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Combinatorial interventions in aging

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Abstract

Insight on the underlying mechanisms of aging will advance our ability to extend healthspan, treat age-related pathology, and improve quality of life. Multiple genetic and pharmacological manipulations extend longevity in different species, yet monotherapy may be relatively inefficient and we have limited data on the effect of combined intervention. Here we summarize interactions between age-related pathways and discuss strategies to simultaneously retard these in different organisms. In some cases, combined manipulations additively increase their impact on common hallmarks of aging and lifespan, suggesting they quantitatively participate within the same pathway. In other cases, interactions affect different hallmarks, suggesting their joint manipulation may independently maximize their effects on lifespan and healthy aging. While most interaction studies have been conducted with invertebrates and show varying levels of translatability, the conservation of pro-longevity pathways offer an opportunity to identify “druggable” targets relevant to multiple human age-associated pathologies.

BRIEF SUMMARY

This review provides an overview of combinatorial approaches used to extend lifespan across species. The authors identify frameworks that can be used for analyzing interactions between mechanisms of aging with the goal of finding translatable interventions.

Keywords

aging; longevity; healthspan; combined interventions; systems biology; complexity

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Contributions

A.A.P., E.F., and M.T. wrote the manuscript and prepared the figures.

Competing interests

The authors declare that they have no competing interests.

Editor summary:

Parthitko et al discuss combinatorial approaches targeting underlying mechanisms of aging across species and describe frameworks to analyze these interactions and their cross-species translational potential.

I. A framework for interaction analysis in aging research

There are two goals in aging research: deciphering the mechanisms of aging, and discovering interventions that delay its manifestations. Although many single genetic and pharmacological manipulations are known to slow aging [1,2], it is unlikely that monotherapy will be effective because aging is an outcome of pleiotropic processes. Instead, combined interventions will interact and likely be more effective than any alone. Interaction analysis measures the phenotypic effects of two (or more) simultaneous aging interventions. The effects are measured as manifestations of aging, including the various hallmarks, inflammaging, specific age-associated diseases, age-acquired frailty, and age-dependent mortality. The manipulations include genetic alterations, pharmacology, environmental conditions such as diet and temperature, and physiological/surgical procedures. A substantial body of interaction analyses has developed over recent years, in which each study focuses on one biological specific. Here, we begin to assemble these data in a common framework.

Types of interventions

Perhaps the oldest intervention in aging research involves diet, originally applied to rodents through caloric and protein restriction. More recent studies also include macro-nutrient ratio, specific amino acid content, control of mealtime, or administration of enzymes that degrade specific nutrients [3]. These interventions are now applied to many animal models and to humans. Diet interventions often are combined with genetic manipulations.

Among these combinations, genetic mutations of the insulin/IGF pathway provide a foundation for manipulative studies of aging in *C. elegans* and *Drosophila*. Current technologies have expanded possibilities for genetic interventions through targeted single nucleotide substitution of endogenous genes, tissue and temporal control of gene products, targeted gene ablation, RNA depletion, and more. The genetic approach is central to discovering the basic mechanisms of aging where a key feature of the approach is to study the interaction between two genetic manipulations or between a gene and an extrinsic intervention such as with diet or drugs.

Pharmacological interventions [4] are emerging as a powerful analytical approach, which is greatly enhanced when the work establishes a mechanistic basis. Well-studied examples, each with caveats, include rapamycin, spermidine, vitamin E, metformin, NAD^+ metabolites and curcumin [4]. Their hypothesized mechanisms of action span the range of molecular discovery in aging research. In each case, we can understand the mechanism by which such drugs slow aging by using genetic manipulations to determine what molecules and pathways are required for them to extend lifespan.

Topography of interactions

Interaction analysis can be improved when we consider how manifestations of aging are organized. These topographies identify the relationship between experimental interventions and their phenotypic effects, and ways to interpret mechanism.

One approach builds a hierarchy for aging from the perspective of high-throughput-omics [5] (Figure 1a). In this framework, tiers reflect the flow of biological information from genome

to transcriptome, to proteome, to metabolome and ultimately to the aging phenome - the set of all cellular, tissue, organ and organismal age-associated phenotypes. Within each tier, nodes describe signaling and functional networks. In this approach, interaction analysis manipulates multiple features either within the same level or among two levels. We measure the phenome as the outcome. 'Same level' interventions may involve the transcriptome tier where we overexpress pro-longevity transcription factors FOXO and NRF2, or the metabolome tier where we feed animals various NAD metabolites. Interventions across tiers may include when long-lived *eat-2* mutants (genome level) are supplemented with an H_2S donor FW1256 (metabolome level). One challenge for this topography is the way one level can impact many nodes at the next level. For example, when transcription factors impact the expression of many proteins, it becomes difficult to assign causal direction within and between -omics levels. Hierarchical structures of aging need to also include feedback, which is not readily represented in the simple -omics topography. For example, the universal methyl donor, S-adenosylmethionine (metabolome tier) provides methyl groups to molecules belonging to all other tiers, while each of these layers subsequently feedback upon the methionine cycle and impact SAM [6]. Similarly, glucose can cause non-enzymatic modification detected at the genome and the proteome, while events at these tiers feedback to the metabolome.

A second approach builds hierarchy based on "manifestations of aging", which includes the so-called Hallmarks of Aging [7,8] (Figure 1b). In this view, aging originates from the nonenzymatic interactions between transient metabolites such as glucose, reactive oxygen species, and carbonyls, with slow-turnover, information-dense macromolecules of metabolic control such as DNA, proteins and ribozymes. These chemical processes yield molecular manifestations such as protein damage and misfolding, DNA damage and mutation, and mitochondrial damage. Interactions between these molecular manifestations propagate to produce Hallmarks of Aging (stem cell exhaustion, epigenetic alterations, loss of proteostasis, etc.), which subsequently reverberate to physiological manifestations (sarcopenia, cancer, inflammation) and then to emergent properties such as reserve depletion, functional decline, and accelerated mortality rate. Multiple interventions can be made within and between these levels. We measure how their interaction affects manifestations at similar or higher levels. For example, mitochondrial fusion (mitochondrial dysfunction hallmark) may be altered with age by simultaneously manipulating protein misfolding and nuclear DNA damage (molecular manifestations) [9]. Manipulating protein damage and clearing senescent cells (features of molecular and hallmark manifestations) may alter sarcopenia (physiological manifestation) [10,11].

A key feature of this aging-manifestation topography is how different levels interact and involve feedback [8]. Responses at higher levels intrinsically affect those at lower levels such that relationships across levels cannot be inferred only from analyzing interactions at one tier. For example, altered intercellular communication (Hallmark of Aging) can produce compensatory effects upon both mitochondrial and DNA damage responses, which themselves interact to affect various Hallmarks. Such feedback provides avenues for homeostasis and may also explain why transient stresses such as a burst of ROS produces hormesis. The interconnected structure produces a systems biology that is unique for each species and may determine the propensity for perceived Hallmarks framework, physiological

manifestations, or nominal features such as the mortality rate doubling time characteristic of each species.

Relative to these frameworks, interaction analysis asks: can the simultaneous use of two interventions improve healthy aging more than either intervention alone? What does the impact of combined interventions reveal about the underlying mechanisms of their action? The first question is often addressed empirically: combine two interventions and see if the outcome exceeds the benefit of each individual case. The second question provides a way to predict productive combinations and builds mechanistic insight that will drive future discovery. Table 1 surveys a range of published intervention interactions including manipulations of nutrient sensing, oxygen sensing, mitochondria, redox regulation, lipid metabolism, reproduction, and microbiota.

Interactions among age-interventions are studied through epistasis analysis and gene-by-environment analysis. Epistasis analysis is a genetic approach in which the combined effect of two loci are compared to their independent effects [12]. It is deceptively challenging to design and interpret these approaches. As Gems et al. highlighted, designs are compromised when 1) the interventions do not fully alter the targeted mechanism; 2) the interventions affect aging through multiple mechanisms; 3) interventions have different age-dependent effects on the age-trait; 4) suppressor mutants are deleterious, and 5) reaction norms are based on too few environmental levels. To address these issues, Gems et al. recommend we use null alleles rather than hypomorphs (often not an option), use more than one allele or transgene, and use alleles with few pleiotropic effects. For environmental interventions, Gems et al. suggested testing multiple treatment levels to identify the dose that produces the maximum lifespan. All these designs require appropriate statistical analysis and interpretation [13].

A further question asks whether the interaction observed in a model organism is universal for all organisms. Many examples exist where a manipulation is beneficial in one animal yet causes a devastating disease in humans. For instance, ribosomal downregulation may extend lifespan in worms but leads to ribosomopathy in humans [14]; loss of OPA-1 (eat-3 in worms) leads to lifespan extension in worms but optic atrophy in humans [15]; SOD-1 loss, which increases lifespan in worms, leads to ALS in humans [16]. This problem is compounded in interaction analysis where one component negates the positive effects of others.

In the following we sample the space of published interaction analyses to illustrate these themes and to provide a foundation for further analyses of the complexity.

II. Interactions within Hallmarks

The Hallmarks of Aging are proposed categories of universal cellular and physiological aging [7,17]. This expanding system identifies underlying molecular manifestations that impact complex phenotypes including morbidity and mortality. We illustrate Hallmark interactions by focusing on nutrient sensing and mitochondrial function, with additional cases compiled in Supplementary Table 1.

Interactions in Nutrient Sensing Hallmarks

Insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) and the target of rapamycin (TOR) (Figure 3) are conserved nutrient-sensing pathways shown to regulate lifespan [18,19]. In *C. elegans*, double mutants of hypomorphic mutant allele for insulin-like growth factor 1 receptor (*daf-2(e1370)*) and a deletion mutation of the TOR-regulated S6K (*rsks-1(ok1255))* synergistically increase lifespan compared to single mutants [20]; their joint effect is greater than the sum of their individual effects. Analysis with a third genetic component revealed this two-factor interaction also required DAF-16, AMPK, HSF-1, and germline signaling. These gene interactions regulate lifespan via CYC-2 translation in the germline, which nonautonomously upregulates UPRmt and AMPK in the intestine to extend lifespan [21]. Interactions between multiple components in nutrient sensing pathways are represented in Figure 3, and are detailed in Table 1.

Translation is also studied relative to nutrient sensing. Downregulated ribosomal subunits and elongation factors (*RPS-15, RPS-22, S6K, eIF2β, or eIF4G)* extend the lifespan of long-lived *C. elegans eat-2* mutants, which have limited food intake by reducing pharyngeal pumping. Yet, downregulation of *TOR/let-363* alone extends survival but does not increase *eat-2* survival [22]. Thus, different elements of translational control appear to be either dependent (*let-363*) or independent (elongation factors) of food intake (*eat-2*).

Direct manipulation of diet reveals how nutrients interact – or not – with regulatory mechanisms. *C. elegans daf-2(e1370*) long-lived mutants have been evaluated on five methods of diet manipulation [23]. In each case, DR extended longevity independent of *daf-2*. Further studies manipulated the IIS transcription factor FOXO, encoded by *daf-16* in *C. elegans*. Genetic interaction analysis has broadly established that *daf-16* is required for the increased longevity of *daf-2* mutants [24], although the interpretations do not account for the inherently shortened lifespan of the *daf-16* mutant. A similar negative outcome was seen in *Drosophila*, where the longevity of *dfoxo* mutants was evaluated across a range of diet concentrations [25,26].

Ames dwarf mice can live 50% longer than their normal siblings. Ames have a defect in pituitary development (*Prop1*) that decreases circulating growth hormone (GH), prolactin (PRL), and thyrotropin (TSH) [27]. The longevity of Ames mice appears independent of DR because restriction additively increases their longevity [28]. DR, however, does not increase lifespan in long-lived growth hormone (GH) receptor knockout mice (GHRKO) [29]. As in Drosophila, the effect of DR on mouse lifespan can be dissected from insulin signaling. Mice lacking adipose mTORC2 are insulin resistant, yet DR increases their lifespan as much as it does in wild-type mice [30]. The impact of DR on lifespan also appears to be independent of Sirtuin3 (SIRT3), contrary to some expectations. Sirt3 knockout mice have wild-type lifespan, which can be increased by DR [31]. Paradoxically, these interaction data collected across animal models provide little evidence to suggest that diet restriction modulates aging through insulin/IGF signaling.

Many studies evaluate whether drugs slow aging by inducing mechanisms of nutrient sensing [32,33]. The TOR inhibitor rapamycin extends lifespan in yeast, worms, *Drosophila* and mice [34,35]. However, rapamycin appears to not mediate longevity through IIS because

the survival of *daf-2* mutants treated with rapamycin is approximately additive to the impact of each intervention alone [20]. Similarly, rapamycin extends the lifespan of long-lived *Drosophila* insulin receptor substrate *chico* heterozygote mutants [36]. Although it is unknown whether rapamycin and insulin/IGF interact in mouse aging, these interventions have striking differences upon fat mass, insulin sensitivity [37], liver transcriptome and metabolome [38], and white adipose tissue [39]. Once again and contrary to expectation, there is surprisingly scant evidence to support whether and how DR, IIS and TOR interact to modulate aging.

Interactions within the Mitochondrial Hallmark

Mitochondrial decline is a Hallmark of Aging associated with mtDNA mutations, elevated ROS and decreased oxidative phosphorylation (OXPHOS) [40]. Knockdown of individual mitochondrial ETC and ATP synthase subunits extend *C. elegans* lifespan [41–43], but joint manipulations of these components have complex outcomes (Figure 2). Worm lifespan is increased by mutation of ISP-1 (*isp-1(qm150)*), a catalytic subunit of mitochondrial complex III [41]. Similarly, lifespan is extended by mutation of NUO-6 (*nuo-6(qm200)*), a subunit of mitochondrial complex I (NDUFB4/B15) [44]. The lifespan of *nuo-6, isp-1* double mutants was not different from that of single mutants, suggesting these subunits act within the same longevity pathway. However, survival induced by RNAi of either *nuo-6* or *isp-1* was additive to lifespan of *isp-1*(qm150, and *nuo-6(qm200)* mutants, suggesting these subunits act independently [44]. The discrepancy among these inferences may arise because RNAi adds a temporal feature to the interaction, or because the partial penetrance afforded by RNAi uncovers unique, alternative aging-regulatory networks. This interaction demonstrates the importance of temporal regulation as well as the choice of genetic tools in order to detect an interaction between different components.

Kayser et al. [45] demonstrated an interesting interaction between *gas-1* and *T26A5.3,* orthologs of mitochondrial membrane respiratory chain NADH dehydrogenase (complex I) subunits. *gas-1* and *T26A5.3* mutant worms are individually short-lived, whereas *T26A5.3*; *gas-1(fc21)* double mutant worms are extremely long-lived, representing an interesting class of interactions in which the combination of two short-lived mutants results in an extended lifespan.

Fission and fusion dynamics control mitochondrial homeostasis [46]. Mitochondrial fusion can be inhibited when *eat-3* (worm orthologue of mammalian OPA-1) or *fzo-1* (worm orthologue of human mitofusins MFN1 and MFN2) are depleted. Lifespan is synergistically increases when either of these interventions is combined with RNAi to inhibit mitochondrial translation (RNAi against *mrp-5*, mitochondrial ribosomal protein 5) [47]. These interactions localize HLH-30 to the nucleus where the transcription factor induces lysosome biogenesis and autophagy. In this example, targeting different mitochondrial processes leads to a combined effect on lifespan.

Proteins involved in post-transcriptional modification of mitochondrial tRNAs also interact to control longevity [48]. *C. elegans* MTCU-2 is the orthologue of human MTO1, and MTTU-1 is the orthologue of TRMU. These proteins respectively taurino-methylate and thiolate mt-tRNALys, mt-tRNAGlu, and mt-tRNAGln [49]. Mutations of *mttu-1* or *mtcu-2* mildly reduce mitochondrial respiration. While *mttu-1* mutants moderately increase lifespan,

lifespan was increased more than 50% by the *mtcu-2;mttu-1* double mutant. Interestingly, knockdown of *daf-2* or *rict-1* (a component of the target of rapamycin complex 2 (TORC2)) further extended the lifespan of the *mtcu-2;mttu-1* double mutant. Impaired mitochondrial can further interact with IIS or TORC2 to modulate lifespan [48].

III. Interactions between different mechanisms/Hallmarks of aging

In the view of Geroscience, common mechanisms drive the progression of multiple Hallmarks of Aging [7,17]. This hypothesis can be explored by interventions that affect the interaction of different hallmarks/mechanisms of aging (Figure 3). We illustrate this process through two combinations: Nutrient sensing with Reproduction, and Nutrient sensing with Redox. Other combinations are highlighted in Figure 4 and Supplementary Table 1.

Nutrient sensing with Reproduction

Many studies show that lifespan is synergistically extended by the interaction of insulin and IGF-1 signaling (IIS) with reproduction. Lifespan is extended nearly four-fold in *C. elegans* when depletion of *daf-2* is combined with germline knockout [50,51]. This synergy suggests the longevity mechanisms underlying nutrient sensing and reproduction interact to amplify higher order manifestations of *C. elegans* aging. In contrast, *Drosophila* homozygous for null mutants of insulin receptor substrate homolog *chico* are sterile and increase median lifespan by about 50%, yet heterozygous *chico* females are equally as long-lived but are abundantly fertile [52,53]. As well, inhibition of reproduction via the *ovoD* mutation [54] increases lifespan by ~14% [55] but flies heterozygous for both *ovoD* and *chico* only increase lifespan by 36%. In *Drosophila* it seems that nutrient sensing and reproduction act through common mechanisms to modulate lifespan. Results of interaction studies that are inconsistent between species may still illuminate some mechanisms, providing researchers with useful information about interactions in aging; however, these combinations may also have limited translatability across species.

Steroid hormones are key regulators of reproduction and aging. DAF-12 is the *C. elegans* ortholog of vertebrate vitamin D and liver X receptors. DAF-12 regulates developmental commitment to reproductive growth versus dauer, while in adults it mediates how signals from the gonad impact longevity [56]. Although *daf-12* mutation slightly shortens adult lifespan, lifespan is doubled when mutants of daf-12 are combined with a mutation of the insulin receptor [57]. This synergy was completely suppressed by mutation of the foxo transcription factor *daf-16*, indicating that *daf-16* acts downstream of both insulin and reproductive-steroid signaling.

Steroid sulfotransferases and sulfatases modulate sulfated hormones. Sulfatation impairs the ability of hormones to activate their receptors. In *C. elegans*, inhibition of steroid sulfatase by *sul-2* mutation or pharmacological inhibition (using the specific STS inhibitor STX64) increased lifespan and ameliorated protein aggregation [58]. These effects required the *daf-16*, *daf-12*, *kri-1*, *tcer-1*, and *daf-36* factors, which function to extend lifespan through ablation of the germline. As well, *sul-2* mutations further extended the lifespan of *daf-2* mutant worms, and increased the lifespan of long-lived *daf-10(m79)* mutant worms, which are defective in sensory cilia formation [58].

Together these examples illustrate in *C. elegans* that reproduction and nutrient sensing have synergistic effects on lifespan.

Nutrient sensing with Redox regulation

Lifespan across various species can be extended by numerous antioxidant interventions based on genetics and pharmacology [59]. A variety of natural compounds possess antioxidant properties and delay aging in model organisms, but these likewise affect multiple targets such that it is difficult to ascribe a specific mechanism. The topography of interactions is complicated by their pleiotropy of downstream targets and their potential cross-interactions.

One systematic example involves *Brachionus manjavacas* (Rotifera), which has a two-week long lifespan that otherwise displays typical patterns of animal aging [60]. Among 20 single antioxidants tested, none alone extended lifespan. However, among 60 two-way combinations, seven produced a significant effect on rotifer lifespan: trolox and beta-carotene (10% lifespan increase), trolox and L-carnosine (13% lifespan increase); trolox and N-acetyl cysteine (16% lifespan increase); trolox and EUK-8 (14% lifespan increase); N-acetyl cysteine and quercetin (14.5% lifespan increase); EUK-8 and L-carnosine (10% lifespan increase); and EUK-8 and indole-3-propionic acid (14% lifespan increase). Interestingly, none of the 20 three- and four-way antioxidant combinations resulted in significant rotifer lifespan extension. This analysis represents one of the rare systematic studies evaluating all possible interactions between most available antioxidants. The potential topography and feedback among these interactions illustrates the complexity of understanding whether or how the added lifespan was caused specifically by antioxidant activity.

Antioxidant and nutrient sensing interventions have also been combined. Pyrroloquinoline quinone (PQQ) is a polyphenolic antioxidant that enhances resistance to oxidative stress and extends *C. elegans* lifespan [61,62]. PQQ further extends lifespan of long-lived IIS pathway mutants (*daf-2(e1370)* and *age-1(hx546)*, as well as that of *eat-2* mutants), suggesting the mechanism for PQQ longevity differs from that of nutrient sensing. Another natural antioxidant, the root extract of Rhodiola rosea (RS), possesses antioxidant properties and has been shown to protect flies and human cells against oxidative stress, and to extend lifespan in yeast, worms, and flies [63–67]. As with PQQ, RS extended lifespan of *Drosophila* with reduced nutrient sensing (wt/*chico*^{1}) [67].

Superoxide dismutases (SODs) catalyze the conversion of superoxide into oxygen and hydrogen peroxide. In contrast to yeast, flies, and mice, where deletion of either cytoplasmic or mitochondrial SOD decreases lifespan; eliminating any of the five individual *sod* genes present in worms does not affect lifespan despite their increased sensitivity to oxidative stress. Instead, deletion of *sod-2* extends lifespan even when *sod-2* is deleted with other *sod* genes in double or triple *sod* mutants [68]. Mutants of *sod2* differentially interact with other Hallmarks: it is synergistic with the long-lived *clk-1* mutant (Mitochondria), but only slightly increases the lifespans of long-lived *eat-2* (Nutrient Sensing) and *glp-1* (Reproduction) mutants. The observed synergy with *clk* suggests there are mechanistic interactions between redox signaling and mitochondrial function [68].

IV. High order interactions

Interaction analysis can simultaneously involve many targets (Table 2 and Table 3). Yen et al. manipulated four interventions known to extend *C. elegans* lifespan: low temperature, DR, reduced IlS, and mutations in mitochondrial genes [69]. Double and triple interactions produced additive effects on lifespan, while DR and cold temperature were synergistic, implying that these two interventions extend lifespan via independent mechanisms [69]. The strongest increase in the extension of lifespan was observed in the combined *clk1*/low temperature/DR or *clk-1*/*daf-2*/low temperature/DR interventions.

Aging interactions can also be probed using genes from multiple species at the same time [70]. *C. elegans* lifespan is extended by overexpressing *hsf-1* (heat shock transcription factor), *aakg-2* (gamma subunit of AMP-activated protein kinase), *sod-1* (cytosolic superoxide dismutase), and Imp-2 (chaperone-mediated autophagy). Introduced genes from zebrafish likewise extended lifespan, including *ucp2* (mitochondrial uncoupling) and *lyz* (anti-bacterial enzyme). Combining *aakg-2*(*sta2*) and *ucp2* extended lifespan ~80%, while *hsf-1* and *lyz* extended lifespan ~60%. Combinations of three genes extended lifespan about 100% (*aakg-2*, *ucp2*, *lyz* or *aakg-2*, *hsf-1*, *lyz)*. Combining all four genes together impressively extended lifespan ~135%, and these worms were more resistant to paraquat than those expressing two or three genes together [70]. Downregulation of HOX co-factor *unc-62* (Homothorax) (quintuply-modified *C. elegans* strain) further extended lifespan by 15% relative to the quadruple strain and by 160% relative to the control strain [71]. Downregulation of *unc-62* exerted several beneficial effects on lifespan. It prevented the accumulation of yolk proteins in old worms, the age-dependent overall decline of transcription in the intestine, and the age-dependent upregulated expression of neuronal genes in the intestine [71].

While dietary restriction has surprisingly weak interactions in two-way analyses, it produces connections when studied with higher order approaches. Three regulatory modules were identified in *C. elegans* undergoing calorie restriction or intermittent fasting*: rheb-1_let-363/tor, aak-2_tax-6_xbp-1,* and *daf-16_glp-1* [72]. Downregulating all three modules simultaneously additively extended lifespan (175%) and the lifespan of these worms remained insensitive to DR [72].

Higher order combinations can also be studied in mammals. Davidsohn et al. queried fibroblast growth factor 21 (FGF21), αKlotho, and transforming growth factor-β1 (TGFβ1) [73], which are secreted factors with beneficial roles in aging [74–76]. Each was tested alone and together through adeno-associated virus (AAV)-mediated gene therapy for their impact on age-related diseases: obesity, type II diabetes, heart failure, and renal failure. FGF21 alone reversed obesity and diabetic phenotypes. The combination of AAV:sTGFβR2 combined with FGF21 prevented renal medullary atrophy (unilateral ureteral obstruction was used as a model of progressive renal fibrosis, which is a feature of many forms of progressive renal diseases). Ascending aortic constriction was used to simulate heart failure, mimicking age-related hypertrophy caused by systemic hypertension. TGFβR2 with either FGF21 or αKlotho moderated heart failure. Overall, TGFβR2 with FGF21 successfully treated all four age-related diseases at once [73].

V. Pharmacological interactions

Beyond how interacting mechanisms coordinate through the Hallmarks of Aging, pharmacological interaction analysis empirically addresses how simultaneous interventions maximize successful aging (Table 4). One approach uses drugs already recognized for the effects on conditions with known age-associated progression, such as diabetes or cardiovascular disease. A broader strategy uses unbiased or targeted pharmacological screens based on model organism lifespan. In both cases, drug testing adds the ability to explore treatments limited to adults, or even at post-reproductive stages. On the other hand, compounds often target multiple pathways, and here it will be important to evaluate the combined drug treatments with specific genetic mutants in a higher order analysis.

Dasatinib and quercetin

Based on a wealth of single intervention treatments, eliminating senescent cells extends mouse healthspan [77]. The combination of dasatinib (inhibitor of multiple tyrosine kinases) and quercetin (a natural flavonol), both approved for use in humans, was more effective in eliminating senescent cells than either of these drugs alone [78]. Likewise, treating a Ercc1$^{-/\Delta}$ mouse model of accelerated aging with dasatinib and quercetin weekly significantly reduced senescence markers and frailty, and extended healthspan [78]. Biweekly administration of dasatinib and quercetin to wild-type mice starting at 24–27 months increased posttreatment lifespan by 36%, lowered mortality hazard, and improved healthspan [79]. Currently, clinical trials in humans are testing how various age-related diseases respond to dasatinib combined with quercetin (ClinicalTrials.gov).

Rapamycin and metformin

Metformin is a widely prescribed FDA-approved oral antidiabetic drug that may target several molecular mechanisms associated with aging [80]. To date there are mixed results on whether metformin can slow aging based on rodent models. It modestly extends the lifespan of tumor-prone HER2/neu mice by 8% [81], and of male C57BL/6 mice when begun at middle age [82]. However, in the NIA Interventions Testing Program, treating mice with 0.1% metformin in the diet alone did not significantly extend lifespan [83]. Yet, because metformin has protective age-associated effects in diabetics, trials are envisioned to measure its potential to delay normal human aging [84]. In this context, dual interventions may be promising. Notably, metformin combined with rapamycin robustly extended mouse lifespan compared to an independently tested cohort treated with rapamycin alone [83]. However, it is unclear whether the lifespan with rapamycin and metformin would be extended compared to the single treatments in a properly controlled experiment.

Rapamycin and acarbose

Acarbose is an FDA-approved anti-diabetic drug. In UM-HET3 mice it increased male longevity, decreased liver degeneration in males and females, decreased lung tumors in males, and improved rotarod performance in aged females [85]. The combination of rapamycin and acarbose administered to male mice starting at 9 months of age resulted in a significant increase in lifespan across three separate study sites, and this increase was higher than historical data for treatment with rapamycin alone [86]. A further combination

of rapamycin, acarbose, and phenylbutyrate improved age-related phenotypes including body composition, cognition, strength, endurance, and tissue resilience [87]. As expected, rapamycin can have negative side effects including glucose intolerance and insulin resistance [88,89]. The antidiabetic drugs metformin and acarbose may alleviate these detrimental effects [90], and thereby enhance how rapamycin impacts lifespan.

Simvastatin and ramipril

Simvastatin and ramipril are routinely used to treat cardiovascular disease (CVD) [91]. Simvastatin reduces the biosynthesis of the isoprenoids used for cholesterol biosynthesis, while ramipril reduces the biosynthesis of angiotensin and the activity of the angiotensin receptors. Long-lived, B6C3F1 mice fed simvastatin along with ramipril increased mouse lifespan by 9%, while simvastatin or ramipril alone were ineffective. The combination also promoted weight loss despite eating the same number of calories as controls [91].

Valproic acid and trimethadione

Several anticonvulsants (ethosuximide, trimethadione, and 3,3-diethyl-2-pyrrolidinone/DEABL) extend worm healthspan and lifespan [92]. Longevity conferred by ethosuximide and trimethadione appears to be independent of DR. Similarly, trimethadione additively extended lifespans of long-lived worms carrying loss of-function mutations of genes important for the function of sensory neurons (*osm-3* and *tax-4*), for neurotransmission (*unc-31*, *unc-64*, and *aex-3*), as well as *daf-2* (insulin/IGF-1 receptor) mutant worms [92]. The antiepileptic drug Valproic Acid (VA) was also demonstrated to extend worm lifespan up to 35%, as does structurally similar Valpromide (VPD). While Ethosuximide and trimethadione (TRI) extended lifespan of *daf-16* mutant worms, VA does not [93]. This is consistent with how worms treated with a combination of VA and TRI lived longer than mono-treatment, suggesting these drugs act via separate mechanisms [93]. In addition, although VA might inhibit bacterial proliferation and extend worm lifespan via inhibition of bacterial pathogenicity, the combination of VA and kanamycin had a partially additive effect on extension of lifespan, implying a mechanism independent of bacterial proliferation inhibition [93].

Allantoin, rifampicin, rapamycin, and psora-4

Admasu et al. defined drug combinations by initially testing five drugs individually that were previously shown to extend *C. elegans* lifespan: rapamycin, rifampicin, metformin, psora-4, allantoin, [94]. Using transcriptional profiling and principal component analysis, they found that rifampicin, rapamycin, and psora-4 cause the most distinctive effects. While each extends lifespan individually, rifampicin with rapamycin, and rifampicin with psora-4 increased survival more than the largest effect of any drug alone. When allantoin was added to these combinations, worm lifespan was further extended by about 90% [94]. Rifampicin with psora-4 also extended the lifespan of *eat-2(ad1116)* worms, implying this combination acts independently of DR. Each synergistic drug combination likewise improved movement of aged worms, and had higher resistance to thermal and oxidative stress (paraquat). Similar to worms, the longevity of male Drosophila was synergistically increased by the combinations of rapamycin with rifampicin, and rapamycin with rifampicin and allantoin [94].

Trametinib (MEK inhibitor), rapamycin (mTORC1 inhibitor), and lithium (GSK-3 inhibitor)

Trametinib is a MEK inhibitor used to manage cancer, while lithium is a GSK-3 inhibitor used to treat mood disorders. Trametinib and lithium each increase Drosophila lifespan alone and further extends the lifespan of flies lacking the insulin-like peptides 2, 3, and 5 (*dilp2–3,5*) [95,96]. Double combinations of lithium and rapamycin, lithium and trametinib, or rapamycin and trametinib produced greater lifespan extension than any single interventions, and longevity was increased nearly 50% when all three drugs were combined [97].

Rapamycin and wortmannin

Danilov et al. tested rapamycin and other anticancer agents for their ability to extend *Drosophila* health- and lifespan. They tested the effects of rapamycin (mTORC1 inhibitor), wortmannin (PI3K inhibitor), 1400W (iNOS inhibitor), pyrrolidin dithiocarbamate (NF-kB inhibitor), and QNZ (NF-kB inhibitor) as single agents or in combination. The most effective lifespan-expanding intervention was the combination of rapamycin and wortmannin, which extended *Drosophila* lifespan by 23.4% [98].

Rapamycin and myriocin

Lipid metabolism plays a key role in the regulation of aging and longevity [99–101]. Myriocin is a potent inhibitor of serine palmitoyltransferase, the first step in sphingosine biosynthesis. When combined with a low dose of rapamycin, chronological lifespan was synergistically increased in both *S. cerevisiae* and *S. pombe* [102 103]. This dual treatment likewise increased resistance to heat and oxidative stress, and increased genomic stability [102].

Metformin with SRT1720 reduce lifespan

Paradoxically, molecules that individually extend healthspan and lifespan may reduce survival when combined. Two SIRT1 activators (SRT2104 and SRT1720) improve the survival and age-associated health of mice on high fat diet that induces traits of type 2 diabetes [104–107]. Yet, such mice treated with both metformin and SRT1720 dramatically lose body weight and experience reduced survival. On the other hand, these mice were leaner and performed significantly better on the accelerating rotarod relative to age-matched controls [108].

VII. Conclusion and open questions

Dozens of genetic and pharmacological interventions interact to affect Hallmarks of Aging and lifespan. We need to address outstanding questions to synthesize these nodes into systems and networks.

How do we define the topology of multiple and varied interactions?

We started our review by discussing different ways to structure the complexity of aging across levels of interaction. “Hallmarks of Aging” [7,17] features as a common level for many studies, although aging research has yet to fix what qualifies or not as a Hallmark. We still require a clear classification of aging-related processes or how to hierarchically structure

their interactions. We also require better concepts of feedback, both positive and negative, within interaction networks.

How do we predict which combined interventions will optimally slow aging?

To predict prospective cooperation between different targets, we seek similarity in downstream effectors of lifespan. For example, different lifespan-extending interventions in mice that inhibited protein translation [109], and the size of nucleoli [110] and flux of 1C metabolism [111] were affected by manipulations of different pro-longevity pathways in worms. Our challenge is to *a priori* select combinations of interventions that will synergistically amplify their individual effects upon these common targets, and thereby efficiently manage aging.

What new genetic tools are needed to test high-order combinations?

Most interactions to date have been described using *C. elegans* because strains are coisogenic and bacterial dsRNA offers easy combinatorial gene knockdown. To study multi-feature interactions in Drosophila or mice requires great care to manage genetic background and tools to overcome the constraints of transgenes. One solution might multiplex several RNAi [112] or gRNA lines [113] into one cassette with an easily identifiable genetic marker, as we previously applied to target several cancer-related genes simultaneously [114].

How do we sample or create a matrix of all pairwise interactions?

Norris et al. developed the CRISPR/Cas9-based Synthetic Genetic Interaction (CRISPR-SGI) approach to systematically generate double-mutants in *C. elegans* [115]. They generated all possible single and double mutations of 14 conserved RNA-binding proteins and measured the effect of each on worm fitness. A similar approach can be applied to a large set of known longevity genes to comprehensively study how they interact to affect aging.

What constrains the potential to further slow aging?

Geroscience proposes that common underlying processes modulate the progression of multiple manifestations or Hallmarks of aging. If targeting two independent underlying processes additively affects aging, what determines when further interactions cease to increase health and lifespan? Targeting all defined hallmarks is unlikely to extend lifespan without limits. Are there “weak” spots that constrain further lifespan? Might these involve trade-offs between biological necessities such as reproduction and cellular integrity? Will multi-interventions produce side effects? As we elaborate the processes driving diverse Hallmarks of aging, can we understand when they become jointly constrained?

How do we predict which combined interventions apply to humans?

One approach might use epigenetic-like clocks, which predict chronological and biological age in humans [116]. One application of such tools is to predict how interventions at younger ages will affect the trajectory of aging. This approach may be extended to how multiple interventions interact to affect aging. For instance, the Thymus Regeneration, Immunorestoration, and Insulin Mitigation clinical trial found epigenetic age was reduced by approximately 1.5 years after one year of treatment with a combination of human growth

hormone (rhGH), dehydroepiandrosterone (DHEA), and metformin [117]. As interaction analyses expand from model systems to applications for humans we will need robust approaches to evaluate effectiveness and safety, and all within constrained timeframes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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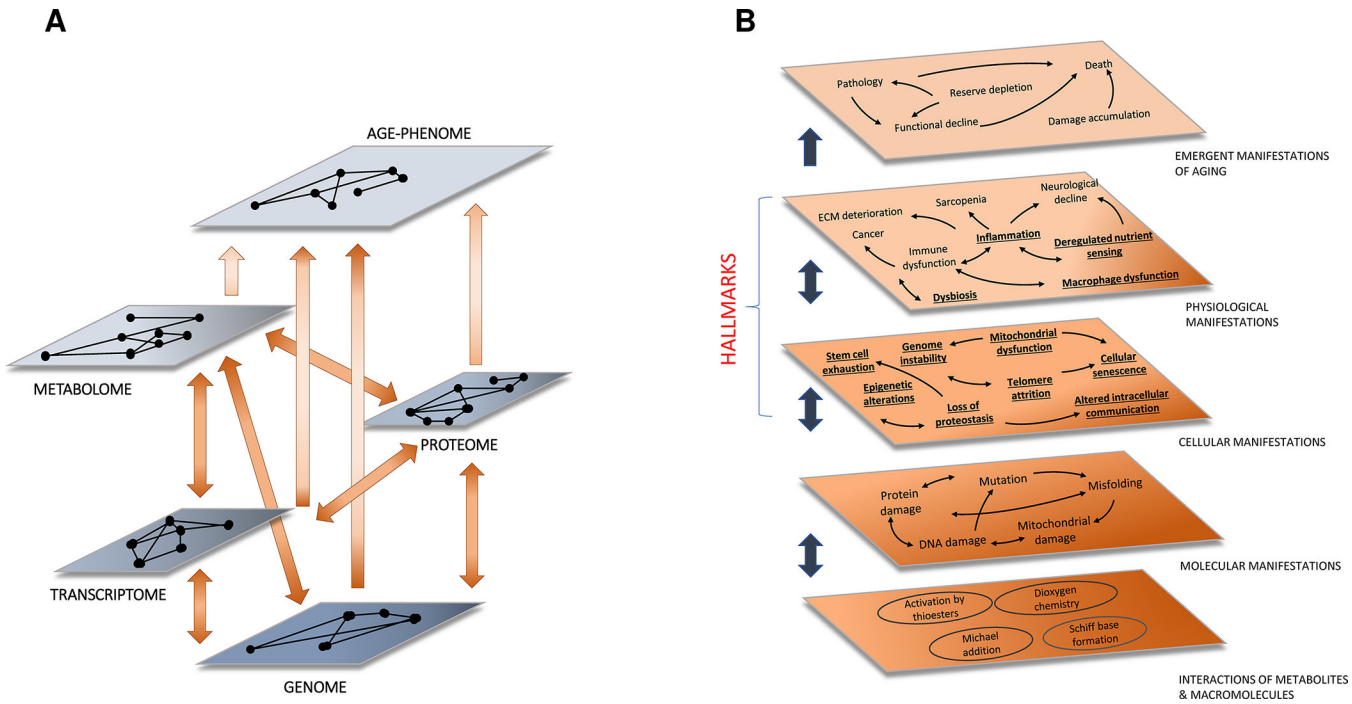

Figure 1. Hierarchical frameworks for aging interaction analysis.

a. Systems biology considers high-throughput-omics as a set of tiers that contain functional and signaling networks within levels. The effects of manipulations of features within an -omic tier or between two tiers can be observed at the level of the age-phenome, the set of all cellular, tissue, organ and organismal age-associated phenotypes. This approach can map aging-related interactions among levels, but is complicated when interventions impact multiple nodes upon the next level. Figure modified from [5]. **b.** The ‘nominal aging’ hierarchy of Golubev [8]. Asymmetry in chemical dynamics provides the underlying basis of aging. We observe increasingly complex, manifestations that are built upon processes of lower interactions. Hallmarks of Aging (bolded and underlined) are observed as specific molecular, cellular, and physiological manifestations of aging. By considering manipulations of processes at the cellular level via genetic or pharmacological studies, interactions among Hallmarks generate emergent manifestations of aging such as morbidity, frailty and mortality.

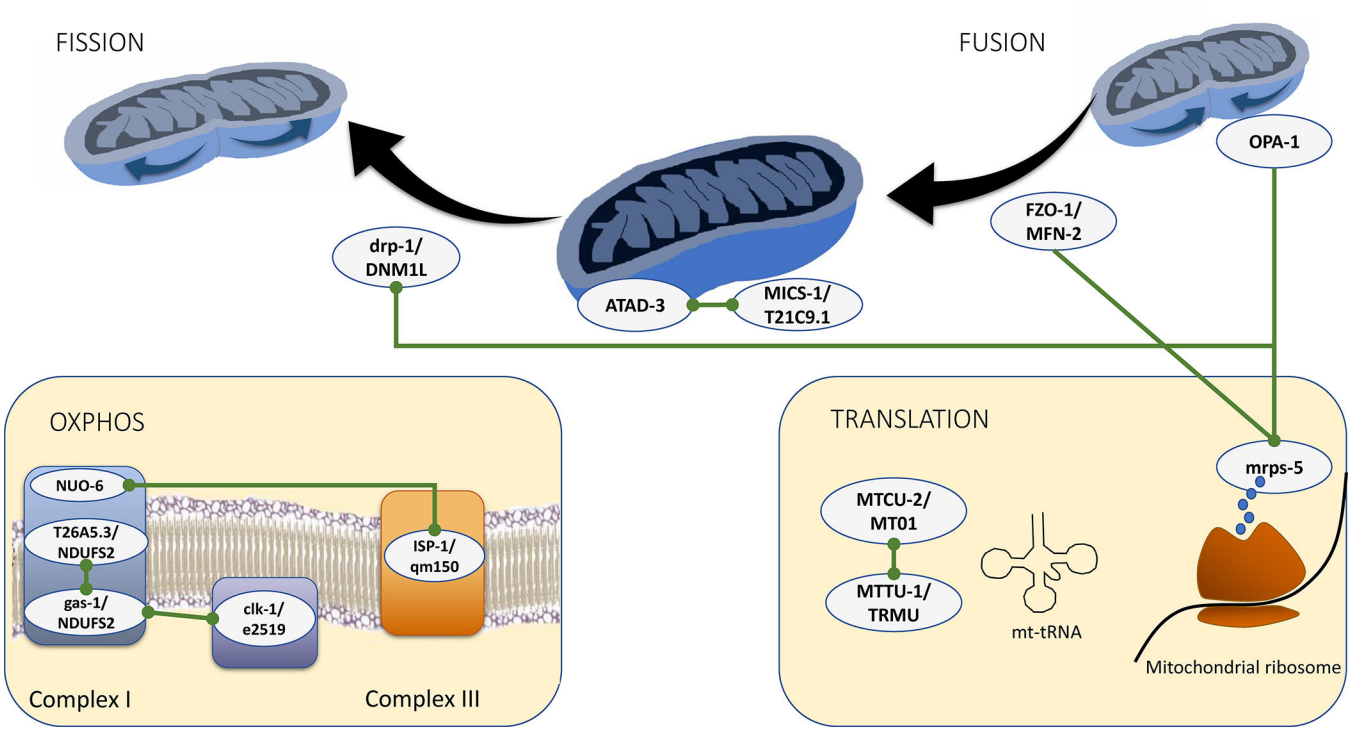

Figure 2. An example of interactions within a Hallmark: Mitochondria-related mechanisms.

Mitochondrial dynamics and function degrade with age: mitochondrial dysfunction is an identified Hallmark of Aging. Interactions between key aspects of mitochondrial function, including fission, fusion, oxidative phosphorylation, and translation have been studied in the context of aging. Green lines represent interactions identified to impact aging in at least one species.

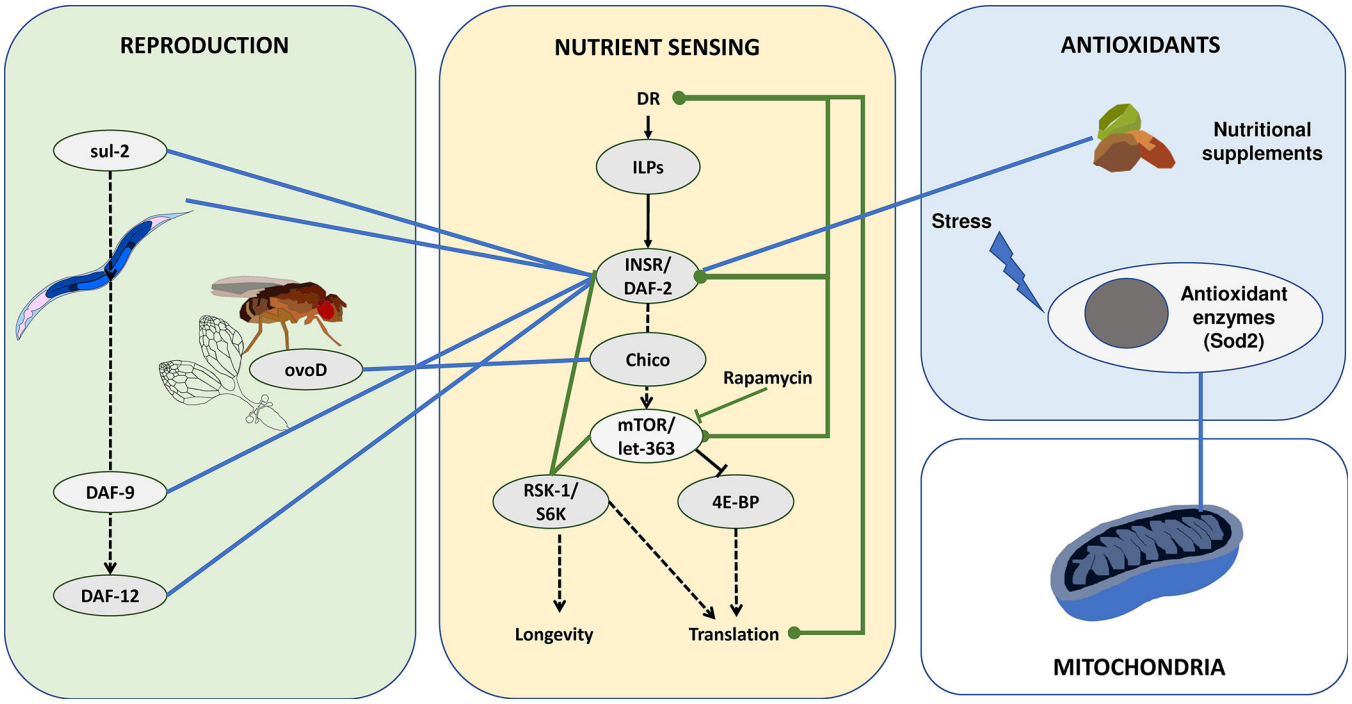

Figure 3. Select Interactions between mechanisms/Hallmarks of aging.

Studies have investigated many interventions among multiple mechanisms or Hallmarks of aging, measuring outcomes upon higher levels in -omics and manifestation hierarchies. The manipulations include food supplementation, trans-genes, mutants, diet restriction, and drugs. We illustrate a select subset (not exhaustive) of interactions within and between mitochondrial, oxidative stress, nutrient sensing, and reproductive decline processes. Blue lines depict data for interactions between processes of two or more mechanisms. Green lines illustrate interactions within nutrient sensing for the widely studied subnetwork of insulin/IGF signaling.

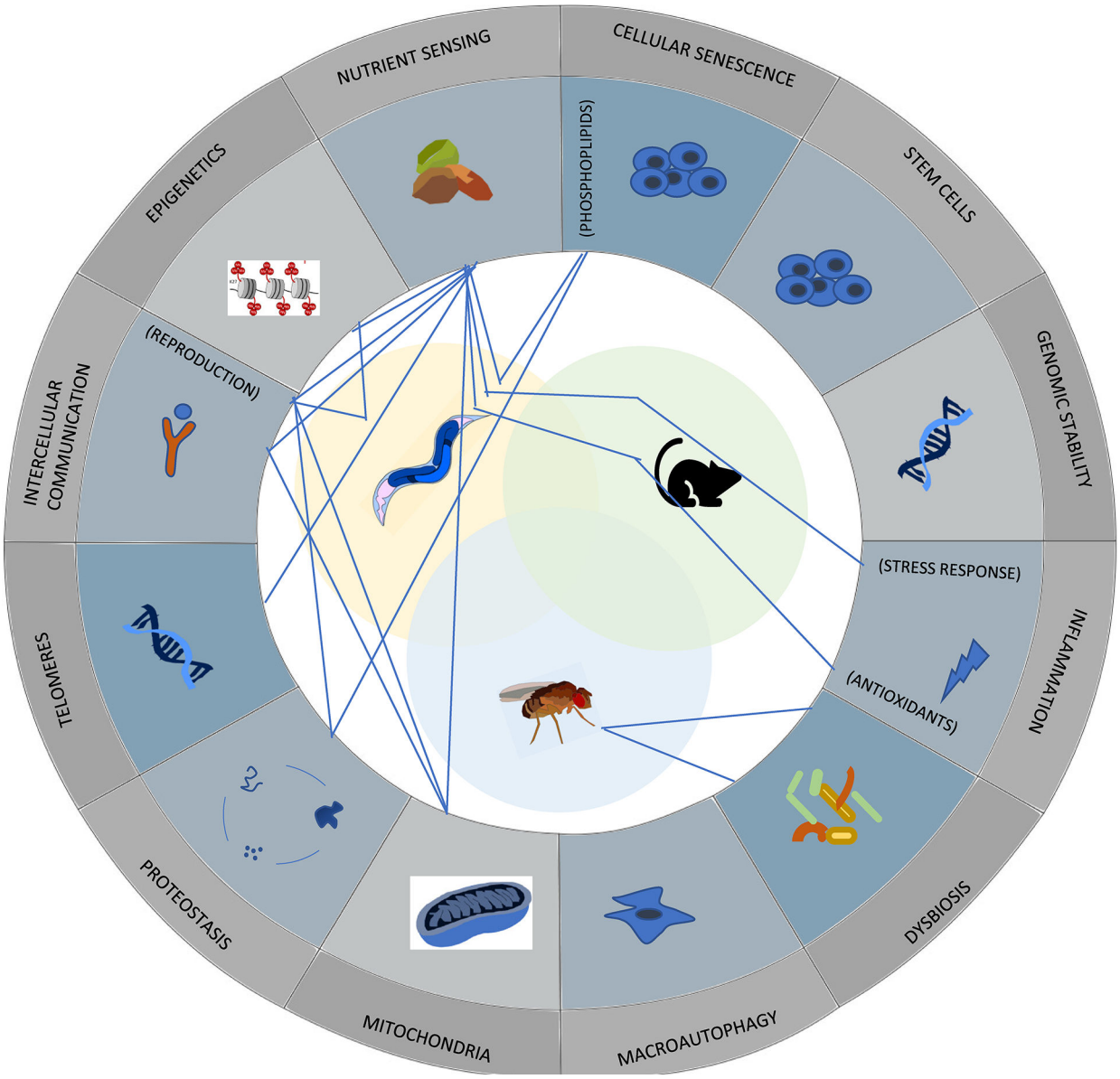

Figure 4. Overview of interactions between different processes (hallmarks) of aging.

Various studies have combined approaches from different Hallmarks of Aging to test for additive or synergistic effects. This wheel, modified from the Hallmarks of Aging framework[7,17], shows examples of various aging approaches that have been previously combined to further extend lifespan in three species: *C. elegans, Drosophila*, and mice. Blue lines represent combinatorial approaches that have shown promise in aging studies in at least one species. While most studies have been conducted in worms, targeting these combinations in multiple species can highlight promising approaches from a translational standpoint.

Table 1.

Dual interventions for extension of lifespan.

Studies that have manipulated two nodes within an individual hallmark of aging or two different hallmarks and shown an additive, partial, or synergistic effect on lifespan. Select studies of interest are summarized here, with extent of effect added where relevant. Note that lifespan effects were included in the table only when explicitly specified in the original publication as mean or median lifespan extension.

Organism	Intervention I	Signaling pathway (Hallmark of aging) I	% lifespan increase (Intervention I)	Intervention II	Signaling pathway (Hallmark of aging) II	% lifespan increase (Intervention II)	% lifespan increase (combination)	Reference
C. elegans	*daf-2(e1370)*	Deregulated nutrient sensing	169%	*rsks-1(ok1255)*	Deregulated nutrient sensing	20%	454%	20
C. elegans	*daf-2(e1370)*	Deregulated nutrient sensing	111%	Rapamy cin (TOR inhibitor)	Deregulated nutrient sensing	26%	187%	20
C. elegans	*daf-2(e1370)*	Deregulated nutrient sensing		LET-363/CeT OR	Deregulated nutrient sensing			20
C. elegans	*cyc-2.1*	Mitochondrial dysfunction	64%	*rsks-1*	Deregulated nutrient sensing	14%	54%	21
C. elegans	*cyc-2.1*	Mitochondrial dysfunction	64%	*daf-2*	Deregulated nutrient sensing	114%	252%	21
C. elegans	*RPS-15*	Deregulated nutrient sensing		*eat-2*	Deregulated nutrient sensing			22
C. elegans	*RPS-22*	Deregulated nutrient sensing		*eat-2*	Deregulated nutrient sensing			22
C. elegans	*rsks-1/S6K*	Deregulated nutrient sensing		*eat-2*	Deregulated nutrient sensing			22
C. elegans	*eIF2β*	Deregulated nutrient sensing		*eat-2*	Deregulated nutrient sensing			22
C. elegans	*eIF4G*	Deregulated nutrient sensing		*eat-2*	Deregulated nutrient sensing			22
C. elegans	*TOR/let-363*	Deregulated nutrient sensing		*S6K/ rsks-1(sv31)*	Deregulated nutrient sensing			22
C. elegans	*TOR/let-363*	Deregulated nutrient sensing		*eIF4E/ife - 2(ok306)*	Deregulated nutrient sensing			22
C. elegans	*isp-1(qm150)*	Mitochondrial dysfunction		*rps-15*	Deregulated nutrient sensing			22
C. elegans	*isp-1(qm150)*	Mitochondrial dysfunction		*rps-22*	Deregulated nutrient sensing			22
C. elegans	*isp-1(qm150)*	Mitochondrial dysfunction		*S6K/rsks-1*	Deregulated nutrient sensing			22
C. elegans	DR (limiting bacterial food)	Deregulated nutrient sensing	37%	*daf-2(e1370)*	Deregulated nutrient sensing	105%	205%	118
D. melanogaster	Rapamycin	Deregulated nutrient sensing	10%	*Chico* heterozy gous flies	Deregulated nutrient sensing	7%	20%	36

Organism	Intervention I	Signaling pathway (Hallmark of aging) I	% lifespan increase (Intervention I)	Intervention II	Signaling pathway (Hallmark of aging) II	% lifespan increase (Intervention II)	% lifespan increase (combination)	Reference
D. melanogaster	Rapamycin	Deregulated nutrient sensing		DR	Deregulated nutrient sensing			36
D. melanogaster	Rapamycin	Deregulated nutrient sensing	12–14%	Wortmannin	Deregulated nutrient sensing	8%	2.4-23.4%	98
C. elegans	*isp-1*	Mitochondrial dysfunction		*nuo-6*	Mitochondrial dysfunction			44
C. elegans	*mrps-5*	Mitochondrial dysfunction	18%	*eat-3*	Mitochondrial dysfunction	12%	55%	47
C. elegans	*mrps-5*	Mitochondrial dysfunction	18%	*fzo-1*	Mitochondrial dysfunction	7%	36%	47
C. elegans	*drp-1*	Mitochondrial dysfunction		*fzo-1*	Mitochondrial dysfunction			47
C. elegans	*T21C9.1/ MICS-1*	Mitochondrial dysfunction	54%	*ATAD-3*	Mitochondrial dysfunction	29%	83%	119
C. elegans	*mtcu-2*	Mitochondrial dysfunction		*mttu-1*	Mitochondrial dysfunction		50%	48
C. elegans	*gas-1*	Mitochondrial dysfunction		*T26A5.3*	Mitochondrial dysfunction			45
C. elegans	*gas-1*	Mitochondrial dysfunction		*clk-1(e2519)*	Mitochondrial dysfunction			45
Rotifera	Trolox	Antioxidant		beta-carotene	Antioxidant		10%	60
Rotifera	Trolox	Antioxidant		L-carnosine	Antioxidant		13%	60
Rotifera	Trolox	Antioxidant		N-acetyl cysteine	Antioxidant		16%	60
Rotifera	Trolox	Antioxidant		EUK-8	Antioxidant		14%	60
Rotifera	N-acetyl cysteine	Antioxidant		quercetin	Antioxidant		14.5%	60
Rotifera	EUK-8	Antioxidant		L-carnosine	Antioxidant		10%	60
Rotifera	EUK-8	Antioxidant		indole-3-propionic acid	Antioxidant		14%	60
C. elegans	*ule-4*	Uterinelumen-expressed proteins		*ule-5*	Uterine lumen-expressed proteins		15%	120
C. elegans	*daf2*	Deregulated nutrient sensing	100%	Germline removal	Reproduction	~60%	~300%	50,51
C. elegans	*daf-2*(e1370)	Deregulated nutrient sensing	253%	*daf12*(m20)	Nuclear receptor for dafachronic acid	−27%	387%	57
C. elegans	*daf-2*(e1370)	Deregulated nutrient sensing		*daf-9*	Reproduction			121
C. elegans	*daf-2*	Deregulated nutrient sensing	92%	*sul-2*	Steroid hormone production	32%	124%	58
C. elegans	*daf-10(m79)*	Cilia formation	34%	*sul-2*	Steroid hormone production	32%	89%	58
C. elegans	*daf-2(e1370)*	Deregulated nutrient sensing	106%	Pyrroloquinoline quinone (PQQ)	antioxidant	29%	148%	62

Organism	Intervention I	Signaling pathway (Hallmark of aging) I	% lifespan increase (Intervention I)	Intervention II	Signaling pathway (Hallmark of aging) II	% lifespan increase (Intervention II)	% lifespan increase (combination)	Reference
C. elegans	*age-1(hx546)*	Deregulated nutrient sensing	36%	Pyrroloquinoline quinone (PQQ)	antioxidant	36%	79%	62
C. elegans	*eat-2*	Deregulated nutrient sensing	22%	Pyrroloquinoline quinone (PQQ)	antioxidant	26%	42%	62
C. elegans	*hif-1(ia4)*	Oxygen sensing	18%	Pyrroloquinoline quinone (PQQ)	antioxidant	20%	30%	62
D. melanogaster	*chico*[1]	Deregulated nutrient sensing		Rhodiola rosea (RS)	antioxidant		9–14%	67
D. melanogaster	DR	Deregulated nutrient sensing		Rhodiola rosea (RS)	antioxidant		13–36%	67
C. elegans	*sod-2*	Mitochondrial function		*clk-1*	Mitochondrial function		165%	68
C. elegans	*sod-2*	Mitochondrial function		*eat-2*	Deregulated nutrient sensing			68
C. elegans	*sod-2*	Mitochondrial function		*glp-1*DR	Deregulated nutrient sensing			68
Rotifera	Rapamycin	TOR inhibitor	16%	SP600125	JNK inhibitor	20%	33%	122,123
Mice	dasatinib	inhibitor of multiple tyrosine kinases		quercetin	a natural flavonol		36%	79
Mice	metformin		No effect	rapamycin	mTORC1 inhibitor		23%	83
Mice	Rapamycin	mTORC1 inhibitor		acarbose	Alpha-glucosidase inhibitor		28–34%	86
Mice	Simvastatin	cholesterol biosynthesis inhibitor		ramipril	Angiotensin system inhibitor		9%	91
Mice	Ames dwarf mice			DR	Deregulated nutrient sensing			28
C. elegans	ethosuximide	anticonvulsant		*eat-2(ad465)*	Deregulated nutrient sensing			68,92
C. elegans	ethosuximide	anticonvulsant	*13%*	*osm-3*	Sensory neuron function	21%	32%	92
C. elegans	ethosuximide	Anticonvulsant		*tax-4*	Sensory neuron function			92
C. elegans	ethosuximide	Anticonvulsant	*13%*	*unc-31*	neurotransmission	37%	64%	92
C. elegans	ethosuximide	Anticonvulsant	*13%*	*unc-64*	neurotransmission	35%	46%	92
C. elegans	ethosuximide	Anticonvulsant	*17%*	*aex-3*	neurotransmission	1 6%	31%	92
C. elegans	ethosuximide	anticonvulsant	*17%*	*daf-2*	Deregulated nutrient sensing	107%	135%	92

Table 2.

Triple interventions for extension of lifespan.

Studies that have manipulated three nodes within one, two, or three Hallmarks of Aging and have shown an additive, partial, or synergistic effect on lifespan. Select studies of interest are summarized here, with the extent of effect added where relevant.

Organism	Intervention I	Signaling pathway (Hallmark of aging) I	Intervention II	Signaling pathway (Hallmark of aging) II	Intervention III	Signaling pathway (Hallmark of aging) III	% lifespan increase (combination)	Reference
C. elegans	*mtcu-2*	Mitochondrial dysfunction	*mttu-1*	Mitochondrial dysfunction	*daf-2*	Deregulated nutrient sensing	~70%	48
C. elegans	*mtcu-2*	Mitochondrial dysfunction	*mttu-1*	Mitochondrial dysfunction	*rict-1*	TORC2	~80%	48
C. elegans	*glp-1*	Reproduction	*mrps-5*	Mitochondrial dysfunction	*eat-3*	Mitochondrial dysfunction		9
C. elegans	*ule-4*	Uterine lumen-expressed proteins	*ule-5*	Uterine lumen-expressed proteins	*far-6*	Uterine lumen-expressed proteins	10–20%	120
C.elegans	*daf-2*	Deregulated nutrient sensing	*clk-1*	Mitochondrial function	Low temperature		~230%	69
C.elegans	DR	Deregulated nutrient sensing	*clk-1*	Mitochondrial function	Low temperature		~315%	69
C.elegans	*daf-2*	Deregulated nutrient sensing	*rsks-1*	Deregulated nutrient sensing	*tax-6*	Calceneurine complex		72
C.elegans	*eat-2 (ad1116)*	Deregulated nutrient sensing	rifampicin	antibiotic	psora-4	Kv1.3 channel blocker		94

Table 3.
Quadruple interventions for extension of lifespan.

Studies that have manipulated four nodes within multiple Hallmarks of Aging and have shown an additive, partial, or synergistic effect on lifespan. Select studies of interest are summarized here, with the extent of effect added where relevant.

Organism	Intervention I	Intervention II	Intervention III	Intervention IV	Signaling pathways			
C. elegans	ule-4	ule-5	ule-3	ule-2	Uterine lumen-expressed proteins	15–20%		120
C. elegans	*aakg-2(sta2)*	ucp2	lyz	hsf-1	AMP activated protein kinase, mitochondrial uncoupling, heat shock transcription	~130%	70	
C. elegans	*aakg-2(sta2)* + lyz	ucp2	*unc-62*	hsf-1	AMP activated protein kinase, mitochondrial uncoupling, heat shock transcription, neurotransmission neurotransmission	~160%	71	
D. melanogaster	*Dark*	18 °C *Dark*	*DR*	3G	maintaining in the dark, DR, low temperature, combination of rapamycin, berberine, and fucoxanthin	~120%	124	

Table 4.
Pharmacological interventions for extension of lifespan.

Studies that have explored pharmacological interventions, particularly those that are already approved for use in humans, are appealing due to their translational potential. Several promising candidate combinations are summarized here, with the extent of effect added where relevant.

Organism	Intervention I	Signaling pathway (Hallmark of aging) I	Intervention II	Signaling pathway (Hallmark of aging) II	Intervention III	Signaling pathway (Hallmark of aging) III	% lifespan increase (combination)	Reference
C. elegans	Valproic acid	antiepileptic	trimeth adione (TRI)	anticonvulsant			63%	93
C. elegans	Valproic acid	antiepileptic	kanamycin	antibiotic			41%	93
C. elegans	Allantoin		rifampicin	antibiotic	rapamycin	mTO RC1 inhibitor	89%	94
C. elegans	Allantoin		rifampicin	antibiotic	psora-4	Kv1. 3 channel blocker	96%	94
D. melanogaster	trametinib	MEK inhibitor	rapamycin	mTORC1 inhibitor	lithium	GSK-3 inhibitor	48%	97
s. cerevisiae	rapamycin	mTORC1 inhibitor	myriocin	sphingolipid synthesis inhibitor				102
S. pombe	rapamycin	mTORCI inhibitor	myriocin	sphingolipid synthesis inhibitor				103