissue repair immediately after injury or in a subsequent injury	Wang et al.,[28] Ocampo et al.,[63] Browder et al.,[64] Chen et al.,[65] Hishida et al.,[66] Rodríguez-Matellán et al.,[67] Gao et al.,[68] and Doeser et al.[69]
	mouse	AAV2-driven expression of OSK in the eye	restoration of visual acuity to old mice and to mice with glaucoma. Improved repair of crushed optical nerve	Lu et al.[70]
Altered intercellular communication	mouse	dilution of blood from old mice with saline/albumin	rejuvenation in multiple tissues	Mehdipour et al.[71]
	mouse	blood transfusion	improved muscle repair; reduced liver steatosis and fibrosis	Rebo et al.[72]
	mouse	human umbilical cord plasma	improved hippocampal neurogenesis	Castellano et al.[73]
	mouse	heterochronic parabiosis	rejuvenation in multiple tissues	Ma et al.[74] and Pálovics et al.[75]
	mouse	CCL3/MIP1α administration	HSC rejuvenation	Ma et al.[74]
	mouse	TIMP2 i.v. administration	hippocampus rejuvenation	Castellano et al.[73]
	mouse	IL-37 injection into old mice	improved metabolism and endurance exercise	Ballak et al.[76]
	mouse	GDF11 i.v. administration	rejuvenation of brain, muscle and pancreas, but pro-fibrotic effects	Frohlich and Vinciguerra[77]
	mouse	transgenic overexpression of VEGF	improved health- and lifespan, enhanced liver and muscle repair	Grunewald et al.[78]
	mouse human cells	YAP expression	rejuvenation of old cells, prevention of emergence of aging features	Sladitschek-Martens et al.[79]
	human cells	ECM from young fibroblasts	rejuvenation of aged senescent cells	Choi et al.[80]
	mouse	chondroitin 6-sulfo-transferase overexpression	improved memory in old mice	Yang et al.[81]
Chronic inflammation	mouse	blockade of TNF-α with etanercept from 16 to 18 months of age in C57BL/6 mice	prevention of sarcopenia and increased lifespan (f)	Desdin-Mico et al.[82]; Sciorati et al.[83]
	rat	blockade of TNF-α with etanercept from 24 to 26 months of age in male Wistar albino rats	prevention of cognitive deficits, endothelial dysfunction, peripheral and neuro-inflammation	Gocmez et al.[84]
	mouse	knockout of prostaglandin E_2 receptor EP2 in myeloid cells or treatment of C57BL/6 mice with EP300 inhibitors	improved cognition and reduced age-associated inflammation	Minhas et al.[85]

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Table 1. ***Continued***

Hallmark	Species/model	Intervention	Outcome	Ref
	mouse	knockout of NLRP3 in C57BL/6 mice	improved glucose tolerance, cognition, motor performance and female fertility due to reduced ovarian aging	Marín-Aguilar et al.[86]
	human	treatment of patients with a history of myocardial infarction and high hsCRP with canakinumab (phase 3 trial)	reduced incidence of hypertension and diabetes; reduced frequency of recurrent myocardial infarction and non-small cell lung cancer	Ridker et al.[87]
Dysbiosis	HGPS mouse	fecal microbiota transplantation from WT mice; *Akkermansia muciniphila* administration	enhanced healthspan and lifespan	Bárcena et al.[88]
	SAMP8 mouse	*Lactobacillus plantarum* GKM3	longevity promotion and alleviation of age-related cognitive impairment	Lin et al.[89]
	mouse	microbiota transplantation from young mice to aged host	improved maintenance of brain health and immunity	Boehme et al.[90]
	mouse	fecal microbiota transplantation	improved ovarian function in aged mice	Xu et al.[91]
	mouse	fecal microbiota transplantation	improved germinal centre reactions in lymph nodes	Stebegg et al.[92]
	mouse	indole metabolites	reduction of inflammation during aging	Krishnan et al.[93]
	mouse	short-chain fatty acids	restored microglial function in aged mice	Cryan et al.[94]
	human	oral administration of *Akkermansia muciniphila* (randomized phase 1/2 trial)	improved metabolic parameters in obese or diabetic patients	Depommier et al.[95]

accelerated aging and reduced lifespan associated with deletions rather than point mutations in mtDNA[101] (Table 1). Overall, these data suggest that the avoidance, attenuation, or correction of mtDNA mutations might contribute to extend healthspan and lifespan. Nevertheless, as in the case of nuclear DNA mutations, experimental evidence demonstrating deceleration of aging by gain of function in mtDNA repair mechanisms is still largely missing.

Nuclear architecture

Defects in the nuclear lamina, which constitutes a scaffold for tethering chromatin and protein complexes, can generate genome instability.[102] Accelerated aging syndromes such as the Hutchinson-Gilford and the Néstor-Guillermo progeria syndromes (HGPS and NGPS, respectively) are caused by mutations in genes *LMNA* and *BANF1* encoding protein components of nuclear lamina. Alterations of the nuclear lamina and production of an aberrant prelamin A isoform called progerin are also characteristics of normal human aging, and lamin B1 levels decline during cellular senescence.[18] Animal and cellular models have facilitated the identification of the response mechanisms and stress pathways elicited by nuclear lamina aberrations caused by aging and progeria, including activation of tumor suppressor protein p53 (TP53), deregulation of the somatotrophic axis, and attrition of adult stem cells.[18]

The causal implication of nuclear lamina abnormalities in premature aging has been corroborated by the observation that decreasing prelamin A or progerin levels delays the onset of progeroid features and extends lifespan in mouse models of HGPS. This can be achieved by systemic injection of antisense oligonucleotides, farnesyltransferase inhibitors, a combination of statins and aminobisphosphonates, restoration of the somatotrophic axis, or blockade of NF-κB signaling.[103] Some of these interventions have been already approved for use in progeria patients.[104] Moreover, gene editing strategies have been recently developed to correct *LMNA* mutations in cells from HGPS patients and in animal models of this disease.[105,106] Hopefully, these approaches will be clinically implemented for the future treatment of progeria, but to date, no evidence is available showing that reducing progerin would delay normal aging.

TELOMERE ATTRITION

DNA damage at the end of chromosomes (telomeres) contributes to aging and age-linked diseases.[107] Replicative DNA polymerases are unable to complete the copy of telomere regions of eukaryotic DNA. Accordingly, after several rounds of cell division, telomeres undergo a substantial shortening that induces genomic instability and finally leads to either apoptosis or cell senescence. These deleterious effects can be prevented by the reverse-transcriptase activity of telomerase, an active ribonucleoprotein that elongates telomeres to maintain their adequate length.[108,109] However, most mammalian somatic cells do not express telomerase, which leads to the progressive and cumulative erosion of telomere sequences from chromosome ends throughout life. There are several examples in which telomere attrition attenuates carcinogenesis through limiting the replicative lifespan of malignant cells. Hence, in contrast to genomic instability which unambiguously favors oncogenesis, telomere attrition may antagonize malignancy. For this reason, we consider telomere attrition as a hallmark of aging that is separable from genomic instability.[110]

Telomerase deficiency in humans is associated with premature development of diseases such as pulmonary fibrosis, aplastic anemia, and dyskeratosis congenita, all of which hamper the regenerative capacity of the affected tissues.[111] Telomere shortening is also observed during normal aging in many different species, including humans and mice.[112] The telomeric attrition rate is influenced by age, genetic variants, lifestyle, and social factors; depends on the proliferative activity of the affected cells; and predicts lifespan in a wide variety of species.[112] Telomere uncapping can also result from deficiencies in shelterins, a group of proteins that block the DNA damage response at chromosome ends and modulate telomere length. Several loss-of-function models for shelterin components indicate a decline of tissue regenerative capacity and accelerated aging, even in the presence of telomeres with a normal length.[113]

Genetically modified animal models have revealed causal links between telomere attrition, cellular senescence, and organismal aging. Mice with shortened or lengthened telomeres exhibit decreased or increased lifespan, respectively.[19] Notably, the premature aging of telomerase-deficient mice can be reverted when telomerase is genetically reactivated[20] (Table 1). Moreover, normal aging can be delayed in mice by pharmacological activation or systemic viral transduction of telomerase,[21] whereas mice with hyperlong telomeres show increased lifespan and metabolic health improvement[22] (Table 1). Likewise, mice engineered to maintain physiological levels of telomerase in adult neurons preserve the survival of these cells and maintain cognitive function in Alzheimer's disease models[23] (Table 1). Thus, aging can be modulated by telomerase activation.

Telomerase activation to decelerate aging and treat telomere diseases

In humans, many studies have provided evidence for causal associations between short telomere length and age-related diseases.[114] In particular, generation of mouse models with short telomeres has demonstrated that telomeric attrition is at the origin of telomere syndromes[115] and prevalent age-associated diseases, such as pulmonary and kidney fibrosis.[24,116] These links between telomere dynamics and organismal aging have resulted in the design of new interventions to delay aging and age-related diseases. As an example, telomerase activation using a gene therapy strategy has shown therapeutic effects on mouse models of pulmonary fibrosis and aplastic anemia.[24,25]

EPIGENETIC ALTERATIONS

The large variety of epigenetic changes that contribute to aging include alterations in DNA methylation patterns, abnormal post-translational modification of histones, aberrant chromatin remodeling, and deregulated function of non-coding RNAs (ncRNAs) (Figure 2B). These regulatory and often reversible changes impact on gene expression and other cellular processes, resulting in the development and progression of several

age-related human pathologies, such as cancer, neurodegeneration, metabolic syndrome, and bone diseases. A vast array of enzymatic systems is involved in the generation and maintenance of epigenetic patterns. These enzymes include DNA methyltransferases, histone acetylases, deacetylases, methylases, and demethylases, as well as protein complexes implicated in chromatin remodeling or in ncRNA synthesis and maturation.

DNA methylation

The human DNA methylation landscape accumulates multiple changes with the passage of time.[117] Early studies described an age-associated global hypomethylation, but further analyses revealed that specific loci, including those of several tumor suppressor genes and Polycomb target genes, are hypermethylated with age. Cells from patients and mice with progeroid syndromes also exhibit DNA methylation changes that partially recapitulate those found in normal aging.[118] The functional consequences of most of these age-related epimutations are uncertain, as the majority of changes affect introns and intergenic regions.[119]

Epigenetic clocks based on DNA methylation status at selected sites have been introduced to predict chronological age and mortality risk as well as to evaluate interventions that may extend human lifespan.[119] This has been demonstrated with protocols aimed at thymus regeneration, which resulted in improved risk indices for many age-related diseases and a mean epigenetic age approximately 1.5 years less than baseline after 1 year of treatment. Moreover, predictions of human morbidity and mortality showed a 2-year decrease in epigenetic versus chronological age, which persisted 6 months after discontinuing treatment.[27] Likewise, α-ketoglutarate supplementation for 7 months turned back the epigenetic clock by 8 years.[26] In summary, DNA methylation changes are associated with aging, but there is no definitive evidence that they actually cause aging. Further studies will be necessary to demonstrate that defective maintenance of DNA methylation produces accelerated aging and that improved fidelity in maintenance of DNA methylation patterns extends longevity. It will be also necessary to identify the molecular drivers responsible for the modulation of changes occurring in the aged human methylome.

Histone modifications

Global loss of histones and tissue-dependent changes in their post-translational modifications are also closely linked to aging. Increased histone expression extends lifespan in *Drosophila*,[120] whereas increased histone H4K16 acetylation or H3K4 trimethylation and decreased levels of H3K9 or H3K27 trimethylation are found in fibroblasts from aged individuals and progeroid patients. These histone modifications can lead to transcriptional changes, loss of cellular homeostasis, and age-associated metabolic decline.[121] Of note, loss of heterochromatic marks at telomeres has been shown to lead to telomere lengthening.[122]

Histone demethylases modulate lifespan by targeting components of key longevity routes such as the insulin/insulin growth factor-1 (IGF-1) signaling pathway. Other histone-modifying enzymes such as members of the SIRT family of protein deacetylases and ADP-ribosyltransferases also contribute to healthy aging.[29] Transgenic overexpression of SIRT1 improves genomic stability and metabolic efficiency during aging in mice, although without increasing longevity.[29] Overexpression of mitochondrial SIRT3 reverses the regenerative capacity lost in aged hematopoietic stem cells (HSCs) and can mediate the beneficial effects of dietary restriction in longevity.[123] Similarly, *Sirt6* ablation in mice results in accelerated aging,[124] whereas *Sirt6* overexpression extends lifespan.[16] The underlying mechanisms derive from the fact that Sirt6 is a multitask protein with ability to interconnect chromatin dynamics with metabolism and DNA repair.[125] Finally, *Sirt7* deficiency induces global genomic instability, metabolic dysfunctions, and premature aging.[29] Together, these findings are consistent with the idea that a decrease in deacetylase activity would result in chromatin relaxation, increased exposure to DNA damaging agents, and enhanced genomic instability.[126] Conversely, genetic inactivation of the histone acetyltransferase KAT7 in human stem cells decreases histone H3K14 acetylation and alleviates cell senescence features.[28] Moreover, intravenous injection of lentiviral vectors encoding Cas9/sg-Kat7 ameliorates hepatocyte senescence and liver aging and extends lifespan in both normal and progeroid mice.[28] Inhibitors of histone acetyltransferases also ameliorate the premature aging phenotype and extend lifespan of progeroid mice, whereas histone deacetylase activators promote longevity in part via upregulation of SIRT1 activity.[127] Together, these findings suggest that histone-modifiers should be further explored as part of therapeutic strategies against age-associated cognitive decline, although it is still unclear whether these interventions influence aging and longevity through purely epigenetic mechanisms, by impinging on DNA repair and genome stability or via transcriptional alterations affecting metabolic or signaling pathways.

Chromatin remodeling

Besides DNA- and histone-modifiers, several chromosomal proteins and chromatin remodeling factors, such as the heterochromatin protein 1α (HP1α) and Polycomb group proteins which are implicated in genomic stability DNA repair, may modulate aging.[128] Alterations in these epigenetic factors result in profound changes in chromatin architecture, including global heterochromatin loss and redistribution, which are common events in aged cells.

The causal relevance of these chromatin alterations in aging has been largely studied in invertebrates in which loss-of-function mutations in HP1α decrease longevity, whereas its overexpression expands healthspan and lifespan[129] (Table 1). Similar studies in mammals are still limited, but most studies indicate that heterochromatin relaxation contributes to aging and aging-related pathologies, whereas maintenance of heterochromatin promotes longevity. For example, loss of PIN1—a prolyl isomerase essential to preserve heterochromatin is associated with premature aging and neurodegeneration in different species from *Drosophila* to mammals[130] (Table 1). Nevertheless, experiments aimed at extending vertebrate longevity by gain of function of chromatin remodeling factors are still missing.

Non-coding RNAs

The large and growing universe of ncRNAs, including lncRNAs (such as telomeric RNAs or TERRA), microRNAs (miRNAs), and circular RNAs, has emerged as epigenetic factors with ability to influence aging. ncRNAs modulate healthspan and lifespan by post-transcriptional targeting of components of longevity networks or by regulating stem cell behavior.[131] A circular RNA mediates the effect of the insulin/IGF-1 signaling pathway on *Drosophila* lifespan,[132] but most studies have focused on miRNAs, and there is still debate on the extent to which other ncRNAs may derive from transcriptional noise, with their regulatory roles in human physiology and pathology only circumscribed to few specific cases.[133]

Gain- and loss-of-function studies first confirmed the capacity of several miRNAs to modulate longevity in invertebrates. Subsequent studies in mice have provided causal evidence on the functional relevance of miRNAs in aging (Table 1). For example, miRNA-188-3p expression is upregulated in skeletal endothelium during aging and contributes to vascular problems associated with the passage of time. Depletion of miR-188 in mice alleviates the age-related decline in beneficial bone capillary subtypes, whereas endothelial-specific overexpression of this miRNA decreases bone mass and delays bone regeneration.[30] Conversely, depletion of miR-455-3p in mice exhibits deleterious effects on mitochondrial dynamics, cognitive behavior, and lifespan, whereas its overexpression preserves these functions and extends lifespan.[31] Overall, these findings suggest that miRNAs may causally contribute to aging and aging-related pathologies and represent potential therapeutic targets for delaying or ameliorating these conditions.

Derepression of retrotransposons

Recent studies have unveiled the role of retrotransposons in aging of complex metazoans, including humans.[134] These retrotransposable elements are mobile genetic units that can move from one genomic location to another, using a molecular mechanism that involves an RNA intermediate. Retrotransposons consist of long interspersed nuclear elements (LINEs), which encode the required proteins for retrotransposition, and SINEs, which are short, non-coding RNAs that hijack the LINE protein machinery. Retrotransposons are reactivated in senescent cells and during lifetime and generate deleterious effects through genetic and epigenetic changes or by activation of immune pathways triggered after identification of retrotransposon nucleic acids as foreign DNA.[134] Mechanistically, epigenetic derepression of LINE-1 RNA inhibits the epigenetic reader Suv39H1,2 resulting in global reduction of H3K9me3 and heterochromatin,[135] whereas reverse transcription of LINE-1 RNA results in double-stranded cDNA that activates the cGAS/STING/interferon pathway.[136]

Treatments with nucleoside reverse-transcriptase inhibitors (NRTIs), which suppress or attenuate retrotransposition, extend lifespan of *Sirt6*-null mice and improve healthspan, ameliorating bone and muscle phenotypes (Table 1). Likewise, treatment of aged wild-type mice with NRTIs reduces the levels of DNA damage markers.[32] Moreover, *in vivo* targeting of retrotransposons with antisense oligonucleotides increases the lifespan of progeroid mice.[135] Notably, a rare *SIRT6* variant in centenarians is a stronger suppressor of LINE1 retrotransposons, enhances genome stability, and can more robustly kill cancer cells than wild-type *SIRT6*.[137] Collectively, these findings suggest that retrotransposons causally contribute to the aging process and that interventions that oppose retrotransposon activity might improve healthy longevity. Further clinical studies in aged populations with drugs targeting the different functions of retrotransposons may delineate novel intervention strategies on aging and aging-related pathologies.

Gene expression changes

The mechanisms underlying the effects of all the above epigenetic factors converge at the modulation of gene expression levels. Aging causes an increase of the transcriptional noise and an aberrant production and maturation of many mRNAs.[138,139] Microarray-based comparisons of young and old tissues from human and other species have identified age-related transcriptional signatures that result from epigenetic changes occurring during aging. Environmental exposures also cause alterations in gene regulation via DNA methylation alterations and histone modifications and promote aging-related epigenetic changes including the acceleration of epigenetic clocks.[140]

Single-cell transcriptomic and plasma proteomics of multiple cell types and organs at several ages across the entire mouse lifespan have unveiled remarkable gene expression shifts during aging.[138] These changes specially affect certain biological processes, such as inflammation, protein folding, extracellular matrix (ECM) regulation, and mitochondrial function, which are widely deregulated in aging.[141] The common expression patterns observed during aging in different tissues may help to guide future interventions aimed at improving healthspan and lifespan (Table 1). Likewise, the observed decline in transcriptional and post-transcriptional efficiency and fidelity in the course of aging, and its negative consequences on the proteome health may also open new opportunities for prolongevity strategies.[139]

LOSS OF PROTEOSTASIS

Aging and several age-related morbidities, such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, and cataract, are associated with impaired protein homeostasis or proteostasis, leading to the accumulation of misfolded, oxidized, glycated, or ubiquitinylated proteins that often form aggregates as intracellular inclusion bodies or extracellular amyloid plaques.[142]

Proteostasis collapse

Intracellular proteostasis can be disrupted due to the enhanced production of erroneously translated, misfolded or incomplete proteins (Figure 3). Genetic manipulation of the ribosomal protein RPS23 to improve the accuracy of RNA-to-protein translation extends lifespan in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Drosophila melanogaster*,[143] whereas a mutation in RPS9 that favors error-prone translation causes premature aging in mice.[144] Another mechanism driving the collapse of the proteostasis network resides in slowed translation elongation and cumulative oxidative damage of proteins, increasingly distracting the chaperones from folding healthy proteins

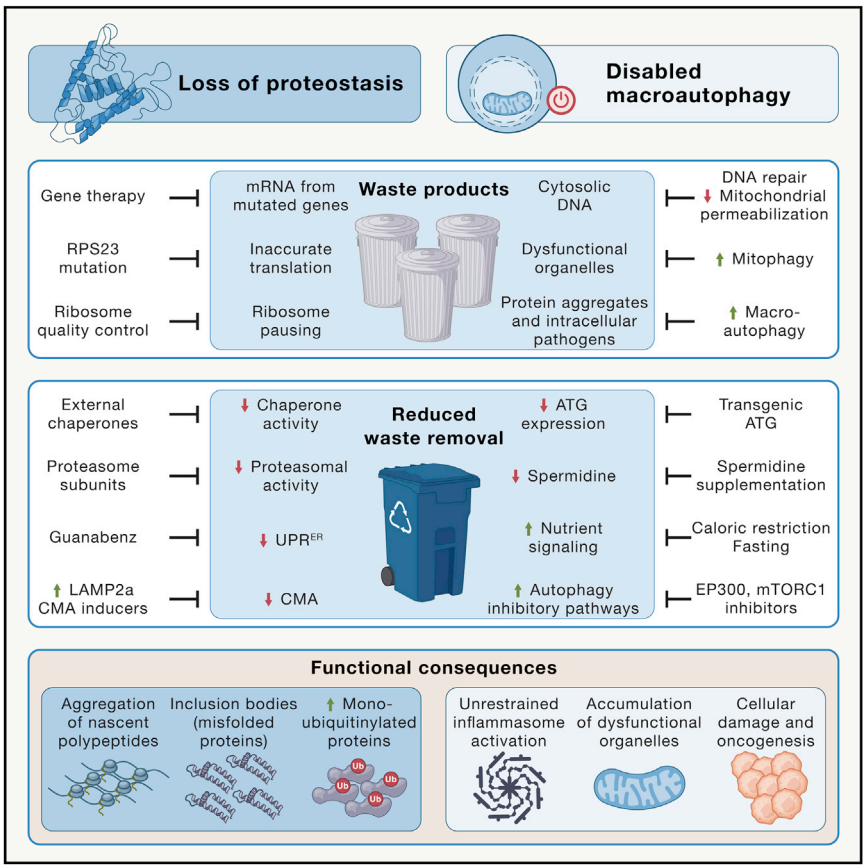

Figure 3. Loss of protein and organellar turnover

Loss of proteostasis and disabled macroautophagy are characterized by a deviation from the young equilibrium state in which an accumulation of waste products results from a variety of age-associated alterations and simultaneously waste removal is compromised through a variety of mechanisms. The functional consequences of these alterations are listed. Some strategies for reestablishing proteostasis and autophagy are exemplified on the left and on the right.

required for cellular fitness.[145] In addition, numerous age-related neurodegenerative diseases including ALS and Alzheimer can be caused by mutations in proteins that render them intrinsically prone to misfolding and aggregation, hence saturating the mechanisms of protein repair, removal, and turnover that are required for maintenance of the healthy state.[146]

The proteostasis network also collapses when mechanisms assuring quality control fail, for instance, due to reduced function of the unfolded protein response (UPR) in the endoplasmic reticulum (ER),[147] when stabilization of correctly folded proteins is compromised or when mechanisms for the degradation of proteins by the proteasome or the lysosome become insufficient (Figure 3). Reduction of proteasome activity has been observed in aged organs including the brain of the short-lived fish *Nothobranchius furzeri*.[148] Moreover, some mono-ubiquitinylated proteins accumulate in aging tissues from flies, mice, monkeys, and humans, as documented for histone 2A.[149]

The degradation of proteins by the lysosome can be achieved in a specific fashion, through chaperone-mediated autophagy (CMA), wherein proteins exposing a pentapeptide motif resembling KFERQ first bind to heat shock protein HSC70 and then to lysosome-associated membrane protein type 2A (LAMP2A), which facilitates the translocation of the client protein into the lumen of the lysosome.[150] Hepatic LAMP2A expression declines with age in mice, and its transgenic re-expression reduces liver aging.[151] Protein aggregates can also be removed by macroautophagy upon their inclusion in two-membrane vesicles, the autophagosomes, for their later fusion with lysosomes.[152] Since autophagosomes can envelop non-proteinaceous structures, this process will be discussed separately from proteostasis in the next hallmark section (disabled macroautophagy). Nonetheless, stimulation of autophagy constitutes a valid strategy for the elimination of intracellular protein aggregates.[153]

Proteostasis, aging, and longevity

Perturbation of general proteostasis accelerates aging. For example, feeding *D. melanogaster* with advanced glycation end products (AGEs) or lipofuscin (an aggregate of covalently cross-linked proteins, sugars, and lipids) causes the accumulation of AGE-modified and carbonylated proteins with a reduction of healthspan and lifespan that is further accentuated upon knockdown of the lysosomal protease cathepsin D.[154] Loss of the protease ZMPSTE24 abolishes the normal proteolytic maturation of prelamin A and causes a progeroid syndrome in mice, phenocopying that observed in humans with loss-of-function mutations of ZMPSTE24.[18] In mice, knockout of LAMP2A (essential for CMA) in neurons profoundly affects the proteome, yielding similar changes as found in Alzheimer patients. Indeed, inhibition of CMA in mice exacerbates experimental Alzheimer's disease, whereas its stimulation by a pharmacological CMA activator attenuates the pathology.[155]

Experimental amelioration of proteostasis can retard the aging process (Table 1). Intranasal application of recombinant human HSP70 protein to mice enhances proteasome activity, reduces brain lipofuscin levels, enhances cognitive functions, and extends lifespan.[35] Similarly, administration of the chemical chaperone 4-phenylbutyrate to aged mice reduces ER stress in the brain and improves cognition.[36] In nematodes and flies, transfection-enforced overexpression of isolated proteasome subunits improves proteostasis and increases lifespan.[156] In mice, stimulation of CMA by transgenic expression of LAMP2a HSCs improve the survival of the targeted cell populations,[33] in line with the observation that pharmacological enhancement of CMA attenuates Alzheimer's pathology and

arteriosclerosis.[155,34] Hence, activation of CMA may constitute a valid strategy for delaying the aging process.

A phase 3 clinical trial has revealed that in patients with recent ALS diagnosis, administration of the antihypertensive guanabenz inhibits progression to the life-threatening bulbar stage.[37] Guanabenz may act to stimulate the phosphorylation (or to inhibit the dephosphorylation) of eukaryotic translation initiation factor 2α (eIF2α), which occurs in the context of the "integrated stress response (ISR)" as part of the UPR,[157] although it remains under debate to what extent the actions of guanabenz are mediated by the stimulation of the ISR.[158] Importantly, eIF2α phosphorylation causes a switch from 5′ cap-dependent to 5′ cap-independent RNA translation, knowing that the latter is enhanced by several longevity-extending manipulations.[159] Moreover, eIF2α phosphorylation is essential for the induction of stress granules, which are required for longevity extension by dietary restriction in worms.[160] Finally, eIF2α phosphorylation is indispensable for the induction of autophagy,[161] which is a major anti-aging mechanism (see below), suggesting a crosstalk between UPR and autophagy in prolongevity pathways. Future studies must determine whether the capacity of guanabenz to attenuate neurodegeneration is mediated via ISR stimulation or alternative mechanisms. Indeed, it has been proposed that inhibitors of ISR might be also used for the treatment of neurodegenerative diseases.[162]

DISABLED MACROAUTOPHAGY

Macroautophagy (that we will refer to as "autophagy") involves the sequestration of cytoplasmic material in two-membrane vesicles, the autophagosomes, which later fuse with lysosomes for the digestion of luminal content.[152] Thus, autophagy is not only involved in proteostasis but also affects non-proteinaceous macromolecules (such as ectopic cytosolic DNA, lipid vesicles, and glycogen) and entire organelles (including dysfunctional mitochondria targeted by "mitophagy," and other organelles leading to "lysophagy," "reticulophagy," or "pexophagy"), as well as invading pathogens ("xenophagy").[152] An age-related decline in autophagy constitutes one of the most important mechanisms of reduced organelle turnover, justifying its discussion as a new hallmark of aging. As a note of caution, genes and proteins that participate in the autophagic process are also involved in alternative degradation processes such as LC3-associated phagocytosis of extracellular material,[163] and the extrusion of intracellular waste (e.g., dysfunctional mitochondria) in the form of exospheres for their subsequent removal by macrophages.[164] That said, there is strong evidence that the core process of autophagy is relevant to aging (Figure 3).

Accelerated aging due to autophagy inhibition

In humans, the expression of autophagy-related genes, such as *ATG5*, *ATG7*, and *BECN1*, declines with age.[165] $CD4^+$ T lymphocytes isolated from the offspring of parents with exceptional longevity show enhanced autophagic activity compared with age-matched controls.[166] Decreased autophagy in circulating B and T lymphocytes from aging donors is accompanied by a reduction of the pro-autophagic metabolite spermidine.[167,168] Similarly, in rodents, a progressive deterioration of autophagy has been described for some organs, pleading in favor of the idea that autophagic flux is compromised with age. Reduction of autophagic flux may participate in the accumulation of protein aggregates and dysfunctional organelles, reduced elimination of pathogens, and enhanced inflammation because autophagy eliminates proteins involved in inflammasome and their upstream triggers.[169]

Genetic inhibition of autophagy accelerates the aging process in model organisms. This process is partially reversible, as illustrated in mice in which *Atg5* is downregulated by a doxycycline-inducible shRNA. *Atg5* knockdown causes the premature degeneration and senescence of multiple organ systems leading to premature death.[170] Upon withdrawal of doxycycline, autophagy restoration is accompanied by attenuated systemic inflammation and segmental reduction of aging. Of note, in this model, the transient inhibition of autophagy is followed by a major increase in the incidence of malignancies. Hence, autophagy apparently acts as a tumor-suppressive mechanism, which may involve cell-autonomous processes and cancer immunosurveillance.[153] In patients, loss-of-function mutations of genes that regulate or execute autophagy have been causally linked to a broad spectrum of cardiovascular, infectious, neurodegenerative, metabolic, musculoskeletal, ocular, and pulmonary disorders, many of which resemble to premature aging at the histopathological and functional levels.[152,153]

Autophagy stimulation for decelerated aging

There is ample evidence that stimulation of autophagic flux increases healthspan and lifespan in model organisms (Table 1). For example, increasing autophagy solely in the enterocytes of the intestine increases *Drosophila* lifespan.[120] In mice, transgenic overexpression of *Atg5* under the control of a ubiquitously expressed promoter is sufficient to extend lifespan and to improve metabolic health and motor function.[38] Moreover, knockin mutation of beclin 1 ($Becn1^{F121A/F121A}$) to reduce its inhibition by Bcl-2 causes an increase in autophagic flux, as well as an extension of lifespan. This effect is coupled to a reduction of age-associated pathologies and spontaneous tumorigenesis,[39] as well as to increased neurogenesis.[40]

Oral supplementation of spermidine to mice induces autophagy in multiple organs and extends longevity by up to 25%, accompanied by reduced cardiac aging. This latter effect is lost upon cardiomyocyte-specific knockout of *Atg7*, suggesting that it relies on autophagy.[41] Mechanistically, the pro-autophagic effects of spermidine have been linked to an inhibition of the acetyl transferase EP300 (resulting in reduced acetylation of several core autophagy proteins)[171] or to the hypusination of eIF5A, which is essential for the synthesis of the autophagy transcription factor TFEB.[167] Among these factors, EP300 is the target of the longevity-enhancing drugs nordihydroguaiaretic acid[43] and salicylate.[42] Pharmacological inhibition of EP300 with C646 mimics the stimulatory effects of spermidine on autophagy and cancer immunosurveillance.[172] When circulating B lymphocytes or $CD8^+$ T cells from aged human donors are cultured in the presence of spermidine, the cells recover juvenile levels of TFEB and eIF5A, coupled to a normalization of autophagic flux.[167,168] Moreover, in *Drosophila*, hypusination deficiency due to a heterozygous mutation or knockdown of deoxyhypusine synthase abolished lifespan extension by

spermidine supplementation.[173] Deoxyhypusine synthase deficiency in murine T cells triggers severe intestinal inflammation coupled to epigenetic remodeling and rewiring of the tricarboxylic acid cycle,[174] whereas spermidine treatment of wild-type mice protects against colitis and colon carcinogenesis.[175] Hence, both EP300 inhibition and eIF5A hypusination appear plausible targets to explain the *in vivo* effects of spermidine.

Pharmacological agents that induce mitophagy and have a positive impact on murine healthspan include NAD^+ precursors (such as nicotinamide, nicotinamide mononucleotide, and nicotinamide riboside)[176] and urolithin A.[177] Clinical trials have demonstrated the efficacy of NAD^+ precursors in the chemoprevention of non-melanoma skin cancer,[46] in reversing insulin resistance in prediabetic women,[44] and in reducing neuroinflammation in patients with Parkinson's disease.[45] Moreover, a phase 3 trial has revealed the capacity of urolithin A to improve muscle strength and to reduce C-reactive protein (CRP).[47]

DEREGULATED NUTRIENT-SENSING

The nutrient-sensing network is highly conserved in evolution. It includes extracellular ligands, such as insulins and IGFs, the receptor tyrosine kinases with which they interact, as well as intracellular signaling cascades. These cascades involve the PI3K-AKT and the Ras-MEK-ERK pathways, as well as transcription factors, including FOXOs and E26 factors, which transactivate genes involved in diverse cellular processes. The mechanistic target of rapamycin (MTOR) complex-1 (MTORC1) responds to nutrients, including glucose and amino acids, and to stressors such as hypoxia and low energy to modulate the activity of numerous proteins including transcription factors such as SREBP and TFEB. This network is a central regulator of cellular activity, including autophagy, mRNA and ribosome biogenesis, protein synthesis, glucose, nucleotide and lipid metabolism, mitochondrial biogenesis, and proteasomal activity. Network activity responds to nutrition and stress status by activating anabolism if nutrients are present and stress is low or by inducing cellular defense pathways in response to stress and nutrient-shortage. There is extensive intracellular crosstalk and feedback within the network, and between it and other intracellular signaling pathways. Genetically reduced activity of components of the nutrient-sensing network can increase lifespan and healthspan in diverse animal models[178,179] (Table 1). Moreover, genetic association studies in humans have implicated the FOXO3 transcription factor[180] and genetic variants encoding components of the network in human longevity.[178] Epigenetic age is also associated with nutrient-sensing in human cells.[181] In youth, activity of this signaling network thus functions to promote beneficial anabolic processes, but during adulthood, it acquires pro-aging properties (Figure 4).

The somatotrophic axis—the first one historically implicated in the control of aging—is a growth-stimulatory cascade that, at its apex, involves growth hormone (GH) produced by the hypophysis. GH acts on the GH receptor of hepatocytes to stimulate the secretion of IGFs, in particular IGF1, which promotes growth and development via the IGF1R to stimulate trophic signals through activation of PI3K-AKT and the MTORC1 network.[182] In multiple model organisms, spontaneous or engineered mutations of this pathway enhance lifespan and retard facets of age-associated deterioration (Table 1). Innate defects in the somatotrophic axis cause dwarfism, but inhibition of this axis from early adulthood has beneficial effects on organismal health (Figure 4).

Another signaling pathway involved in nutrient-sensing relies on the receptor tyrosine kinase ALK (Figure 4), which, in mice, is induced in the hypothalamus by feeding[183] and responds to the ligands augmentor α and β (Augα and Augβ).[184] In *Drosophila*, knockdown of ALK decreases triglyceride levels and the expression of several insulin-like peptides, whereas genetic or pharmacological inhibition of ALK extends healthspan and lifespan, mostly in females.[183] In mice, body-wide or hypothalamus-specific deletion of ALK, as well as double knockout of Augα and Augβ, promotes resistance against diet-induced obesity, and in humans, a loss-of-function mutation of ALK is associated with leanness.[183,184] Hence, this pathway may offer additional targets for interventions on metabolic aging.

Drugs targeting diseases such as cancer and metabolic disease often engage the nutrient-sensing network, thus such drugs are candidates for repurposing as geroprotectors. Rapamycin and rapalogs, which disrupt the MTORC1 complex, have proved to extend lifespan in model organisms even with treatment starting late in adulthood.[185] In mice, rapamycin can increase diverse aspects of health, although it exacerbates some age-related traits such as cataract, and it is protective in models of neurodegenerative and other age-related diseases.

Elderly humans are susceptible to viral respiratory infections. Pre-treatment with MTORC1 inhibitors increased the immune response of elderly volunteers to immunization against influenza[186] and reduced viral respiratory infections in the ensuing winter,[187] thus pointing to a potential strategy for reverting age-related immunosenescence.

Mechanisms

In humans, IGF1 peaks during the second decade of life but declines with aging. Inhibition of the GH/IGF1 pathway in adult or late life extends lifespan in model organisms, including mice.[48] Inhibition of cardiac IGF1R by expression of a dominant negative p110α isoform of PI3K increases maximum lifespan of male mice and improves heart function in aged mice.[188] Moreover, enzymatic inhibition of IGF1R with tyrosine kinase inhibitors improves anticancer immunosurveillance requiring autophagy induction in malignant cells.[189] Long-term administration of an anti-IGF1R antibody enhances the longevity of female (but not male) mice, although reducing inflammation and tumor development. These findings suggest that the IGF1/IGF1R signaling axis may constitute a target for anti-aging interventions. In favor for this conjecture, in elderly women ($\geq$95 years), as well as in a mixed population of older adults (mean age 76 years), low IGF1 levels correlate with a low probability of cognitive impairment and death.[190] Moreover, in a large cohort from the UK Biobank, significant positive correlations were noted between the hazard associated with high IGF-1 and age for dementia, diabetes, vascular disease, osteoporosis, and overall mortality.[191] In centenarians, the concentrations of IGF1BP2 and IGFBP6 are elevated.[192] Future will tell whether yet-to-be-developed antibodies or small molecules that selectively inhibit IGF1R signaling without affecting other receptor tyrosine kinases (and in particular

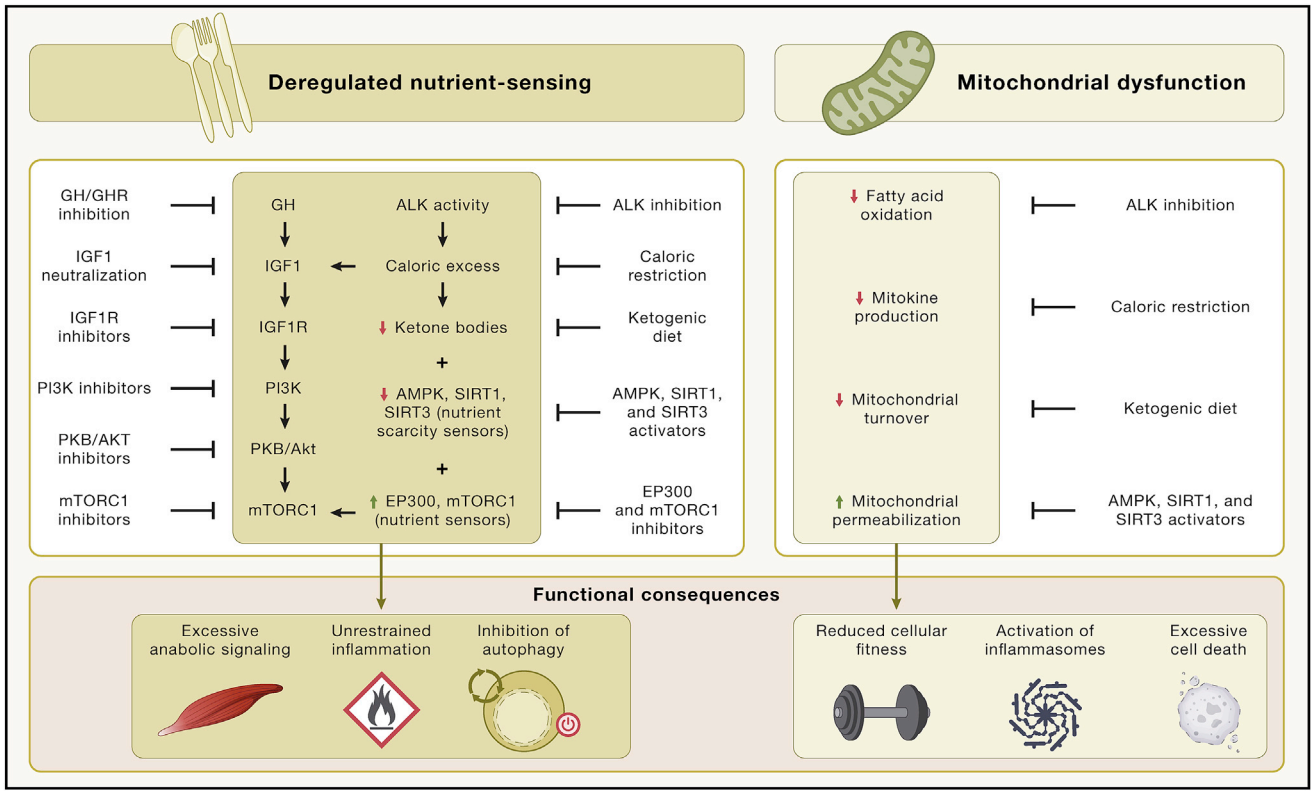

Figure 4. Metabolic alterations

A simplified version of intertwined trophic pathways is shown for deregulated nutrient-sensing with their possible countermeasures to restore nutrient-sensing. Of note, the reduced activity of the nutrient-sensing network influences numerous processes beyond metabolism modulation during aging, including resistance to diverse stressors, activation of repair mechanisms, autophagy stimulation, or inflammation control. Similarly, for mitochondrial dysfunction, a series of age-associated alterations with their possible antidotes are listed. The functional consequences of age-associated metabolic alterations, some of which are relevant to other hallmarks of aging, are exemplified in the lower part of the graph.

the insulin receptor) might be used for the pulsatile inhibition of the somatotropic axis to achieve health benefits with acceptable side effects.

Effects of nutrition

Diet is one of the most practical targets for interventions into human aging. Mechanistically, overnutrition: (1) triggers intracellular nutrient sensors, such as MTORC1 (activated by leucine and other amino acids), and the acetyltransferase EP300 (activated by acetyl coenzyme A); (2) inhibits sensors that detect nutrient scarcity, such as AMP-activated kinase (AMPK) and the deacetylases SIRT1 and SIRT3 (which respond to NAD^+); and (3) abolishes catabolic reactions (glycogenolysis, proteolysis for gluconeogenesis, and lipolysis coupled to ketogenesis) with consequent suppression of adaptive cellular stress responses, including autophagy, antioxidant defense, and DNA repair. Conversely, fasting and dietary restriction inhibit MTORC1 and EP300; activate AMPK, SIRT1, and SIRT3; and stimulate adaptive cellular stress responses as they suppress the somatotrophic axis and extend longevity in multiple model organisms including primates.[193]

Nutrient sensors constitute targets for potential longevity drugs (Figure 4), but health benefits and extended lifespan might also be achieved by dietary restrictions. Mechanistically, this is possible via reduction of overall caloric intake, manipulation of the dietary composition,[194,195] or time-restricted feeding.[196] Dietary restriction regimens are particularly successful in extending lifespan in male C57BL/6J mice, if the animals are completely deprived from nutrients during daytime.[49] However, dietary restriction regimens do not extend lifespan in all mouse strains, supporting the contention that they must be adapted to the genetic makeup of each individual.[197] In humans, clinical assays based on dietary restriction are complicated by poor compliance, yet suggest positive effects on immunity and inflammation.[50]

Intermittent fasting (e.g., 1 day without nutrients, followed by 1 day of *ad libitum* feeding) can avoid long-term weight loss induced by caloric restriction, yet increases lifespan in mice[195] and improves biomarkers of health in clinical trials.[198,199] Life time extension of a similar intermittent fasting regimen in flies has been attributed to the nighttime-specific upregulation of autophagy-stimulatory genes,[200] but this has not yet been investigated in mammals. Rapamycin-induced longevity extension (which in flies partially depends on autophagy induction) can be obtained by constant-long term exposure, as well as by intermittent regimens,[201] suggesting that pulsatile inhibition of this axis is sufficient to obtain the benefits of lifespan extension. The optimal interval for such intermittent treatments has not

yet been determined for clinical use, although partial caloric restriction for 4–7 days every 3–4 weeks may be sufficient to improve metabolic syndrome and anticancer immunosurveillance.[202]

Another potentially beneficial regimen is ketogenic diet, which is a low-carbohydrate, high-fat, and adequate protein diet. Both fasting and ketogenic diet increase the production of ketone bodies (in particular 3-hydroxybutyrate), which are synthesized from acetyl coenzyme A in the liver in an autophagy-dependent fashion, can reach millimolar concentrations in the plasma and replace glucose as an essential fuel, for instance, for the maintenance of brain function.[203] Permanent but not cyclic administration of 3-hydroxybutyrate in the drinking water increases lifespan and healthspan in mice.[51] This strongly suggests that this ketone body mediates some of the beneficial effects of ketogenic diet. Mechanistically, 3-hydroxybutyrate induces vasodilatation and activates immune responses acting on GTP protein coupled receptor 109A,[203] whereas it directly inhibits the NLRP3 inflammasome,[204] indicating a potential pleiotropic mode of action.

MITOCHONDRIAL DYSFUNCTION

Mitochondria are not only the powerhouses of the cell but also constitute latent triggers of inflammation (when reactive oxygen species [ROS] or mtDNA leak out of the organelle causing activation of inflammasomes or cytosolic DNA sensors, respectively) and cell death (when activators of caspases, nucleases, or other lethal enzymes are released from the intermembrane space).[146] With aging, mitochondrial function deteriorates due to multiple intertwined mechanisms including the accumulation of mtDNA mutations, deficient proteostasis leading to the destabilization of respiratory chain complexes, reduced turnover of the organelle, and changes in mitochondrial dynamics. This situation compromises the contribution of mitochondria to cellular bioenergetics, enhances the production of ROS, and may trigger accidental permeabilization of mitochondrial membranes causing inflammation and cell death.[182] Logically, the function of mitochondria is primordial for the maintenance of health, and its progressive deterioration contributes to the aging phenotype (Figure 4).

Mitochondrial function and longevity

Healthspan-extending interventions can stimulate the function of mitochondria. For instance, placebo-controlled trials have revealed positive effects of L-carnitine supplementation on both pre-frail subjects and elderly men[57] (Table 1). The effect is possibly mediated by counteracting age-related declining L-carnitine levels which may limit fatty acid oxidation by mitochondria.[205] Paradoxically, in model organisms, lifespan can be improved by compromising mitochondrial function, which induces a hormetic response ("mitohormesis"), provided that this inhibition is partial and occurs early during development. In *C. elegans*, partial inhibition of mitochondrial protein synthesis or import enhances lifespan through a mechanism involving the mitochondrial UPR (UPR^{mt}).[206] In *Drosophila*, muscle-specific knockdown of complex I subunit NDUFS1/ND75 extends longevity in an UPR^{mt}-dependent fashion.[207] Mild inhibition of mitochondrial ATP synthesis with TPP-thiazole can improve metabolic health in aging mice, reducing visceral fat and improving glucose tolerance, mitochondrial quality, and oxidative metabolism.[52] Partial uncoupling of hepatic mitochondria by means of a controlled release mitochondrial protonophore (CRMP) also reverses age-related metabolic syndrome in mice with high-fat diet-induced obesity.[53] In non-human primate models including spontaneously obese rhesus macaques and high-fat, high-fructose-fed cynomolgus macaques, CRMP reverses signs of metabolic syndrome and improves fatty acid oxidation.[55] These effects are coupled to a reduction of hepatic acetyl-coenzyme A levels, a phenomenon known to stimulate autophagy.[208] Protonophores induce mitophagy,[209] which might explain their positive effects on metabolism as well. Metformin, an antidiabetic considered as a weak complex I inhibitor, has been discussed as a possible anti-aging drug.[210] However, thus far, there is no evidence that challenging mitochondria can increase healthspan or lifespan in humans.

Increased mitochondrial membrane permeability (MMP) due to the absence of serum/glucocorticoid regulated kinase-1 decreases lifespan, which is further compromised when autophagy is enhanced but normalized when autophagy is inhibited by knockdown of essential autophagy-relevant genes in *C. elegans*.[211] Hence, MMP may constitute a life-threatening condition that is aggravated by autophagy. A modified tetrapeptide, elamipretide, has been developed to target cardiolipin in the inner mitochondrial membrane (IMM) and then turned out to bind to the IMM protein adenine-nucleotide translocator-1 to inhibit the mitochondria permeability transition, which is one particular mechanism leading to MMP.[54] Elamipretide has positive effects on multiple aging-related phenotypes in mice and has yielded positive results in a clinical trial on patients with Barth syndrome[56] (Table 1). It will be important to understand whether elamipretide can be advantageously combined with other lifespan-enhancing drugs including autophagy enhancers. In addition to these works, there are also several preclinical and clinical studies evaluating the potential beneficial effects of the antioxidant lipophilic cations MitoQ and SkQ1.[212] Further research will define the utility of all these compounds in the context of other interventions aimed at ameliorating age-associated mitochondrial dysfunctions.

Mitochondrial microproteins and aging

Plasma levels of the microprotein humanin, which is encoded by mtDNA, decline with age. However, centenarians and their offspring exhibit high levels of humanin.[213] Notably, humanin levels negatively correlate with IGF1 in humans and treatment of patients with GH-insufficiency, with GH or IGF1, reduces circulating humanin.[214] Transgenic expression of humanin in *C. elegans* extends longevity through autophagy induction, and treatment of middle-aged mice with the humanin analog HNG improves metabolic healthspan and reduces systemic inflammation.[213] Another mtDNA-encoded microprotein, MOTS-c, declines with age but can be induced by exercise.[215] MOTS-c favors the production of the metabolite 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR), which acts as an endogenous AMPK agonist, thereby preventing age-dependent and high-fat-diet-induced insulin resistance, as well as diet-induced obesity.[215] Hence, mitochondrial microproteins emerge as potential anti-aging factors that link organellar function to organismal homeostasis.

CELLULAR SENESCENCE

Cellular senescence is a response elicited by acute or chronic damage.[216] In humans, senescent cells accumulate in multiple tissues at different rates, from 2- to 20-fold when comparing young (<35 years) to old (>65 years) healthy donors,[217] mainly affecting fibroblasts, endothelial cells, and immune cells, although all cell types can undergo senescence during aging,[218] a process that is triggered at least in part by telomere shortening with aging.[109] In fact, even post-mitotic or slowly proliferating tissues, such as the brain or the heart, may harbor senescent cells.[219] In addition, focal or tissue-specific accumulation of senescent cells occurs in many diseases.[220] The most compelling evidence for the causal role of cellular senescence in aging is that continued genetic or pharmacological elimination of senescent cells extends the healthspan and longevity of naturally aged mice.[59] Also, genetic or pharmacological elimination of senescent cells is therapeutic in many diseases modeled in mice,[221] and at least 3 clinical trials have been completed and 15 clinical trials are ongoing or planned to target senescence for a variety of indications.[222]

The types of damage that trigger primary senescence include oncogenic signaling, genotoxic damage, critically short telomeres, mitochondrial damage, viral or bacterial infection, oxidative damage, nutrient imbalance, and mechanical stress.[216] In addition, secondary or paracrine senescence can be triggered by extracellular mediators of inflammation and fibrosis including CCL2, IL-1β, IL-6, IL-8, and TGF-β.[223] There is evidence suggesting that primary and secondary senescence differ in relevant biological aspects, but the molecular basis of this distinction remains elusive. Historically, the most salient feature of cellular senescence is a stable proliferative arrest mediated by the activation of the tumor suppressors TP53 and CDKN2A/p16, and their downstream effectors CDKN1A/p21 and retinoblastoma-1 (RB1) family proteins, respectively. Together, these proteins inhibit cyclin-dependent kinases (CDKs) and transcriptional activators (E2F family) that drive the cell cycle.[216] Another important event during senescence is the depletion of lamin B1 from the nuclear envelope. This results in the loss of lamin-associated heterochromatin and *de novo* formation of heterochromatin rich in H3K9me3, a process that can be visualized as HP1α foci or senescence-associated heterochromatin foci (SAHFs).[224] The net result is a long-term and viable proliferative arrest with a low rate of spontaneous escape. Depending on their molecular makeup, cancer cells exposed to genotoxic therapy may undergo a canonical senescence response with a highly stable cell cycle arrest or can undergo a senescence-like response with a highly reversible arrest or can even completely bypass senescence.[225] Of note, senescence also plays a role during embryogenesis in the programmed elimination of specific cells and structures.[226]

Senescence and human diseases

Cellular senescence is implicated in multiple non-proliferative diseases, including lung fibrosis, kidney diseases, liver steatosis, obesity-associated metabolic syndrome, type I and II diabetes, atherosclerosis, as well as Alzheimer's and Parkinson's diseases.[220] The pathogenic role of cellular senescence in these diseases can be explained by the senescence-associated secretory phenotype (SASP). SASP results from three features of senescent cells: (1) the transcriptional derepression of endogenous retroviruses, most notably LINE-1, which causes cytosolic leakage of double-stranded DNA and activates the cGAS/STING and TLR pathways;[136] (2) the mitochondrial overproduction of ROS; and (3) the perturbation of the autophagy-lysosomal system leading to an expansion of lysosomal content that facilitates the histochemical detection of lysosomal senescence-associated beta-galactosidase (SABG).[227]

SASP is highly heterogeneous, depending on the cell type-specific activation of innate immunity signaling pathways (cGAS/STING, TLRs, and NLRPs), mTORC1, and transcription factors (NF-κB, CBPs, GATA4, and others). SASP usually has simultaneous and partially conflicting consequences on the microenvironment: (1) to recruit and activate immune cells through the secretion of chemokines (CCL2, CXCL2, and CXCL3) and cytokines (IL-1β, IL-2, IL-6, and IL-8); (2) to suppress the immune system through the secretion of TGF-β; (3) to trigger fibroblast activation and collagen deposition through pro-fibrotic factors (TGF-β, IL-11, and PAI1); (4) to remodel the ECM through the secretion of matrix metalloproteases; (5) to trigger the activation and proliferation of progenitor cells through the secretion of growth factors (EGF and PDGF); and (6) to trigger paracrine senescence in neighboring cells (TGF-β, TNF-α, and IL-8). In many diseases, the net effect of SASP is chronic inflammation and progressive fibrosis.[228]

Although there is not a single unequivocal marker of cellular senescence, this process can be identified by the co-existence of a combination of features that, together, are specific and provide a molecular definition to the phenomenon:[216] (1) lysosomal expansion, detectable by SABG; (2) upregulation of CDK inhibitors, particularly p16 and/or p21; (3) loss of LMNB1 from the nuclear envelope; (4) loss of the chromatin component HMGB1 from the nucleus and its extracellular release as an alarmin; (5) heterochromatic foci, visualized as HP1γ nuclear foci or SAHFs; (6) high levels of ROS; (7) exacerbated DNA damage, visualized as γH2AX nuclear foci; and (8) high levels of SASP factors, notably IL-6, TGF-β, PAI1, and others.

Given the association between cellular senescence and multiple pathologies, the question arises about the biological purpose of such a cellular response. Cellular senescence is a potent tumor suppressor mechanism, but mounting evidence has linked cellular senescence to tissue repair processes in which senescent cells promote localized fibrosis and the recruitment of immune cells that then remove damaged and senescent cells. In this regard, tissue repair can be considered a two-step process: cellular senescence followed by immune recruitment and immune clearance of senescence (Figure 5A). In this scenario, senescence is a temporally restricted response that programs its self-elimination with a beneficial outcome.[229] The pathological consequences of senescence only become visible when the second step of immune clearance is not achieved, and the accumulation of senescent cells and the SASP effects on the tissue microenvironment eventually result in fibrosis.

Senolytics

The strong association between cellular senescence and multiple pathologies has spurred the search for small chemical compounds that selectively kill senescent cells and that are referred to as "senolytics.[230]" Of note, senolysis (elimination of senescent cells) is

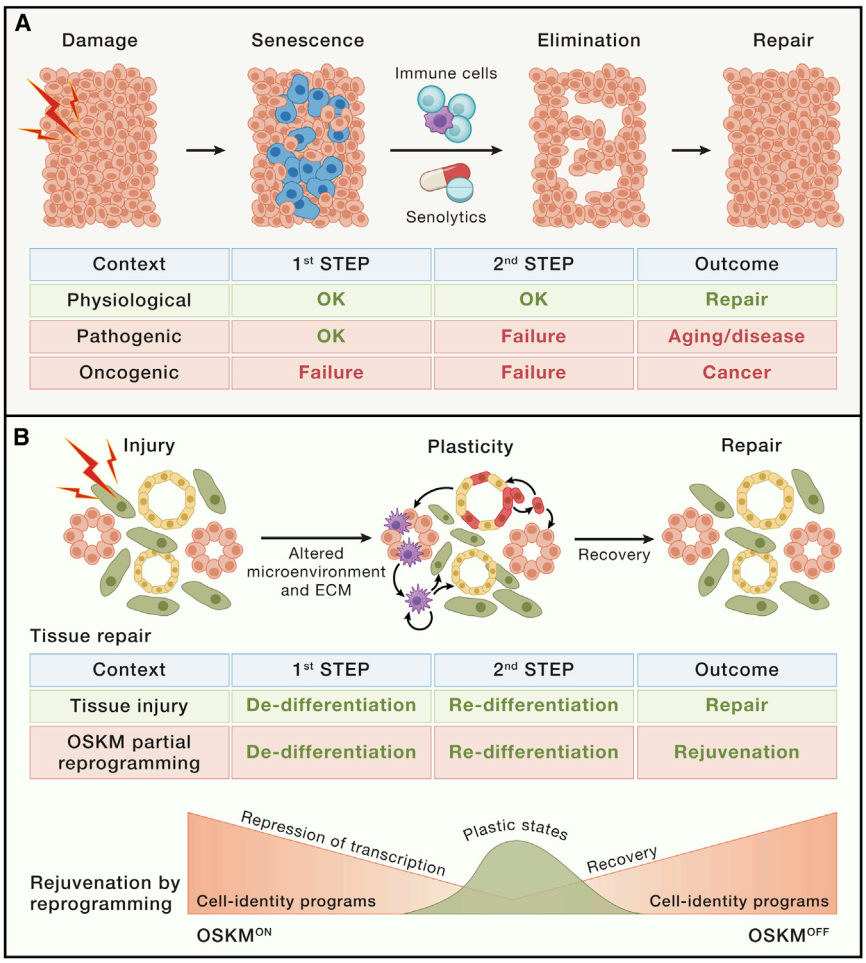

Figure 5. Cellular senescence and stem cell exhaustion

(A) Cellular senescence usually promotes tissue repair after injury and protects the organism from oncogenic damage. This is achieved in two steps: (1) establishment of senescence and (2) recruitment of immune cells that will eliminate the senescent cells, thereby promoting tissue repair. If any of these steps fails, the organism is prone to develop diseases.

(B) Stem cell exhaustion results from the loss of cellular plasticity required for tissue repair. Tissue repair requires a modified microenvironment through the secretion of cytokines (in part due to the senescence-associated secretory response), growth factors and modulators of the extracellular matrix (ECM) that favors the de-differentiation and plasticity of cells from different tissue compartments. These injury-induced plastic cells may acquire multipotent progenitor function. Transient expression of OSKM factors represses the transcription of cell identity programs causing global de-differentiation ($OSKM^{on}$) and the acquisition of plasticity. For rejuvenation, the process must be interrupted at this point ($OSKM^{off}$) to allow cells to re-differentiate and to restore their original cell identities.

very different from the cancellation of the senescence response, which can result, for example, from mutation of p16 or p21. Senolysis does not prevent the execution of senescence but rather recapitulates the natural immune clearance of senescent cells (Figure 5A). In support of this, mice subjected to long-term genetic-induced or pharmacologically induced senolysis present extended longevity without increased cancer incidence or signs of defective tissue repair.[59,58]

The number of senolytic therapies is still limited, but some have been extensively used in preclinical models of disease, as exemplified by navitoclax, dual treatment with dasatinib and quercetin (D/Q), fisetin, cardiac glycosides, and others.[221] The survival and apoptotic resistance of senescent cells strongly depends on the BCL2 family of proteins, specially BCLXL, but also BCL2 and BCLW. This renders senescent cells highly vulnerable to navitoclax, which targets these three proteins.[231] Navitoclax has been evaluated in clinical trials for antitumor activity and it is expected that this drug (or derivatives lacking toxicity on platelets) will enter clinical trials for senescence-associated diseases.[232] Other potential senolytic treatments such as D/Q[230] and fisetin[60] are approved for human use and are being tested in various clinical trials for multiple indications. The mechanistic basis for their action remains unclear. Dasatinib is a promiscuous kinase inhibitor, and quercetin and fisetin are natural flavonoids with multiple targets. D/Q has been tested in clinical trials with promising results in the case of lung and kidney fibrosis.[62,61] Cardiac glycosides inhibit the plasma membrane Na^+/K^+-ATPase present in all cells causing a cationic imbalance and lowering the intracellular pH.[233] The mechanism of senolysis by cardiac glycosides is likely connected to the vulnerability of senescent cells to low intracellular pH. Thus, chemical inhibition of glutaminase deprives cells of a mechanism to counteract low pH and results in senolysis.[234] All the above-discussed senolytic compounds exert therapeutic activity in a wide range of murine disease models associated with senescence. Senolysis can also be achieved by immunological approaches that target proteins appearing on the surface of senescent cells. In particular, antibodies directed against the glycoprotein NMB (GPNMB)[235] and CAR T cells directed against the receptor uPAR[236] attenuate senescence-associated disease models in mice.

In summary, cellular senescence is an important response to stress and damage that, in normal physiology, is followed by immune clearance, but that upon aging or chronic damage fails to be eliminated by immune mechanisms and hence is pathogenic due to the abundant secretion of pro-inflammatory and pro-fibrotic factors. Therapeutic strategies aimed at killing senescent cells have been extensively explored in animal models and are now in clinical trials (Table 1).

STEM CELL EXHAUSTION

Aging is associated with reduced tissue renewal at steady state, as well as with impaired tissue repair upon injury, with each

organ having its own strategy for renewal and repair.[237] For example, in skeletal muscle, one single-cell type, the satellite cell, is placed at the apex of a unipotent and unidirectional hierarchy, both for renewal and repair. In skin epidermis, which is characterized by high renewal and exposure to injury, there are multiple stem cell niches, particularly in association to the hair follicles, each one generating its progeny and territory. However, upon injury, multiple cells can acquire stem cell properties and subvert territorial boundaries. Other organs like liver, lung, or pancreas exhibit rather low renewal rates under normal conditions, contrasting with the acquisition of stem cell properties including proliferation and multipotency by different cell types (Figure 5B). Indeed, tissue repair is believed to rely to a large extent on injury-induced cellular de-differentiation and plasticity. For example, in the intestine, brain, and lung, injury induces de-differentiation of non-stem cells, which reactivates normally silent embryonic and stemness transcription programs, thus acquiring the plasticity needed for tissue repair.[238–240] Injury-induced plasticity (and its progressive loss with aging) may be more relevant for aging than the plasticity of resident stem cells under normal homeostatic conditions. Stem and progenitor cells are all subject to the same hallmarks of aging as are cells without stem potential, and for this reason, we do not discuss here the abundant literature about the impact of each hallmark of aging on stem cell function. Instead, we will focus on a general strategy to counter the decline of stem cell function with aging based on the concept of "cellular reprogramming." This process is thought to act in a cell-autonomous manner on multiple cell types; however, its impact on stem and progenitor cells is considered of higher relevance because of its long-term impact.

Rejuvenation of tissue repair by reprogramming

Cellular reprogramming toward pluripotency consists in the conversion of adult somatic cells into embryonic pluripotent cells (known as induced pluripotent stem cells or iPSCs) by the concomitant action of four externally transduced transcription factors, namely, OCT4, SOX2, KLF4, and MYC (OSKM).[241] The process of reprogramming usually requires several weeks during which cells first lose their differentiated phenotype by transcriptional repression of cell identity genes and subsequently transactivate pluripotency genes.[242] Full reprogramming not only implies a change of cellular identity but also cellular rejuvenation, characterized by a number of aging features that are reset to the embryonic state, as indicated by p16 reduction,[243] extension of telomeres,[244] and resetting of the DNA methylation clock.[245] Interestingly, rejuvenation occurs in a progressive fashion starting shortly after the initiation of de-differentiation.[246] Indeed, it is possible to initiate reprogramming with OSKM, interrupt the process at an intermediate state, and allow cells to return to their original identity. This transient cellular perturbation, variously known as "partial," "transient," or "intermediate" reprogramming, is able to rejuvenate cellular markers of aging such as the DNA methylation clock, DNA damage, epigenetic patterns, and aging-associated changes in the transcriptome, both *in vitro* and *in vivo*.[63,64,70,246,247] Therefore, it can be proposed that the processes of de-differentiation and rejuvenation are coupled. Specifically, de-differentiation implies the erasure of epigenetic and transcriptional programs, and this may also erase aging-associated alternations. Upon interruption of partial reprogramming, cells re-stablish their original epigenetic and transcriptional status in a process of re-differentiation that, interestingly, does not re-stablish the erased aging-associated changes and therefore resets the epigenome and transcriptome to a younger state.

Transient reprogramming in mice confers repair capacity to old tissues so that a subsequent damage is repaired as efficiently as in young individuals. This increased repair capacity has been shown for models of tissue damage in the endocrine pancreas,[63] skeletal muscle,[63] nerve fibers,[70] eye,[70] skin,[64] heart,[65] and liver.[66] Also, tissue dysfunctions characteristic of natural aging, such as reduced visual acuity[70] and the loss of adult neurogenesis in the hippocampus and long-term memory,[67] can be partially reversed by transient reprogramming. There are a few instances in which transient reprogramming is beneficial also during the process of tissue repair (and not only prior to the injury). This is the case for traumatic brain injury[68] and skin wound healing.[69] Finally, it should be mentioned that the lifespan of progeroid mice can be extended by transient reprogramming,[63] although extension of longevity by OSKM has not yet been reported for wild-type mice.

Partial reprogramming recapitulates features of natural tissue repair (Figure 5B). In both cases, cells undergo a transient process of de-differentiation, acquisition of embryonic and progenitor features, and subsequent re-differentiation. Thus, de- and re-differentiation could explain tissue rejuvenation, in line with the observation that transient de-differentiation of myocytes, followed by their re-differentiation, induces rejuvenation of the transcriptome.[248] The natural process of tissue repair may imply some degree of cellular rejuvenation, in accord with the finding that the epigenetic methylation clock accelerates soon after tissue injury and partially reverses during tissue repair.[249] Moreover, tissue damage reportedly creates a tissue microenvironment that is highly permissive for IL-6-driven reprogramming.[250] Finally, cyclic expression of the transcription factor FOXM1 extends the longevity of progeroid mice and wild-type mice.[251] Although the detailed mechanism is still unexplored, FOXM1 is induced in the kidney upon injury and participates in triggering de-differentiation and proliferation of tubular epithelial cells during the repair process.[252] Thus, several features of natural tissue repair and artificial reprogramming may converge, perhaps allowing refinement of strategies for restoring repair capacity in aging tissues.

ALTERED INTERCELLULAR COMMUNICATION

Aging is coupled to progressive alterations in intercellular communication that increase the noise in the system and compromise homeostatic and hormetic regulation. Thus, aging involves deficiencies in neural, neuroendocrine, and hormonal signaling pathways, including the adrenergic, dopaminergic, and insulin/IGF1-based and renin-angiotensin systems, as well as sex hormones commensurate with the loss of reproductive functions.[182,253] Although the primary causes of such alterations are cell intrinsic, as this is particularly well documented for the SASP, these derangements in intercellular communication ultimately sum up to a hallmark on its own that bridges the

cell-intrinsic hallmarks to meta-cellular hallmarks including the chronification of inflammatory reactions coupled to the decline of immunosurveillance against pathogens and premalignant cells, as well as the alterations in the bidirectional communication between human genome and microbiome, which finally results in dysbiosis. A number of studies in this regard have focused on the search for blood-borne systemic factors with pro-aging or prolongevity properties, the role of diverse communication systems between cells, and the evaluation of the functional relevance of ECM disruption during aging.

Pro-aging blood-borne factors

A single transfusion of old blood induces features of aging in young mice within a few days,[72] and the simple dilution of the blood of old mice with saline buffer containing 5% albumin induces rejuvenation in multiple tissues,[71] indicating the existence of circulating factors that favor the aging process. Among the pro-aging blood-borne factors, the chemokine CCL11/eotaxin and the inflammation related protein β2-microglobulin reduce neurogenesis,[254,255] IL-6 and TGF-β impair hematopoiesis,[256] and the complement factor C1q compromises muscle repair.[257] Theoretically, the neutralization of these factors might have potent anti-aging effects. Indeed, several among the aforementioned factors are secreted in the context of SASP and may be co-responsible for the phenomenon of "contagious" aging, which also involves extracellular vesicles.[258] Thus, so-called "senomorphics" might be used to repress SASP and slow down aging.

Anti-aging blood-borne factors

Soluble factors present in the blood of young mice effectively restore renewal and repair capacity in old mice[259] (Table 1). Heterochronic parabiosis experiments followed by extensive single-cell transcriptomics have confirmed the capacity of young blood to rejuvenate multiple tissues[74] and to restore age-associated reduction in general gene expression, in particular that of mitochondrial genes involved in the electron transport chain.[75] The chemokine CCL3/MIP-1α acts as a rejuvenating factor for hematopoietic stem and progenitor cells;[74] the metalloproteinase inhibitor TIMP2 has been implicated in rejuvenating the hippocampus;[73] the anti-inflammatory interleukin IL-37 (which declines in monocytes from aged humans) improves increased endurance exercise and ameliorates whole-body metabolism in old mice;[76] the cytokine GDF11 rejuvenates some tissues, such as muscle, brain, and endocrine pancreas, although it impairs the function and repair of other tissues due to its pro-fibrotic side effects;[77] and finally, mice with transgene-enforced VEGF overexpression exhibit enhanced liver and muscle repair, improved general health and an extension in average longevity by ~40%.[78]

Long-range and short-range communication systems

The central nervous system controls multiple facets of aging affecting peripheral organs, explaining how brain-specific gene manipulations like overexpression of SIRT1, UCP1, or knockout of IKBKB and TRPV1 can enhance mouse longevity (Table 1). The precise mechanisms of these long-range activities are yet to be determined.[260] Of note, intercellular communication also involves the interaction among short-lived extracellular molecules (such as ROS, nitric oxide, nucleic acids, prostaglandins, and other lipophilic molecules), soluble factors that are released from various tissues including white adipose tissue (adipokines), brown adipose tissue (baptokines), heart (cardiokines), liver (hepatokines) and skeletal muscles (myokines, including exerkines produced in response to exercise), cell-bound ligands, and receptors on other cells (as exemplified by IL-1α that can remain cell-bound), as well as direct cell-to-cell interactions mediated by tight junctions or gap junctions. All these communication systems may be altered during aging and hence are being scrutinized for their potential pro- and anti-aging properties.[258]

Extracellular matrix

Aging causes numerous damages in the long-lived protein components of the ECM, including AGEs, carbonylation and carbamylation, elastin fragmentation, and collagen crosslinking,[261] thus leading to tissue fibrosis (fibroaging).[262] This deleterious process is in part due to the excessive release of TGF-β and other growth factors, and the nuclear translocation of TAZ and YAP transcription factors, which act as mechanotransducers and trigger the expression of pro-fibrotic genes such as transglutaminase-2, lysyl oxidase (LOX), and LOX-like enzymes.[262] ECM stiffness also affects the function of senescent cells, which in turn secrete matrix metalloproteases that amplify the damage of the ECM,[263] and proteolytically generate damage-associated molecular patterns to activate pro-senescent, pro-fibrotic, and pro-inflammatory pathways.[262] The increasing stiffness of the aging matrix may also favor WNT signaling to induce fibroblast activation and expression of pro-fibrotic genes.[264] This pathway exhibits extensive crosstalk with other pro-fibrotic pathways, such as NOTCH, RAS, TGF-β/SMAD, and hedgehog/GLI, thereby demonstrating the complexity and interconnections of mechanisms underlying the development of age-linked fibrosis.[262] Of note, mechanical change caused by matrix stiffness is sufficient to cause age-related loss of function of oligodendrocyte progenitor cells in a process mediated by the mechanoresponsive ion channel PIEZO1.[265]

Several studies have provided causal evidence for the contribution of ECM stiffness to aging and have also suggested approaches for improving healthy aging (Table 1). *In vivo* inhibition of Piezo1 using AAV vectors results in rejuvenation of the oligodendrocyte progenitors in the brain of old mice.[265] Genetic inactivation of YAP/TAZ in stromal cells causes accelerated aging, although sustaining YAP function rejuvenates old cells and prevents the emergence of aging features by controlling cGAS-STING signaling.[79] Moreover, mice engineered to produce collagenase-resistant type I collagen (Col1a1r/r) exhibit vascular cell senescence, accelerated aging, and shortened lifespan.[266] The importance of collagen for human longevity has been reinforced by the discovery of rare variants in *COL25A1*—encoding a brain-specific collagen—that may have a protective role against Alzheimer's disease.[267] Moreover, ECM prepared from young human fibroblasts induces a youthful state in aged senescent cells.[80] ECM compounds such as chondroitin sulfate and hyaluronic acid restore the age-related decline of collagen and increase lifespan in nematodes.[268] Conversely, ectopic expression of human hyaluronidase

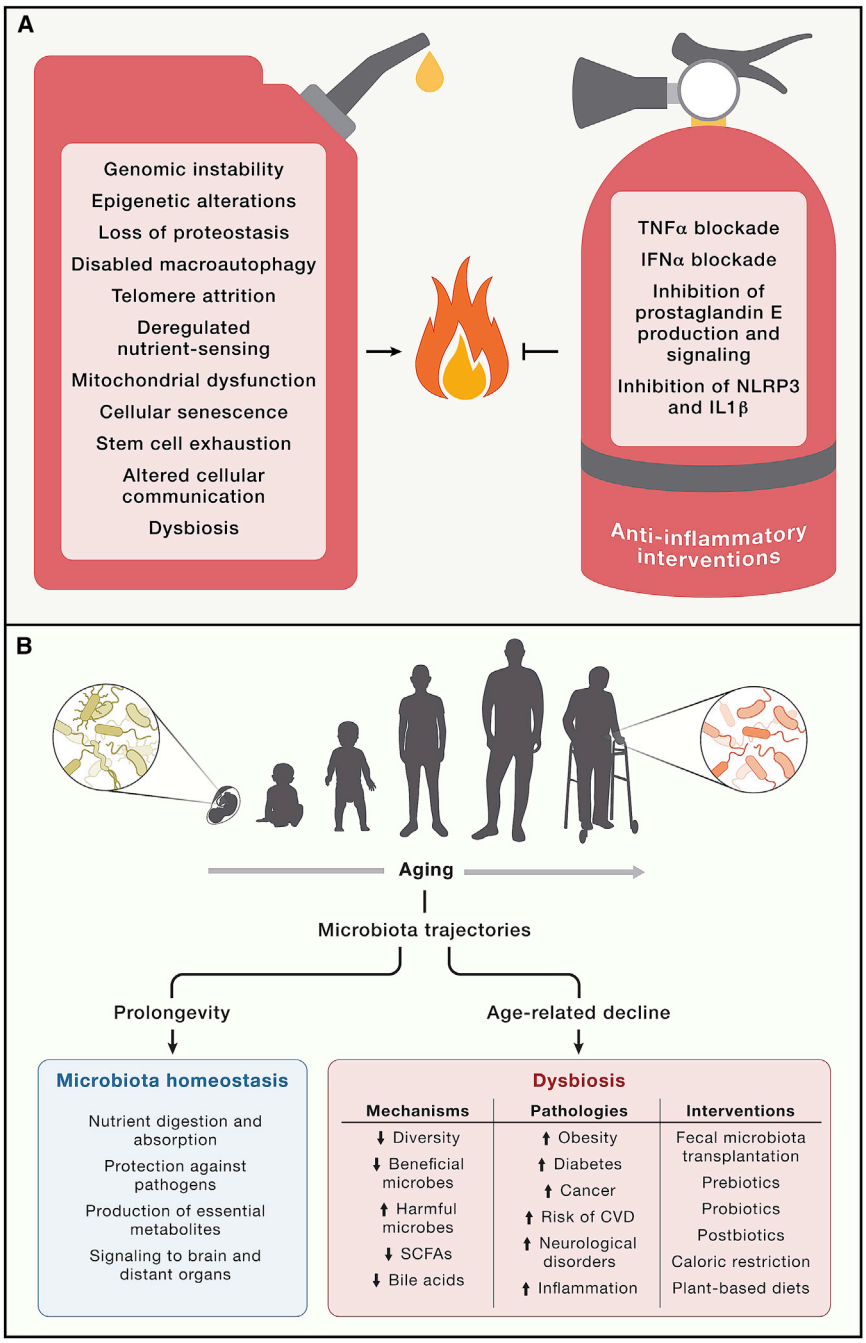

Figure 6. Derangement of supracellular functions

Altered intercellular communication bridges the cell-intrinsic hallmarks to meta-cellular hallmarks including the chronic inflammation, and the alterations in the crosstalk between human genome and microbiome, which finally result in dysbiosis.

(A) Chronic inflammation during aging occurs as a consequence of multiple derangements that stem from all the other hallmarks. Several representative examples of anti-inflammatory interventions with positive effects on healthspan and lifespan are shown in the right part of the figure.

(B) Dysbiosis contributes to multiple pathological conditions associated with aging. The human gut microbiota significantly changes during aging, finally leading to a general decrease in ecological diversity. The main features of the mechanisms underlying these microbiota changes and some examples of interventions on the gut microbiota composition which can promote healthy aging are shown in the lower part of the right panel. CVDs, cardiovascular diseases; SCFAs, short-chain fatty acids.

TMEM2 promotes resistance to ER stress and extends lifespan in *C. elegans* through changes in p38/ERK MAPK signaling.[269] In mice, deletion of chondroitin 6-sulfotransferase results in an abnormal ECM in the brain, early memory loss, and accelerated brain aging, whereas overexpression of this enzyme improved memory in old mice.[81] Retrospective analyses indicate that oral intake of glucosamine/chondroitin sulfate leads to a reduction in all-cause mortality in humans.[270] However, there is no prospective proof thus far that such a prolongevity effect would be mediated through an amelioration of the ECM.

CHRONIC INFLAMMATION

Inflammation increases during aging ("inflammaging") with systemic manifestations, as well as with pathological local phenotypes including arteriosclerosis, neuroinflammation, osteoarthritis, and intervertebral discal degeneration. Accordingly, the circulating concentrations of inflammatory cytokines and biomarkers (such as CRP) increase with aging. Elevated IL-6 levels in plasma constitute a predictive biomarker of all-cause mortality in aging human populations.[271] In association with enhanced inflammation, immune function declines, a phenomenon that can be captured by high-dimensional monitoring of myeloid and lymphoid cells in the blood from patients and from mouse tissues.[272] For example, a population of age-associated T cells—termed Taa cells—is composed of exhausted memory cells that mediate pro-inflammatory effects via granzyme K. Shifts in T cell populations entail the hyperfunction of pro-inflammatory TH1 and TH17 cells, defective immunosurveillance (with a negative impact on the elimination of virus-infected, malignant or senescent cells), loss of self-tolerance (with a consequent age-associated increase in autoimmune diseases), and reduced maintenance and repair of biological barriers, altogether favoring systemic inflammation[273] (Figure 6A).

Links between inflammation and other aging hallmarks

Inflammaging occurs as a result of multiple derangements that stem from all the other hallmarks. For example, inflammation is

triggered by the translocation of nuclear and mtDNA, into the cytosol where it stimulates pro-inflammatory DNA sensors, especially when autophagy is ineffective and hence unable to intercept ectopic DNA.[4] Genomic instability favors clonal hematopoiesis of indeterminate potential (CHIP), with the expansion of myeloid cells that often bear a pro-inflammatory phenotype, driving for instance cardiovascular aging.[274] Intriguingly, the most frequent CHIP-associated mutations affect the epigenetic modifiers DNMT3 (which methylates cytosine residues in DNA) and TET2 (which catalyzes the oxidation of methylcytosine to 5-hydroxymethylcytosine). Mechanistically, CHIP affecting TET2 enhances IL-1β and IL-6 production by myeloid cells and stimulates cardiovascular disease (CVD), which is attenuated among individuals bearing a loss-of-function mutation in the IL-6 receptor or treated with an IL-1β neutralizing antibody.[275]

Overexpression of pro-inflammatory proteins can be secondary to epigenetic dysregulation, deficient proteostasis, or disabled autophagy. Excessive trophic signals resulting in activation of the GH/IGF1/PI3K/AKT/mTORC1 axis trigger inflammation. In addition, inflammation is favored by the SASP secondary to the accrual of senescent cells, as well as by the accumulation of extracellular debris and infectious pathogens, which are not cleared due to senescence, and by exhaustion of myeloid and lymphoid cells. This latter phenomenon involves age-associated thymic involution, abrogating thymopoiesis with the consequent rarefaction of the T cell repertoire and the inability to mount efficient immune responses against novel antigens.[276] Of note, thymopoiesis is improved by CR in humans, and a CR-downregulated gene coding for platelet activation factor acetyl hydrolase A2 group VII (PLA2G7) can be knocked out in mice to combat thymic atrophy.[50] Finally, inflammaging is also exacerbated by perturbations of circadian rhythms and by intestinal barrier dysfunction.[277]

Anti-inflammatory, anti-aging interventions

Although systemic inflammation is mechanistically linked to all the aforementioned age-associated alterations, inflammation constitutes a hallmark on its own. Indeed, specific manipulations of the inflammatory and immune system can accelerate or decelerate the aging process across different organ systems. For example, a T cell-specific defect in the mitochondrial transcription factor A (TFAM) is sufficient to drive cardiovascular, cognitive, metabolic, and physical aging coupled to an increase in circulating cytokines. The TNF-α inhibitor etanercept partially reversed this phenotype.[82] Heterozygous deletion of the DNA repair protein ERCC1 in hematopoietic cells from mice is sufficient to induce immunosenescence and aging of non-lymphoid organs, as well as numerous signs of organ damage coupled to reduced lifespan. This phenotype was alleviated by the senolytic fisetin.[278] These results support the idea that aging of the immune system may drive organismal aging. Of note, adoptive transfer of TFAM-null T cells, young ERCC-deficient splenocytes, or aged wild-type splenocytes into young mice induced senescence, whereas the transfer of young immune cells into ERCC-deficient mice attenuated senescence, pointing to the capacity of immune cells to modulate organismal aging in both positive and negative terms.[82,278]

There are multiple examples of broad healthspan and lifespan-expanding effects of anti-inflammatory treatments (Figure 6A; Table 1). Thus, blockade of TNF-α prevents sarcopenia in mice and improves cognition in aging rats.[83,84] Blockade of the common type 1 interferon receptor (IFNAR1) reverses the accumulation of monocytes in the aging mouse lung.[279] Knockout of the prostaglandin E_2 receptor EP2 in myeloid cells or treatment of aged mice with pharmacological EP2 inhibitors ameliorates cognition.[85] Knockout of the inflammasome protein NLRP3 improves metabolic biomarkers, glucose tolerance, cognition, and motor performance and extends mouse longevity.[86] Pharmacological inhibitors of NLRP3 or of its downstream enzyme caspase-1 have encouraging preclinical effects on normal and accelerated aging models.[280] Most importantly, inhibition of the caspase-1 product IL-1β with canakinumab exemplifies an anti-aging treatment applicable to patients. The phase 3 clinical trial CANTOS evaluated the capacity of canakinumab to prevent recurrent CVD in patients with a history of myocardial infarction and signs of pronounced inflammation. Beyond meeting the primary endpoint of the trial, canakinumab reduced the incidence of diabetes and hypertension, as well as the incidence of, and mortality from, lung cancer.[87] Finally, although long-term use of non-steroidal anti-inflammatory agents such as aspirin may have positive effects on human health—in particular with respect to the prevention of CVD and gastrointestinal cancers—a large phase 3 clinical trial in which aspirin was administered to over 70-year-old subjects yielded negative results.[281] Hence, further studies will be necessary to explore the value of prophylactic treatments with aspirin at a younger age to combine aspirin with other medications or to replace aspirin by less toxic anti-inflammatory drugs.

DYSBIOSIS

Over recent years, the gut microbiome has emerged as a key factor in multiple physiological processes such as nutrient digestion and absorption, protection against pathogens, and production of essential metabolites including vitamins, amino acid derivatives, secondary bile acids, and short-chain fatty acids (SCFAs). The intestinal microbiota also signals to the peripheral and central nervous systems and other distant organs and strongly impacts on the overall maintenance of host health.[146] Disruption of this bacteria-host bidirectional communication results in dysbiosis and contributes to a variety of pathological conditions, such as obesity, type 2 diabetes, ulcerative colitis, neurological disorders, CVDs, and cancer.[282] The progress in this field has generated an enormous interest in exploring gut microbiota alterations in aging (Figure 6B).

Microbiota alterations in aging

The microbial community within the intestinal tract is highly variable among individuals as a consequence of host genetic variants (ethnicity), dietary factors, and lifestyle habits (culture), as well as environmental conditions (geography), which makes difficult to unveil the relationships between microbiota and pleiotropic age-associated disease manifestations. Nonetheless, some meta-analyses have revealed microbiota-disease associations that have been validated across distinct pathologies[283]

and countries.[284,285] Studies in both humans and animal models have provided valuable information on clinical, epidemiological, sociological, and molecular aspects that underlie the complex effects of an aged microbiome on human health and disease.[286] Once bacterial diversity is established during childhood, it remains relatively stable during adulthood. However, the architecture and activity of this bacterial community undergoes gradual changes during aging, finally leading to a general decrease in ecological diversity. Thus, several studies conducted on centenarian populations showed a reduction in core abundant taxa, such as *Bacteroides* and *Roseburia*, but also an increase in several genera such as *Bifidobacterium* and *Akkermansia*, which appear to have prolongevity effects.[287]

These studies have been extended by recent analysis of the gut microbiome and phenotypic data from over 9,000 individuals of three independent cohorts spanning 18–101 years of age.[288] Of note, individual gut microbiomes become increasingly more unique to each individual with age, and this uniqueness is associated with well-known microbial metabolites involved in immune regulation, inflammation, and aging. In older age, healthy participants show continued drift toward a unique microbial composition, whereas this drift is reduced or absent in individuals in worse health. The identified microbiome pattern of healthy aging is characterized by a depletion of core taxa, such as *Bacteroides*, present across most humans. Moreover, in individuals approaching extreme age, retention of high *Bacteroides* levels and a low gut microbiome uniqueness measure are significantly associated with decreased survival. However, findings in microbiota from centenarians and supercentenarians are not always concordant with those derived from elderly populations. The ELDERMET study reported an increased dominance of the core genera *Bacteroides*, *Alistipes*, and *Parabacteroides* in old individuals compared with younger controls. This study also identified age-related shifts in gut microbiota composition linked to frailty, cognition, depression, and inflammation.[289] Another study revealed age-related trajectories of the microbiota shared across populations of different ethnicities, as well as a common age-related decrease in sex-dependent differences in gut microbiota. Of note, older adults exhibit higher abundances of several health-promoting bacterial species, including *Akkermansia*.[290] These results suggest that age-related physiological changes, beyond dietary changes and lifestyle of older adults, may have profound effects on the human gut microbiota.

The heterogeneity of findings in all these studies indicates that there may be multiple gut microbiome trajectories of aging. However, there is an interesting convergence in plasma concentrations of microbiota-produced amino acid derivatives. These metabolites include indoles derived from gut bacterial degradation of tryptophan, and several fermentation products of phenylalanine/tyrosine, such as p-cresolsulfate, phenylacetylglutamine, and p-cresol glucuronide. This finding is consistent with data from the ELDERMET cohort showing that fecal concentrations of p-cresol correlate with increased frailty and may contribute to age-associated decline in this population. Conversely, plasma concentrations of certain indole metabolites correlate with improved fitness in older adults. Indole metabolites increase healthspan and lifespan in mice, at least in part, by attenuation of inflammatory responses through binding of the arylhydrocarbon receptor.[93]

Further metabolomics and functional analysis of the gut microbiome of centenarians have shown its enrichment in some particular bacteria, such as *Alistipes putredinis* and *Odoribacter splanchnicus*. Some of these bacterial species are capable of generating unique secondary bile acids, including isoallo-lithocholic, which exerts potent antimicrobial effects against gram-positive multidrug-resistant pathogens such as *Clostridioides difficile* and *Enterococcus faecium*.[291] Thus, specific bile acid metabolism may be involved in reducing the risk of pathobiont infection and contribute to intestinal homeostasis, thereby decreasing the susceptibility to age-associated chronic diseases.

Fecal microbiota transplantation and aging

Multiomics studies in pathological aging have revealed that two different mouse models of progeria exhibit intestinal dysbiosis mainly characterized by an increase in the abundance of Proteobacteria and Cyanobacteria and a decrease in levels of Verrucomicrobia. Consistent with these findings, human progeria patients with HGPS or NGPS also show intestinal dysbiosis, whereas long-lived humans exhibit a substantial reduction in Proteobacteria and a significant increase in Verrucomicrobia.[88] The causal implications of these changes were demonstrated *in vivo* by fecal microbiota transplantation (FMT). FMT from wild type to progeroid mice recipients enhanced healthspan and lifespan in both accelerated-aging models, whereas administration of the verrucomicrobium *Akkermansia muciniphila* was also sufficient to obtain such effects. Conversely, FMT from progeroid donors to wild-type recipients induced detrimental metabolic alterations. Restoration of secondary bile acids and other metabolites depleted in progeroid mice phenocopied the beneficial effects of reestablishing a healthy microbiome[88] (Table 1).

FMT also revealed the causative role of gut dysbiosis in the chronic systemic inflammation and the decline in adaptive immunity associated with aging and age-related diseases. Transfer of the gut microbiota from old mice to young germ-free mice triggered inflammatory responses characterized by enhanced $CD4^+$ T cell differentiation in spleen, upregulation of inflammatory cytokines, and increased circulation of inflammatory factors of bacterial origin.[292] FMT also provided evidence for the implication or the gut microbiota in the maintenance of brain health and immunity during aging.[90] Microbiota from young mice donors reversed aging-associated differences in hippocampal metabolites and brain immunity and ameliorated age-associated impairments in cognitive behavior when transplanted into an aged host. These works open the possibility of manipulating the gut microbiota with pre-, pro-, and post-biotics to rejuvenate the immune system and the aging brain. Heterochronic fecal transfers confirmed the causal link between age-dependent changes in microbial composition and a decline in the function of the host immune system.[92] Indeed, the defective germinal center reaction in Peyer's patches of aged mice can be rescued by FMT from younger animals without affecting germinal center reactions in peripheral lymph nodes. Finally, FMT from young donor mice improves ovarian function and fertility in aged

mice. These beneficial effects are associated with an improvement in the immune microenvironment of aged ovaries, with decreased macrophages and macrophage-derived multinucleated giant cells, reduced levels of pro-inflammatory IFNγ, and increased abundance of the anti-inflammatory cytokine IL-4.[91]

Other prolongevity interventions on gut microbiota

The probiotic *Lactobacillus plantarum* GKM3 promotes longevity and alleviates age-related cognitive impairment in the SAMP8 mouse model of accelerated aging.[89] Interventions on gut microbiota composition also restored the age-linked decline in microglial maturation and function which causes altered brain plasticity and promotes neurodegeneration. Recolonization experiments or administration of gut microbiota metabolites, such as SCFAs, prevented the age-associated decline of beneficial *Bifidobacterium*, increased *Akkermansia* abundance, and restored microglial function in middle-aged mice.[94] Moreover, caloric restriction diets induce structural changes of the gut microbiome increasing the abundance of *Lactobacillus* and other species that influence healthy aging. The gut microbiota-induced inflammaging and the consequent increase in insulin resistance can also be reversed by restoring abundance of beneficial SCFA-producing bacteria, such as *A. muciniphila*, in aged mice and macaques.[293] Similarly, a randomized, double-blind, placebo-controlled pilot study in overweight/obese insulin-resistant volunteers showed that oral administration of pasteurized *A. muciniphila* improved insulin sensitivity reduced insulinemia and plasma total cholesterol levels.[95] Collectively, these results underscore the causal links between aging and dysbiosis and suggest that interventions aimed at restoring a youthful microbiome may extend healthspan and lifespan.

INTEGRATION OF HALLMARKS

All the 12 hallmarks of aging are strongly related among each other. For example, genomic instability (including that caused by telomere shortening) crosstalks to epigenetic alterations (e.g., through the loss-of-function mutation of epigenetic modifiers such as TET2), loss of proteostasis (e.g., due to the production of mutated, misfolded proteins), disabled macroautophagy (e.g., through the capacity of autophagy to remove supernumerary centrosomes, extranuclear chromatin, and cytosolic DNA), deregulated nutrient-sensing (e.g., because SIRT6 is an NAD^+ sensor involved in DNA repair but also responding to nutrient scarcity), mitochondrial dysfunction (e.g., due to the mutation of mtDNA), cellular senescence (e.g., because DNA damage triggers senescence), altered intercellular communication (e.g., by hampering activation of communication pathways), chronic inflammation (e.g., because CHIP and leakage of DNA into the cytosol induce inflammation), and dysbiosis (e.g., because mutations in intestinal cells favors dysbiosis, whereas specific microbial proteins and metabolites act as mutagens). Similar functional relationships can be listed for most if not all hallmarks of aging, illustrating their formidable interconnectivity.

This entanglement is also visible at the level of experimental anti-aging interventions that often simultaneously target several hallmarks. Thus, SIRT activators including NAD^+ precursors attenuate genomic instability (via DNA repair), epigenetic alterations (via histone deacetylation), loss of proteostasis (via the removal of protein aggregates), disabled macroautophagy (via autophagy enhancement), deregulated nutrient-sensing (via activation of nutrient scarcity sensors), and mitochondrial dysfunction (via an increase in mitophagy-dependent quality control).[176] Spermidine complexes to DNA (hence counteracting genomic instability), affects translation (avoiding loss of proteostasis), stimulates macroautophagy, reverses lymphocyte senescence, prevents the exhaustion of muscle stem cells, maintains circadian rhythms, suppresses inflammation, stimulates cancer immunosurveillance, and is produced by intestinal bacteria.[294] Metformin has a pleiotropic mode of action including induction of autophagy, activation of the nutrient scarcity sensor AMPK, inhibition of mitochondrial respiration, alleviation of adipocyte senescence, suppression of inflammation, and favorable shifts in the gut microbiota.[210] Similarly, maintenance of eubiosis by oral supplementation of *A. muciniphila* stimulates intestinal autophagy, reduces metabolic syndrome, dampens inflammation, and enhances anticancer immune responses.[295] Indeed, a notable feature of effective anti-aging interventions, such as lowered insulin/IGF-1 signaling[296] and disruption of the TORC1 complex,[296,297] is the diversity of mechanisms by which they target different aging hallmarks in different tissues to maintain healthspan of the whole organism.

Although each of the 12 hallmarks of aging can be targeted one by one, yielding tangible benefits for healthspan and lifespan (Table 1), there is some kind of hierarchy among them (Figure 1). Thus, as we initially proposed,[1] the primary hallmarks, which reflect damages affecting the genome, telomeres, epigenome, proteome, and organelles, progressively accumulate with time and unambiguously contribute to the aging process.[298] The antagonistic hallmarks, which reflect responses to damage, play a more nuanced role in the aging process. For example, trophic signaling and anabolic reactions activated by nutrient-sensing have beneficial actions in youth but are largely pro-ageing later on. Thus, in an archetypal case of antagonistic pleiotropy, the nutrient-sensing network contributes to organ development until young adulthood but plays a detrimental role beyond this stage. Additionally, reversible and low-dose mitochondrial dysfunction can stimulate beneficial counterreactions (via mitohormesis), whereas limited and spatially confined levels of cellular senescence contribute to the suppression of oncogenesis and improve wound healing. Finally, the integrative hallmarks arise when the accumulated damage inflicted by the primary and antagonistic hallmarks cannot be compensated any more, resulting in stem cell exhaustion, intercellular communication alterations including ECM damage, chronic inflammation, and dysbiosis, which together dictate the pace of aging.

Recently, we postulated the existence of eight hallmarks of health,[146] which include organizational features of spatial compartmentalization (integrity of barriers and containment of local perturbations), maintenance of homeostasis over time (recycling and turnover, integration of circuitries, and rhythmic oscillations), and an array of adequate responses to perturbation (homeostatic resilience, hormetic regulation, and repair and regeneration). Undoubtedly, aging is linked to progressive deterioration of these eight hallmarks of health, implying a ramping

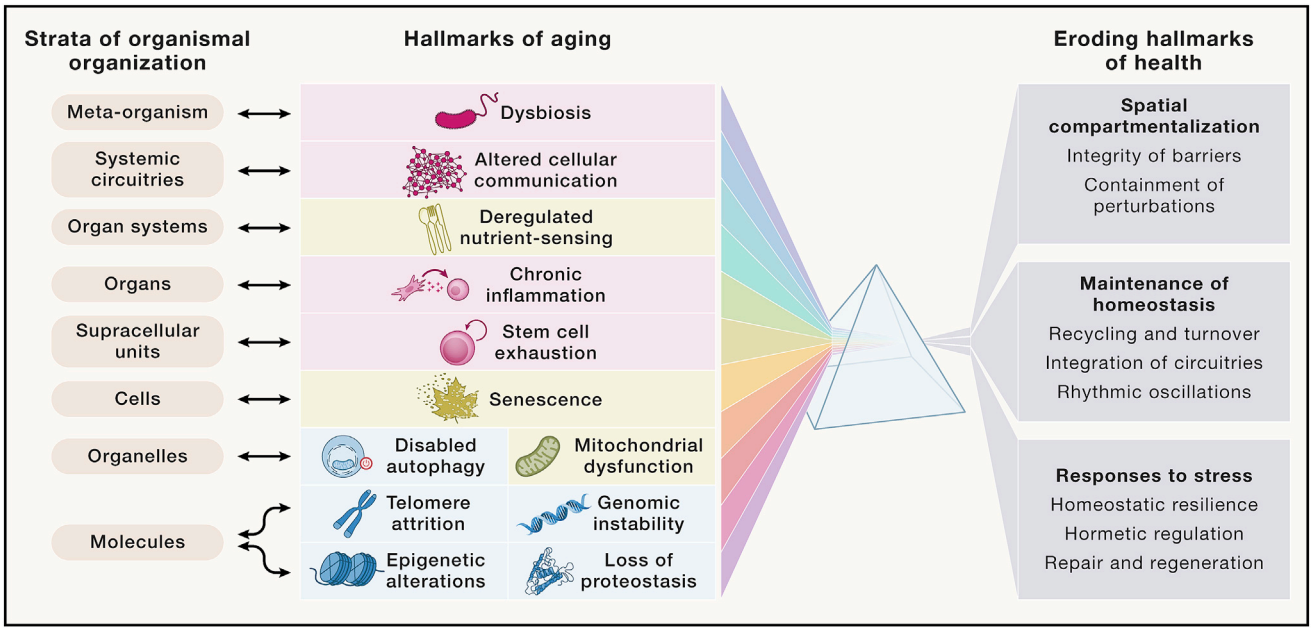

Figure 7. Integration of hallmarks

All the 12 hallmarks of aging proposed in this work are functionally related among each other. These determinants of aging are also interconnected to the eight hallmarks of health, which include organizational features of spatial compartmentalization, maintenance of homeostasis over time, and an array of responses to perturbation.[146] Finally, the hallmarks of aging are interconnected to the eight proposed strata of organismal organization[146] and create a multidimensional space of interactions that may contribute to explain some important features of the aging process.

incapacity to maintain spatial compartmentalization (with the consequent loss of integrity of internal and external barriers, as well as the incapacity to contain perturbations of such barriers in space and time), to assure long-term homeostasis (with reduced capacity of recycling and turnover, inefficient coordination among different systems via integrated circuitries, and desynchronization of ultradian, circadian, or infradian rhythms), and to adequately respond to stress by complete repair and regeneration, homeostatic resilience, and hormetic regulation (Figure 7). This decline affects all eight strata of organismal organization, across different classes of molecules (such as DNA, RNA, proteins, and metabolites), organelles (such as nuclei, mitochondria, and lysosomes), cell types (such as parenchymatous, auxiliary/stromal, and inflammatory/immune cells), supracellular units constituting the minimal functional entities of organs, entire organs within their anatomical boundaries, organ systems (such as the gastrointestinal, respiratory, and urinary tracts), systemic circuitries (with their endocrine, neurological, lymphatic, and vascular connections), as well as the meta-organism (that includes the microbiota). As a result, the 12 hallmarks of aging are interconnected to the eight hallmarks of failing health and the eight strata of organismal organization (Figure 7), creating a multidimensional space of interactions that may explain some features of the aging process.

Heterochronic parabiosis experiments, in which the vascular systems of young and old mice are connected, may illustrate best the importance of systemic regulatory factors (such as hormones and circulating cells) on the aging process. This phenomenon has been extensively characterized at the level of single-cell transcriptomics, yielding a spatiotemporal map of the capacity of the young system to rejuvenate an older one or, vice versa, the ability of pro-aging factors to precipitate the senescence of young cells.[74,75] This type of experiment demonstrates that aging relies on the integration of cell-autonomous and non-cell-autonomous mechanisms that also have been revealed in *Drosophila* (in which stimulation of autophagy in the intestine is sufficient to extend lifespan of the entire organism)[120] and mice (in which injection of a few thousands of senescent fibroblasts is sufficient to trigger invalidating osteoarthritis).[299] Hence, pro-aging and anti-aging mechanisms can be communicated among distinct cell types, perhaps explaining that "normal" aging usually affects multiple organs in a close-to-synchronous fashion, at difference with "pathological" aging in which time-dependent diseases precociously manifest in specific locations, in the form of initially isolated cardiovascular, oncological, or neurodegenerative disorders. However, the distinction between normal and pathological aging is debatable,[300] and some progeroid syndromes manifest signs of incomplete or segmental aging, as exemplified by the absence of a central nervous phenotype in HGPS.

In view of the spectacular progress of developing longevity strategies in mammalian model organisms and initial clinical trials (Table 1), it will be important to develop rational strategies for intervening into human aging. The question arises to which extent strategies for extending human healthspan should be based on the avoidance of age-accelerating environmental factors (such as pollution, stress, inadequate physical activity, and unhealthy diets, often unavoidable in a context of poverty, precariousness, and wartime), the adoption of health-promoting lifestyle factors (such as diet, exercise, regular sleeping patterns,

and social activities), the administration of relatively non-specific, pleiotropic drugs (exemplified by NAD^+ precursors, metformin, spermidine, or MTORC1 inhibitors), or more specific medical interventions. Such specific treatments may involve pharmacological agents—with the prospective of a broad implementation, genetic or cell-based therapies—with rather complex logistics and elevated costs, or bioengineering methods for surgical tissue replacement, which most likely will mainly remain in the realm of experimentation. Given the multiplicity of hallmarks offering therapeutic strategies for decelerating, halting, or reversing aging, it will be interesting to evaluate combination regimens with the scope of maximizing benefits and minimizing side effects. The question remains open whether such healthspan and lifespan extending prophylactic treatments will profit from personalization based on individual patient characteristics determined by the genetic, epigenetic, metabolomic, or phenotypic assessments of aging clocks.

Aging is not yet a recognized target for drug development or for treatment. For this reason, the first clinical trials evaluating anti-aging interventions must deal with the prevention or mitigation of age-associated pathologies rather than aging itself. Although such trials have been designed to target high-risk populations (such as patients with myocardial infarction and laboratory signs of inflammation in the CANTOS trial or patients with frailty or cardiovascular events to be enrolled in future metformin-related trials) and to measure the manifestation of a second cardiovascular event or aggravation of frailty, there is a risk that they are programmed too late, which is of significant concern. Indeed, at this point, academic geroscience may raise or fall as the function of the outcome of the first randomized, double-blinded phase 3 trials. The new directions of the hallmarks of aging may provide an improved framework for the development of effective interventions aimed at the extension of healthy longevity.

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DECLARATION OF INTERESTS

M.A.B. is founder and shareholder of Life Length, SL, which commercializes telomere length measurements for biomedical use. M.S. is shareholder and advisor of Rejuveron Senescence Therapeutics, AG, and Altos Labs, Inc.; and shareholder of Senolytic Therapeutics, Inc., and Life Biosciences, Inc. G.K. has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Sotio, Tollys, Vascage, and Vasculox/Tioma; consulting for Reithera and is on the Board of Directors of the Bristol Myers Squibb Foundation France. G.K. is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics, and Therafast Bio. G.K. is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis, and metabolic disorders and has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Tollys, and Vascage; has been consulting for Reithera; is on the Board of Directors of the Bristol Myers Squibb Foundation France, and is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics, and Therafast Bio. G.K.'s wife, Laurence Zitvogel has held research contracts with 9 Meters Biopharma, Daiichi Sankyo, Pilege, was on the on the Board of Directors of Transgene, is a co-founder of everImmune, and holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota. G.K.'s brother, Romano Kroemer, was an employee of Sanofi and has consulted for Boehringer-Ingelheim. None of the funders had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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