

## Signaling pathways and effectors of aging

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### 1. ABSTRACT

Aging leads to and is associated with aberrant function of multiple signaling pathways and a host of factors that maintain cellular health. Under normal conditions, the pro-longevity, 5' AMP-activated protein kinase (AMPK), is dedicated to the homeostasis of metabolism and autophagy for removal of damaged cellular compartments and molecules. A host of sirtuin family of molecules, that extend life-span, regulate metabolism and repair DNA damage, and possess either mono-ADP-ribosyltransferase, or deacylase activity. Another group of pro-longevity factors, include FOX (forkhead box) proteins, a family of transcription factors that regulate the expression of genes involved in cell growth, proliferation, differentiation, and longevity. Nicotinamide phosphoribosyltransferase

(NAMPRTase or Nampt) catalyzes the condensation of nicotinamide with 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide (NMN), a requisite step for production of NAD<sup>+</sup>, which is known to increase longevity. Loss of Klotho, a transmembrane enzyme that controls the sensitivity of the organism to insulin and suppresses oxidative stress and inflammation, leads to premature aging in mice. Hydrogen sulfide and transsulfuration pathways are crucial to the long life and are required in protection of cells against damage. Aging also leads to the imbalanced activation of other pathways and factors including p53, insulin and IGF signaling, P13K/AKT, mTOR, PKA, RAS, RTK, MEK, ERK, MAPK, CRTC-1/CREB and NFκB. Such aberrant cellular functions, disturb cell metabolism, derail

autophagy and other housekeeping actions, inhibit cell division, induce inflammaging and immunosenescence, cause stem cell exhaustion and induce either senescence, apoptosis or cancer.

## 2. INTRODUCTION

Aging becomes evident in all human beings by loss of the ability to reproduce, and extensive damage and loss of function in organs, tissues, cells. Although, it is not argued that, the age related diseases, are not the cause rather are the consequence of aging, many have argued that, changes and damages that occur at the cellular level, play a causative role in aging. However, the current cell-centric hypotheses of aging merely explain, some but not all, hallmarks of aging in biological systems (1-6). Thus, the most proximal and fundamental causes of aging have remained as major conundrums in biology (7-9). Here, I summarize the signaling pathways and molecules that maintain the cellular homeostasis and health. However, the function of these pathways progressively gets corroded and such aberrant functions, ultimately, lead to an imbalanced signaling and activation of molecules that cause cells to senesce, and if the damage is severe, to induce apoptosis or initiate the processes that lead to tumorigenesis.

## 3. SIGNALING PATHWAYS AND EFFECTORS OF AGING

### 3.1. AMPK

AMPK is a serine/threonine protein kinase and central regulator of cellular and organismal metabolism that resides at the heart of cellular functions including growth, autophagy, polarization, and metabolism in eukaryotes. AMPK senses energy requirement of cells, inhibits anabolic pathways, promotes catabolic pathways and induces ATP production. AMPK is a highly conserved energy sensor comprised of a catalytic  $\alpha$  and regulatory  $\beta$  and  $\gamma$  subunits which are differentially expressed and assembled in mammalian tissues. AMPK is activated by allosteric regulation by the increased levels of AMP, ADP and  $\text{NAD}^+$  which restores and maintains cellular ATP (10-12). AMPK is also activated by

upstream kinases including transforming growth factor- $\beta$ -activated kinase 1 (TAK1) which can activate AMPK by phosphorylating the catalytic  $\alpha$  subunit at Thr<sup>172</sup>, serine/threonine kinase 11 (LKB1), and by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase  $\beta$  (CaMKK $\beta$ ) (13-15).

AMPK is activated in response to normal physiological signals including exercise, hormones, and phytochemicals as well as a host of pathological conditions. Activation of AMPK can be contextual, for example, AMPK can be activated or inhibited by developmental and environmental cues, or by adiponectin, ghrelin and leptin in a tissue specific manner (16-17). AMPK is de-activated by protein phosphatases (PP), such as PP2A, PP2C $\alpha$  and Ppm1E (18-28). During stress, AMPK drives energy production by stimulation of use of glucose and fatty acids and reduces energy consumption by inhibiting protein, cholesterol and glycogen synthesis (10-11).

AMPK also inhibits oxidative stress by induction of mitochondrial UCP2 which represses superoxide production and inflammation (23-26). Activation of AMPK also induces the expression of thioredoxin, a disulfide reductase and prevents the oxidation of cysteine residues in proteins. It has been suggested that anti-oxidative effects of AMPK is also mediated by activation of Nuclear factor erythroid 2-related factor 2 (Nrf2)/SKN-1 signaling and by the induction of expression of anti-oxidative heme oxygenase-1 gene via Nrf2 signaling (27). It appears that AMPK acts, in concert, with Nrf2 and FoxO3a axis in endowing a stress resistant phenotype in long-lived animals.

AMPK and its orthologue in *C. elegans*, AMP-activated kinase-2 (AAK-2) control and extend life-span and health-span by an integrated signaling network that includes metabolic homeostasis, enhancement of stress resistance by FoxO/DAF-16, Nrf2/SKN-1, and Sirt1 signaling pathways, autophagy via the mTOR and ULK1 pathways, inhibition of inflammatory response by inhibition of NF $\kappa$ B signaling and is assigned to cellular housekeeping. Overexpression of AAK-2/AMPK has extended the life-span in *C. elegans* and *D. Melanogaster* (28-32). In mice, the knockout of  $\alpha 1$  (AMPK $\alpha^{1-/-}$ ) and  $\alpha 2$  (AMPK $\alpha^{2-/-}$ ) led to different

outcomes, and only the  $\alpha 2$  catalytic subunit of AMPK, has a negative impact on the health-span. This subunit, which was shown to play a major role as a fuel sensor, leads to high plasma glucose levels, low plasma insulin concentrations, insulin-resistance and reduced glycogen synthesis in the muscle (33).

Unfortunately, such a valuable pathway is eroded by aging, depriving cells from the host of functions that maintain their youthful state, leading to increased oxidative and ER stress, reducing autophagic removal of damaged cellular components, allowing for emergence of inflammation (inflammaging) and loss of energy homeostasis leading to accumulation of hyperglycemia and fat causing a metabolic syndrome including development of insulin resistance, diabetes obesity, and cardiovascular disease (10, 34). This loss of function of AMPK in aging has been tested by AICAR treatment and physical exercise that increased the activity of AMPK $\alpha 2$  in the muscles of young and not old rats (35). Consistent with this, the activation of AMPK induced by muscle contractions, was shown to be repressed in muscles of old mice (36). Similarly, aging impaired AMPK activation and suppressed insulin-stimulated glucose uptake in rat skeletal muscles (37). The deficiency of AMPK exacerbated aging-induced myocardial dysfunction (38). In mouse brain, although, the baseline activity of AMPK was higher in old animals as compared to their younger counterparts, cerebrovascular stroke stimulated an increase in AMPK activity in young mice but not in the old mice (27). Although the precise mechanism that hampers the AMPK activity in aging tissues is not clear, certain factors which are known to diminish this response, include nutritional factors, some hormones and inflammation that is present in aged tissues (39).

### 3.2. FOXO

The FOXO family of transcription factors are characterized by a conserved DNA-binding domain, the so-called 'Forkhead box', or FOX. Based on the sequence similarity, this family includes more than 100 members in humans, classified from FOXA to FOXS (40-43). Invertebrates, have only one FOXO gene (daf-16 in the worm and dFOXO in flies) and mammals have four genes named FOXO1 (FKHRL1), FOXO3

(FKHRL1), FOXO4 (AFX), and FOXO6 (44-45). The four mammalian isoforms of FOXO family appear to have distinct, yet, overlapping functions that can mask the loss of function of the individual FOXO factors (46).

FOXO proteins act primarily as transcriptional activators that bind to the consensus core recognition motif, TTGTTTAC, and their activity is inhibited by the IIS pathway, whereas, the down-regulation of this pathway, in response the harsh environmental conditions, leads to FoxO activation (47-52).

FOXO members interact with many different pathways namely, SIRT1, AMPK, insulin/PI3K/Akt, c-Jun NH2-terminal kinase (JNK), and inhibitor of nuclear factor kappa-B kinase subunit beta (IKK $\beta$ ) pathways. FOXO1, 3 and 4 have several effectors including IIS pathway and PI3K-AKT signaling (47, 53). Insulin or IGF-1 both trigger PI3K and then serine/threonine kinase, AKT, that phosphorylates FOXO factors at three conserved residues. This leads to the exit of FOXO factors from nuclei and their transport to the cytoplasm, an event that leads to a suppression of FOXO-dependent transcription of target genes (54-56). However, in the absence of Insulin or IGF-1 signaling, FOXOs are translocated into the nucleus and activate FOXO dependent gene expression. In addition to PI3K/PKB-AKT, other kinases, including AMPK, JNK, and IKK $\beta$  can also phosphorylate FoxO, establishing their roles as a master-switch, critical to cellular responses (51). The FOXO factors can undergo post-translational modifications such as phosphorylation, acetylation, deacetylation, methylation, or ubiquitination and such changes modify their DNA binding and transcriptional activity (51-52).

One of the first evidence that linked FOXO to longevity was shown for DAF-16 in *C. elegans*, an orthologue of mammalian FoxOs (57-58). DAF-2 pathway, which corresponds to the mammalian insulin/IGF-1 signaling (IIS), down-regulates the activity of FoxO/DAF-16 transcription factor, both in mammals and *C. elegans*. Thus, it became clear that such a pathway might shorten life-span. Indeed, loss-of-function mutations of DAF-2 pathway doubled the life-span of *C. elegans* (57-58). In flies,

overexpression of dFOXO has been shown to be sufficient to increase longevity (46). Following mutations in the insulin/PI3K/Akt pathway, worms that lack daf-16 or flies that lack dFOXO, were viable but did not show an increase in life-span. Several studies have also revealed APOE and FOXOs (FOXO1 and FOXO3) to be “longevity genes” (59-66).

The actions of FOXOs on life-span appear to be rooted in their evolutionarily conserved roles in regulation of glucose and lipid metabolism which allows cells to adapt to stress such as starvation (67-69). Low glucose (low insulin drive), low insulin (reduced strength of the insulin signal) and FoxO activation all induce a similar metabolic shift. FoxOs increase insulin sensitivity and induce expression of the insulin receptor and IRS2 (70). FOXOs appear to be a “master-switch” for adaptation of cells and organisms to food scarcity, ensuring their metabolic stability by opposing many of the functions of IIS pathway and by induction of cell cycle arrest and quiescence, which is reminiscent of the Dauer state in *C. elegans* (71).

Importantly, FOXOs are critically responsive to the oxidative environment in cells by upstream regulatory pathways of FOXO or directly by sensing oxidation and reduction state of cysteine residues, the so-called cellular redox state. In response to this read-out, FOXOs increase the antioxidant capacity of cells through enzymes that degrade reactive oxygen species such as catalase, manganese superoxide dismutase (MnSOD) and GADD45 (72-77). Besides upregulation of antioxidant stress capacity, FoxO activation or low insulin both activate Peroxisome Proliferator-activated receptor Gamma Coactivator 1 $\alpha$  (PPGC-1 $\alpha$ ), a nutrient sensing system that increases mitochondrial biogenesis and induces a shift in metabolism from reliance on carbohydrate towards fat (78). Thus, it is clear why de-activation of this pathway increases the ROS in age related pathologies including atherosclerosis (78-79).

FOXOs also regulate apoptosis and inflammation, endow cell resistance, and regulate, through unknown mechanisms, the protein maintenance, the so-called proteostasis that is

impaired by aging (52, 56, 80-84). Whereas the impairment of PI-3K/AKT-PKB signaling causes FoxO activation, the enhanced PI-3K/AKT-PKB unleashes an inflammatory state by inducing NF $\kappa$ B through activation of the IKK (85).

FOXOs affect the expression of genes involved in autophagy and mitophagy, and more specifically, FOXO1 and FOXO3 have been shown to activate autophagy (52). The importance of autophagy and mitophagy in the function of FOXOs is supported by studies that show that defects of autophagy are associated with premature aging in animal models (86-92).

Ubiquitin-proteasome system, that removes short-lived and regulatory proteins, is also subject to regulation by FOXOs, a process that is impaired in aging and neurodegenerative disorders such as Parkinson's, Alzheimer's, or Huntington's disease (93-96). The mode of action of FOXOs on this system appears to be due to upregulation of ubiquitin ligases and by controlling the composition of the proteasome (97-100). AMPK-induced stimulation of FoxO/DAF-16, Nrf2/SKN-1, and SIRT1 signaling pathways all have been shown to improve cellular stress resistance (101).

Similar to other cells, stem cells are also subject to control by FOXOs and such regulation appears to be significant in the ability of these factors to impact tissue regeneration. For example, deletion of Foxo3a leads to the exhaustion of hematopoietic stem cells as a result of their constant exit from quiescence, an effect that can be prevented by increasing the redox state by administration of N-acetylcysteine (102). Moreover, FOXOs control the major stemness factors, OCT4 and SOX2 that maintain pluripotency of human ESCs (103).

### 3.3. Sirtuins

The life preserving effects of dietary restriction appears to engage the transsulfuration pathway, H<sub>2</sub>S production, Nicotinamide adenine dinucleotide (NAD<sup>+</sup>), sirtuins and AMPK. Exogenous administration of H<sub>2</sub>S has been shown to be beneficial and afforded *C. elegans* health-promoting effects including stress resistance and improved

thermotolerance and led to a 70% increase in life expectancy (104). It was shown that such an effect required NAD<sup>+</sup>-dependent deacetylase, sir-2.1 (105). We showed that while the production of H<sub>2</sub>S decreases with senescence, the replicative senescence can be delayed by exogenous H<sub>2</sub>S in human cells in a NAMPT/SIRT1 dependent manner (106). Increasing the H<sub>2</sub>S to physiological levels, upregulated the *hTERT*, increased telomerase activity and increased population doublings (106).

Members of the Silent information regulator (SIRT) 1 family of NAD<sup>+</sup>-dependent deacetylases act as silencers of gene expression by the deacetylation of histones and control ribosomal DNA recombination, and DNA repair, and confer chromosomal stability and longevity in multiple organisms and are essential to the beneficial effects inducible by dietary restriction (DR) (107-108). SIRT1, the best characterized mammalian sirtuin, deacetylates many non-histone proteins and impacts numerous physiologic processes, including apoptosis, metabolism, and stress resistance (109). There are experimental evidence that have implicated the Sir2 homologs in mammals (SIRT1–SIRT7) as mediators of key effects of caloric restriction (CR) during aging (110-111). SIRT1, SIRT6, and SIRT7 show sub-nuclear localization; SIRT2 is predominantly cytoplasmic; whereas SIRT3, SIRT4, and SIRT5 appear to reside in the mitochondria (112). Mammalian SIRT1 is closely related to Sirt2 which is required in yeast to maintain a silent chromatin state of the ribosomal RNA genes and telomeres. The Sirt2 expression diminishes with replicative aging allowing transcription and recombination of rRNA genes which are known to cause toxicity and to limit replicative life-span in yeast cells (113-114). SIRT1 is an evolutionarily conserved deacetylase that targets histones and several transcription factors, and is known to act as an energy sensor, that is responsive to AMP and NAD<sup>+</sup>, increases the intracellular concentration of NAD<sup>+</sup> by increasing Nampt (115-117). The downstream target of SIRT1 include PGC-1 $\alpha$ , FoxO1 and FoxO3. SIRT1 deacetylates and subsequently increases the activity of LKB1 kinase, an upstream activator of AMPK (118). SIRT1 is stress responsive, and localizes at DNA damage sites such as DNA breaks to repair the damage. Following damage, the trans-localization of

SIRT1, from basal target genes, allows expression of a number of genes whose expression is known to increase with age (119). Thus, continuous damage and environmental stress, vacate normal chromatin occupancy of DNA by SIRT1 and trigger sequential and progressive changes in chromatin state over time, including accumulation of chromosome breaks, mutations, and loss of the youthful gene expression patterns. SIRT1 signaling appears to underlie some of the effects of CR such as stress resistance (120).

Besides SIRT1, the chromatin-associated sirtuin, SIRT6, targets chromatin by transcription factors, maintains telomeres and replicative activity and is required for longevity. SIRT6-deficient mice, although are small and appear relatively normal after birth, exhibit premature aging and show sudden drops in serum glucose and IGF-1 levels, defects in bone mineral density which are reminiscent of osteoporosis, curved spine (kyphosis), loss of subcutaneous fat, lymphocyte depletion, and severe metabolic defects (121-124). SIRT6 directly interacts with NF $\kappa$ B subunit, RelA, and is recruited by RelA to promoters of genes. This leads to deacetylation of H3K9Ac, a key event that promotes removal of RelA and abolishes further NF $\kappa$ B signaling (122). SIRT6 inhibits the expression of several NF $\kappa$ B dependent genes by modulating their chromatin structures whereas its depletion leads to a premature aging phenotype in keratinocytes (122-123). In SIRT6 deficient cells, hyperacetylation of H3K9 at the promoters of the target genes, increases RELA promoter occupancy, and enhances NF $\kappa$ B-dependent modulation of gene expression and leads to cellular senescence and apoptosis. However, such a deficiency, has not been associated with telomeric dysfunction (124). Haplo-insufficiency of RelA has been shown to decrease the early lethality and degenerative syndrome of Sirt6-deficient mice (122). Thus, SIRT6 is thought to lie at the crossroad of aging, rejuvenation, and epigenetics (125).

### 3.4. NAMPT

In *Saccharomyces cerevisiae*, the PNC1 gene is part of the NAD salvage pathway, that encodes a nicotinamidase that depletes cellular nicotinamide by converting NAM to nicotinic acid (vitamin B3). PNC1 gene is thought to be a master



“longevity regulatory gene” that translates a variety of environmental stresses into life-span extension by activating the sirtuin family of longevity deacetylases (126). Overexpression of *PNC1* increases Sir2-mediated silencing and leads to about 50% increase in the replicative life-span in this yeast (127-128). A decrease in glucose concentration, from 2% to 0.5% is sufficient to increase *PNC1* levels by ~4-fold. Interestingly, *PNC1* levels are also increased by more than 4-fold in response to low amino acids, heat stress, and osmotic stress, conditions that are known to extend the life-span in yeast (128). Thus, it is thought that CR induces life extension in yeast through activation of *PNC1* and Sir2 (126). It has been proposed that Nicotinamide phosphoribosyltransferase (Nampt), also known as Pre-B-cell colony-enhancing factor (PBEF) or Visfatin is the functional equivalent of *PNC1* gene in mammals (126, 129-132). Based on the homology between Nampt and the *nadV* gene of *Haemophilus ducreyi*, Rongvaux *et al.* proposed and then confirmed that Nampt acts as a nicotinamide phosphoribosyltransferase (NAMPRTase) (130). Nampt is inducible by nutrient deficiency and stress, regenerates NAD<sup>+</sup>, controls sirtuins, and supports response to damage and increases life-span (132-133).

Nampt is the rate limiting enzyme that salvages NAD<sup>+</sup> from nicotinamide (126, 129-132). Replicative senescence leads to the decreased expression and activity of NAMPT in smooth muscle cells in culture. Loss of NAMPT and synthesis of adequate supply of NAD<sup>+</sup> occur with aging, and this in turn, reduces SIRT1 activity, leading to cellular senescence (134). Artificial inhibition of NAMPT by its inhibitor, FK866, leads to premature senescence whereas forced induction of Nampt, suppresses age dependent increase in p53 expression, increases the rate of p53 degradation, raises the activity of SIRT1, delays senescence and increases life-span in these cells, an effect that can be inhibited by the dominant negative form of SIRT1 (135).

### 3.5. Klotho and FGFs

Klotho gene, is named after the spinner, one of the three goddesses, Klotho, Lachesis, and Atropos that according to the Greek mythology,

control the life-span of every mortals who, respectively, spin, measure, and cut the thread of life (136). *klotho* gene is comprised of 5 exons, giving rise to a single-pass transmembrane protein with a short 10-amino acid-long intracellular domain, that is highly expressed in the brain, kidney, parathyroid and pituitary glands. The ectodomain of Klotho protein, released in a soluble form (sKlotho) to the blood, cerebrospinal fluid and urine, exerts functions that are distinct from the transmembrane protein (137). sKlotho regulates the activity of ion channels and growth factor receptors including insulin/IGF-1 receptors. In 1997, the *klotho* gene, was identified to be mutated in the *klotho* mice, that show pre-mature aging and age related pathology, characterized by hypogonadism, ectopic calcification, impaired bone mineralization, premature thymic involution, skin atrophy, pulmonary emphysema, neuro-degeneration, hearing loss, higher levels of serum phosphorus, calcium, and active vitamin D (1,25-dihydroxyvitamin D3) and lower levels of serum glucose, and an extremely shortened life-span (136-137). Mice homozygous for the mutated *klotho* allele (*KL*<sup>-/-</sup> mice) although initially appear to be normal for 3 to 4 weeks, they start to show premature aging as evidenced by multiple age-related disorders including skin and muscle atrophy, osteoporosis, arteriosclerosis, and pulmonary emphysema, and die prematurely around two months of age (136, 138-139). While, a defect in Klotho gene expression in mice, leads to degeneration of age sensitive processes, the aging phenotypes can be reversed by inhibiting insulin and IGF1 signaling, suggesting, that Klotho-mediated inhibition of insulin and IGF1 signaling, contributes to its anti-aging properties (139).

The evidence in humans supports the action of Klotho as an aging suppressor since single-nucleotide polymorphisms in the human *KLOTHO* gene have been show to be associated with altered life-span, altered risk for coronary artery disease, osteoporosis and stroke (140-147). On the other hand, the overexpression of Klotho extends life-span (139). Mice that carried the *EFmKL46* or *EFmKL48* transgenic alleles of Klotho that were fed *ad libitum*, although did not show a substantial difference in growth from wild-type mice nor changes in blood glucose levels, had higher blood levels of insulin,

likely due to insulin resistance, and lived substantially longer than their wild counterparts and generated fewer offsprings than wild-type breeding pairs (139). Male and not female transgenic mice showed significant reduction in insulin and IGF1 tolerance tests. Moreover, while Klotho peptide did not inhibit the binding of  $(^{125}\text{I})$ insulin or  $(^{125}\text{I})$ IGF1, it suppressed ligand-stimulated autophosphorylation of insulin and IGF1 receptors in a dose-dependent manner, leading to inhibition of intracellular insulin and IGF1 signaling (139). Also, it was shown that inhibition of insulin and IGF1 signaling can rescue  $\text{KL}^{-/-}$  induced phenotypes from age-related pathologies, namely, ectopic calcification, hypogonadism, skin atrophy, pulmonary emphysema, and arteriosclerosis (139).

Fibroblast growth factors (FGF), namely FGF19, FGF21, and FGF23, act in an endocrine fashion and regulate energy and homeostasis of bile acids, glucose, lipid, phosphate, and vitamin D and all require presence of Klotho in target tissues (146). Transmembrane Klotho is an obligate co-receptor for FGF23, a bone-derived hormone that forces secretion of phosphate into urine. In mice, Klotho deficiency leads to reduced klotho levels, hypotrophy of cells in the anterior pituitary gland that secrete GH, and decreased activity of GH/IGF-1 axis and premature aging. Mice, that lack FGF23, retain phosphate and also show a premature-aging syndrome, showing a link between phosphate metabolism and aging. The aging induced phenotype by Klotho is related to ability of klotho to regulate GH, since Ames dwarf and Snell dwarf mice that lack GH live much longer than their normal siblings, and exhibit delayed aging. Targeted disruption of the GH receptor/GH-binding protein gene (GHR-KO mice), the so-called "Laron dwarf mice," are GH resistant and live much longer than their normal counterparts. These mice exhibit increased hepatic sensitivity to insulin, reduced insulin, reduced plasma glucose, lowered hepatic synthesis of IGF-1. They also generate a reduced level of ROS and exhibit an increased resistance to oxidative stress leading to improved antioxidant defense mechanisms, and reduced oxidative damage (147). These long-lived dwarf mice share many phenotypic characteristics that are inducible by CR.

$\beta$ -Klotho protein is also predominantly expressed in the liver, pancreas and adipose tissues where FGF21 regulates metabolic functions by activating AMPK-SIRT1 signaling and adaptive responses to CR (148). FGF21 signals through classic FGF receptors, which act as tyrosine kinases, more preferably, the FGFR1c/ $\beta$ -Klotho complex. FGF21 is strongly induced in the liver by prolonged fasting and plays a key role in eliciting and coordinating the adaptive starvation response including enhancing insulin sensitivity, decreasing triglyceride concentrations, stimulating hepatic gluconeogenesis, fatty acid oxidation, and ketogenesis and weight loss (148).

### 3.6. Hydrogen sulfide and transsulfuration pathways

The trans-sulfuration and its upstream and downstream pathways are critically important in an array of diverse metabolic functions requisite to organismal homeostasis. This involves active transfer of thiol and methyl groups in a large number of biochemical processes that are vital to normal function of DNA, RNA and proteins in mammalian cells. The ancestral trans-sulfuration metabolic pathway in bacteria exists as a forward pathway that transfers thiol groups from cysteine to homocysteine to a reverse pathway which exists only in mammalian cells and involves the transfer of the thiol group from homocysteine back to cysteine. The essential amino acid, methionine, is activated, in an ATP-dependent manner, by methionine adenosyltransferase, to form S-adenosylmethionine (SAM). SAM donates a methyl group by methyltransferase, to yield S-adenosylhomocysteine (SAH), followed by formation of homocysteine (149-150). The homocysteine can either be re-methylated back to methionine using a methyl group donated by methyl tetrahydrofolate (MTHF), or is converted to cysteine via trans-sulfuration pathway and generates cystathionine by conjugating it with serine. This pathway is also crucial to the conversion of cysteine to major cellular antioxidant species, which include GSH, glutaredoxins, thioredoxins, taurine, and peroxiredoxins as well as hydrogen sulfide ( $\text{H}_2\text{S}$ ) which is vital to life due to its antioxidant, anti-inflammatory and other cyto-protective properties.

Due to its paramount importance, H<sub>2</sub>S is formed by at least three enzymatic reactions as well as by chemical means (151). While cystathionine  $\beta$ -synthase (CBS) generates H<sub>2</sub>S and serine as a by-product, cystathionine- $\gamma$ -lyase (CGL, also known as cystathionase, CTH, or CSE), by using cysteine as the substrate, generates H<sub>2</sub>S and produces pyruvate, and NH<sub>3</sub> as by-products of this reaction (152-154). The activity of CBS is enhanced by binding to the carboxy-terminus of SAM and glutathionylation of Cys<sup>346</sup>, while it is suppressed by nitric oxide (NO) and carbon monoxide (CO) that bind to a heme group at its amino-terminus (153-154). A third enzyme, 3-mercaptopyruvate sulfurtransferase (3MPST or 3MST) produces H<sub>2</sub>S by an enzymatic action involving cysteine aminotransferase (CAT), and by virtue of using D-amino-acid oxidase (DAO), generates H<sub>2</sub>S from D-cysteine in presence of thioredoxin (153-154).

Glutathionylated CBS increases the production of cysteine and H<sub>2</sub>S, which, in turn, promotes the production of other antioxidant species. For example, glutathione, a major antioxidant and reducing agent is produced in mitochondria by the metabolism of H<sub>2</sub>S by sulfide quinone oxidoreductase (SQR). Metabolism of H<sub>2</sub>S is also important to the formation of poly-sulfides that act as major cellular reservoirs for H<sub>2</sub>S. Hydrogen sulfide (H<sub>2</sub>S) exists in free form (20%), and dissociates readily in water and produces H<sup>+</sup>, a large (80%) amount of HS<sup>-</sup>, and trace amounts of S<sup>2-</sup> (153-154). H<sub>2</sub>S-derived sulfur sulfhydrates the reactive cysteine residues of target proteins and enzymes and changes their activity. Together, H<sub>2</sub>S and other antioxidants as well as sulfane sulfur protect cells against diverse forms of injuries (155). For example, oxidative stress is a positive regulator of the trans-sulfuration pathway and it activates CBS, promotes conversion of methionine to cysteine and increases synthesis of GSH which protects cells by oxidation, by generating glutathione persulfide (GSSH or GSS<sup>-</sup>). Cys<sup>346</sup> of CBS can also be oxidized to a sulfenic acid which then reacts with glutathione.

H<sub>2</sub>S exhibits a classical U-shaped dose response with negative impact at supra and sub-physiological levels and positive effect at physiological doses ranging from protection from

ischemia-reperfusion injury to life-span extension (153-156). Exposure to a high concentration of H<sub>2</sub>S leads to eye and olfactory irritation, neurotoxicity, inhibition of electron transport chain (ETC), respiratory distress, headache, edema and death (157). Dysregulated endogenous H<sub>2</sub>S metabolism results in a range of pathologies from inflammation to  $\beta$  cell dysfunction and diabetes. Reduced levels of H<sub>2</sub>S are associated with negative consequences. For example, mice with genetic defects in endogenous H<sub>2</sub>S generating enzymes, CGL, or CBS have been shown to be susceptible to hypertension, neurodegenerative disorders, and vascular complications associated with diabetes and osteoporosis (158-161). There are additional evidence that supports the protective effect of H<sub>2</sub>S in organ systems and age induced pathologies including a hypoxia-resistant reduced metabolic rate leading to suspended animation resembling torpor, reducing blood pressure by its action in causing vasodilation, protection against ischemia-reperfusion injury, improving glucose tolerance and insulin sensitivity, delaying cognitive decline in animal models of Alzheimer's disease, and increasing longevity in yeast, worms, and mammalian cells (106, 162-169). The generation of ROS is increased in knockouts of *MPST-1*, a major enzyme that drives the production of hydrogen sulfide in *C. elegans*. This deficit in the short lived animals could be overcome by the administration of H<sub>2</sub>S donor, GY4137, which resulted in an extended life-span (170). This treatment also delayed the onset of detrimental impact of senescence as assessed by pharyngeal contraction and defecation (170).

### 3.7. p53

The p53 is a transcription factor with potent tumor suppressor properties that is known to regulate a large number of genes with effects on stress, metabolism, cell cycle, apoptosis, senescence and autophagy (171-178). p53 regulates mitochondrial energy metabolism and mitochondrial biogenesis (179). p53 also controls the mitochondrial integrity by inducing Miep, a protein which removes oxidized proteins from mitochondria (180).

p53 is a potent inducer of antioxidant defense proteins and decreases aging-associated



oxidative stress (181). Different stresses trigger the phosphorylation of Ser-20 at the trans-activation domain of p53 protein (182). Unfortunately, the transcriptional activity of p53, p53-dependent apoptosis and efficiency of p53 in response to cellular stress, are significantly impaired by aging (183). The reduced p53 functional activity during aging has been attributed to decreased autophagy, and increase in oxidative stress, antagonistic effect of NFkB on p53 function and NFkB induced enhancement of the inflammatory responses (85, 174, 184-186).

In response to energy deficiency, AMPK activates p53 by phosphorylating it at Ser-15 causing cell cycle arrest (187). On the other hand, enforced expression of AMPK $\alpha$ 2 increases the transcription of p53 gene and enhances its phosphorylation at Ser-46 leading to apoptotic cell death (188). Thus, activation of p53 comes at a cost since cells with active p53 undergo either cellular senescence or apoptosis (189).

By virtue of reducing development of cancer, p53 is considered to be an aging suppressor and aids in the extension of the life-span (190-192). The p53-deficient mice do not lend themselves to examine the role of p53 in aging since they die early from malignancy. However, two strains of mice that express full-length p53 along with the C-terminal fragment of p53 have shown premature aging (192). On the other hand, a null mutation in p66Shc, that is associated with an impairment of p53 and p21 stress response showed, a 30% increase in life-span (193). The activation of Arf/p53 pathway, likely by increased expression of antioxidant genes, delayed the aging process and reduced age-related damages in mice (194).

It is thought that some of the effects of p53 on organismal aging are mediated by autophagy (195-196). Cytoplasmic p53 represses autophagy whereas nuclear p53 has the opposite effect and stimulates the transcription of DNA-damage regulated autophagy modulator 1 (DRAM1) and Sestrin 2 proteins (186). It has become clear that while increased autophagy extends the life-span, its repression can lead to the accumulation of damaged molecules with an adverse effect on health-span and life-span (186, 197-198). p53 participates in

autophagy by activation of mTOR and production of ROS (199-200).

Some of the effects of p53 on aging might be directed at IIS and mTOR pathways, known to induce aging or through MDM2, a major component of IIS and PKB/AKT kinase pathway (201). However, transgenic mice with an elevated p53 activity that exhibit high levels of circulating IGF-1 and tissue-activated IIS, have been shown to be both short- and long-lived (192, 202).

### 3.8. Growth hormone, insulin and insulin growth factor (IGF)

The evolutionarily conserved and ancient insulin and insulin-like growth factor (IGF) signaling (IIS) controls longevity and plays a major role in the growth, differentiation and metabolism, in response to changing environmental conditions and nutrient availability. Mutations that limit the extent of insulin/IGF-1 signaling dramatically increase life-span in *C. elegans*, *Drosophila melanogaster* and in several mouse models. After being hatched, *C. elegans* undergoes four successive juvenile (larval) stages before they mature to an adult hermaphrodite worm (203). During food scarcity, crowding and high temperature, the larvae of *C. elegans* exit the cycle of growth and development at the third larval stage, postpone reproduction and form the so called dauer larva which, under laboratory conditions, can survive up to eight times longer than normal (204). Daf mutants are often long-lived and exhibit dauer-like features, such as enhanced resistance to stress and/or changes in the metabolism of carbohydrates, lipids and amino acids. Cloning and sequencing of the daf mutants identified genes that exhibited a strong homology to components of the mammalian insulin and insulin-like growth factor (IGF) signal transduction cascade (IIS) (57-58, 150, 205). In *C. elegans*, in response to food or the sensory perception of food, insulin signaling leads to the secretion of multiple, insulin-like peptides that bind to a common single insulin/IGF-1 like tyrosine kinase receptor (DAF-2). Whereas reduction of function mutations in *daf-18* phosphatase, a homologue of the mammalian phosphatase and tensin homolog, PTEN, abolished the life-span extensions of *daf-2* and *age-1* mutants, the reduction-of-function

mutations in *daf-2*, and the kinase components of the IIS pathway down-regulated IIS cascade in *C. elegans* and these animals remained active and youthful much longer than normal and their life-span was increased in by more than twofold (206).

Although the core of the insulin/IGF-1 signaling pathway is conserved in invertebrates to mammals, the mammalian IIS signaling has greatly increased in its complexity in the latter species (207). This increased complexity has made it difficult to separate the roles of growth hormone (GH), insulin, and IGF-1 in longevity. Yet, genetic and metabolic characteristics that are associated with a healthy life-span suggest that the IIS pathway is involved in setting the mammalian longevity. Reduced GH, insulin and IGF-1 signaling due to various mutations have been associated with long-lived phenotypes in mice (206). *FIRKO* mice, that lack the insulin receptor in adipose tissues, are also long lived and show reduction in fat depots and reduced age related loss of insulin sensitivity (208). Reduced IGF-1 signaling, due to mutation of its receptor, led to increased resistance to oxidative stress and long lived phenotypes in *Igf1<sup>r/r</sup>* females, but not males (209). Whereas, mutation in *Klotho*, shortened life-span in mice, its overexpression, which inhibits IIS pathway, extended their life-span (136, 139).

GH which is released by the anterior pituitary gland controls mammalian growth and regulates the biosynthesis and release of IGF-1 by the liver and peripheral tissues. Four dwarf mouse models *Prop1<sup>df/df</sup>*, *Pit1<sup>dw/dw</sup>*, *GHRHRLit/lit* and *GHR<sup>-/-</sup>* that exhibited reduced IGF-1 production, reduced circulating levels of insulin and glucose and enhanced insulin sensitivity, were are long-lived (210-213). Longest life-extension has been seen in mouse mutants, the so-called GH deficient hypopituitary dwarfs and the GH resistant *GHR<sup>-/-</sup>* dwarfs, that show defective GH/IGF-1 and/or insulin. Enhancement of insulin sensitivity and reduced insulin levels appear to be the primary reasons for the longevity phenotype of these mice as well as in wild type mice that are subjected to caloric restriction (214).

In humans, it has been difficult to show the relationship of the GH/insulin/IGF-1 signaling to the

longevity due to the complexity of these pathways. It can be speculated that low glucose, low insulin and preserved insulin sensitivity may represent key metabolic features of a human longevity phenotype. Defects in insulin signaling has led to insulin resistance and diabetes and defects in GH/IGF-1 caused defects in growth and an increased risk of cardiovascular disease (207, 213). Yet, there are telltale signs that GH/insulin/IGF-1 signaling plays a role in human aging. For example, there are several common polymorphisms in IIS genes that were associated with longevity and in Italian centenarians, genotype combinations at *IGF-IR* and *PI3KCB* genes, were associated with lower free IGF-I plasma levels (207, 214-216). Centenarians of Ashkenazi Jewish heritage that showed overrepresentation of heterozygous mutations in the IGF-1R gene had a small stature and elevated levels of serum IGF-1 (207, 214-216).

### 3.9. P13/AKT

Phosphatidylinositol 3-kinase (PI3K), along with AKT serine/threonine protein kinase and mTOR which is downstream of the insulin/PI3K pathway, are all involved in conveying the metabolic and mitogenic signals. P13Ks are a set of evolutionarily conserved and multi-faceted enzymes in flies to mammals that generate 3' phosphoinositides from phosphatidylinositol in response to growth factors that together with mTOR appear to play a role in aging and life-span (217). The most common form, PI3K IA, is a functional heterodimer comprised of one catalytic and one regulatory unit, encoded by *p85 $\alpha$* , *p85 $\beta$* , and *p55 $\gamma$*  genes (218-220). Adapter proteins, such as insulin receptor substrate proteins (IRS1-4), by binding to the tyrosine residues, activate PI3K and AKT, which regulate downstream targets including GSK3 $\beta$  and mTOR (221-222).

Activated PI3K phosphorylates and activates and localizes its down-stream mediator, AKT, to the plasma membrane (222). AKT, in turn, can activate a number of other factors and pathways including FOXO, and mTOR (222-223). Activation of AKT is essential to the PI3K-dependent regulatory pathways that participate in cellular response to oxidative stress whereas inactivation of DJ-1, a *Drosophila* homologue, impairs phosphatidylinositol

3-kinase/Akt signaling and response to oxidative stress (224-225). The pharmacological inhibition of PI3K, or induced expression of dominant-negative AKT induces cell death during oxidative stress (226-228). High cholesterol intake which impairs insulin signaling, increases serine phosphorylation of IRS1, PI3K and AKT activities, and increases oxidative stress (229).

Mutations in some genes that regulate PI3K have been shown to extend life-span. For example, in *C. elegans*, the mutation in the catalytic sub-unit homologue of mammalian PI3K, Age-1 or loss of CHICO, a *Drosophila* insulin receptor substrate protein, have led to increased life-span (230-232). Reducing the activation of the PI3K/AKT/mTOR pathway significantly increases the longevity in mice as well (233). Common variants of both FOXO3A and AKT1 were associated with longer life-span in three independent Caucasian cohorts (233-234). Residing down-stream from PI3K/AKT, the activity of mTOR appears to be low in centenarian individuals (235). Thus, longevity seems to be intimately linked to the reduced activity of the IIS/PI3K/AKT/mTOR pathways, suggesting that these signaling pathways are important targets for pharmacological manipulation for extension of life (236).

### 3.10. mTOR

mTOR is a 289-kDa serine-threonine kinase that senses cellular nutrient levels. mTOR integrates both intracellular and extracellular signals and serves as a central hub for cell metabolism, growth, proliferation and survival (237). De-regulated mTOR has been described in diverse age related diseases such as type 2 diabetes (238-240). Moreover, there is by now, substantial evidence that mTOR is a negative regulator of life-span. In *Drosophila melanogaster*, life extension can be achieved by the overexpression of TOR suppressors, dTsc1 of dTsc2, or expression of dominant-negative forms of dTOR or dS6K 38. By using genetic manipulation, it was shown that depletion of TOR (*let-363*) or RAPTOR (mTORC1 protein member; *daf-15*) by RNA interference (RNAi) in *C. elegans* or deletion of SCH9, a S6K homologue in

*Saccharomyces cerevisiae*, extends life-span in both models (241-243).

The central role of mTOR along with insulin and insulin growth factor 1 (IGF-1) in regulating life-span is attributable to the actions of these signaling networks as a sensor of nutrients. Such pathways including insulin, IGF-1, P13L/AKT, RAS, RAF, MEK, and ERK, all converge on mTOR, making it central to regulation of amino acid availability, mitochondrial metabolism and biogenesis, rate of protein synthesis and proteostasis, lipid synthesis and energy utilization and homeostasis, cellular senescence, unfolded protein response, autophagy, and proteosomal degradation (244). mTOR is now considered to be essential in delaying the development of age related pathologies and in the life extension by strategies such as calorie restriction (238). One of the prime examples that nutrition has a significant impact on aging has been shown by introducing calorie restriction of the diet without causing malnutrition in animals. Such measures have been able to extend life-span and health-span in rodents to monkeys (245-249). Food scarcity drives the larvae of *C. elegans* to enter a dauer stage that remain metabolically in-active for months until the environmental conditions become hospitable to life. Thus, halting metabolism effectively increases life-span of animals in dauer stage for months (250). Obtaining such metabolic arrest also allows the seeds and spores of bacteria and fungi to gain considerable extension of life for at least 2000 years when preserved in amber and for millions of years in high salt (251-252).

### 3.11. PKA

The multi-unit holoenzyme, Protein Kinase A (PKA), has four regulatory sub-units (RI $\alpha$ , RI $\beta$ , RII $\alpha$ , RII $\beta$ ) and three catalytic sub-units (C $\alpha$ , C $\beta$ , C $\gamma$ ) that show a varied tissue distribution and cellular expression (253-254). In mammals, nutrients are sensed by a G-protein (GEF) that activates adenylyl cyclase (AC) (255). AC produces cAMP, which binds to the regulatory subunits of the PKA, releasing the catalytic subunits which either interact with other signaling proteins, or enter the nucleus and activate gene transcription. Activated PKA phosphorylates serine and

threonine residues and mediates the signal transduction of G-protein-coupled receptors (255).

The regulation of oxidative stress, mitochondrial function, and cell survival appear to require the joint participation of AMPK and PKA signaling (256). The regulation of mitochondrial function and oxidative stress by the mitochondrial-directed scaffold of PKA, requires dual-specificity A-kinase anchoring protein 1 (D-AKAP1)(256). The actions of AMPK and PKA are down-regulated in age related pathologies including diabetes, cardiovascular diseases and ischemia and the protective effect of type II regulatory subunit of PKA (PKA/R11 $\beta$ ) as well as D-AKAP1 diminishes by their reduced mRNA levels in adipocytes and subcutaneous adipose tissues by obesity (257-258). On the other hand, reducing total caloric intake to 20–40% of normal intake which leads to life-span extension, has been attributed to involve down-regulating effect on mTOR-S6 kinase pathway, insulin and insulin-like signaling (IIS) as well as its effect on Ras/cAMP/PKA/Rim15/Msn2/4 and the Tor/Sch9/Rim15/Gis1 pathways (259-262). It is noteworthy that the anti-aging effect caused by the in-activation of both pathways is much more potent than that caused by CR alone (263-264). In yeast, CR and peroxiredoxin promote longevity and H<sub>2</sub>O<sub>2</sub>-resistance through redox-modification of PKA (265). BMH1 14-3-3 protein, which extends chronological life-span in *Saccharomyces cerevisiae*, appears to act by activating the stress response and by virtue of genetically interacting with CR and conserved nutrient-sensing TOR- and PKA-signaling pathways (266).

In eukaryotic cells, intracellular pH is significant to protein folding, enzyme activity, vesicle trafficking, and organellar function and integrity as well as aging. For this reason, the pH is exquisitely and dynamically regulated in tissues and cells by multiple mechanisms such as the plasma membrane H<sup>+</sup>-ATPase, Pma1 and the vacuolar V-ATPase. Both PKA and the TORC1-Sch9 axis regulate the proton pumping activity of the V-ATPase and possibly Pma1 and, in turn, the proton pump acts as a second messenger for availability of glucose by the V-ATPase to PKA and TORC1-Sch9 (267). The replicative and chronological aging in yeast appear to

require three kinases: Sch9, PKA and TOR (268). The stress response proteins, namely transcription factors Msn2 and Msn4, that lead to increased longevity, are associated with decreased activity of either Sch9, PKA, or TOR (268).

The impact of Sir2 on DR-mediated extension of life also depends on cAMP-PKA and casein kinase 2 (CK2) signaling in yeast (269). Sir2 partially represses the transcription of life-span-associated genes, such as PMA1 (encoding an H<sup>+</sup>-ATPase) and many ribosomal genes, an effect that is inhibited by active cAMP-PKA and CK2 signaling (269).

Mutations that decrease the activity of the Ras/Cyr1/PKA pathway extend longevity and increase stress resistance by activating transcription factors Msn2/Msn4 and the mitochondrial antioxidant enzyme superoxide dismutase (Sod2) (270). Additional evidence for the involvement of PKA in aging has been shown in male and not female mice that lack the regulatory R11 $\beta$  subunit (271). These mice have extended life-span, show reduced insulin resistance, and protection against age related pathologies including cardiac dysfunction and hypertrophy, weight gain, and enlargement of liver. These positive impacts of PKA inhibition appear to involve AMPK, and  $\beta$ -adrenergic pathway (271).

Since PKA is part of the signaling cascade that regulates metabolism and aging processes and for this reason is an ideal target in anti-aging strategies. Hydralazine, a FDA-approved drug used for the treatment of high blood pressure and heart failure, has recently been shown to increase the life-span in *C. elegans* in high glucose or stress conditions. These actions of hydralazine appear to involve activation and improved mitochondrial function and metabolic homeostasis via the SIRT1/SIRT5 axis and the NRF2/SKN-1 pathway and by targeting, binding and stabilizing the catalytic sub-unit of PKA (272). Similarly, the life extension by lithocholic acid (LCA) in yeast appears to involve the cAMP/protein kinase A (cAMP/PKA) as well as adaptable target of rapamycin (TOR) and signaling pathways that are under the stringent control of calorie usage (273). LCA extends the life-span by a housekeeping longevity assurance program that is

not purely governed by the adaptable pro-aging TOR and cAMP/PKA pathways. Rather, LCA modulates longevity by reduced lipid-induced necrosis and mitochondrial induced apoptosis, by altering oxidation-reduction processes in mitochondria, enhancing stability of nuclear and mitochondrial DNA and promoting resistance to oxidative and thermal stress (273).

### 3.12. RAS, RTK, MEK, ERK, and MAPK

RAS genes, which encode small 21 kDa (p21) GTPase proteins, were originally identified as viral genes that account for the highly oncogenic properties of RNA tumor viruses and which appear at a high frequency in a large number of human cancers and are risk factors for age related disorders such as cancer, diabetes, as well as cardiovascular and neurodegenerative diseases (274-278). Ras protein family in mammals include N-RAS, H-RAS, K-RAS4A and K-RAS4B. Ras encompasses a large superfamily of proteins that are involved in signal transduction, conveying signals from surface bound receptor tyrosine kinases (RTKs) in response to cytokines, growth factors and hormones and which integrates with RTK, MEK, ERK, and MAPK pathways (279). Ras proteins are binary molecular switches, that cycle through an in-active GDP-bound and active GTP-bound states. The cytoplasmic tail of the activated RTKs recruit the Grb2 adaptor protein which binds to the Ras-GEF, SOS, and this, in turn, localizes Ras to the activated RTK-bound complex. Significant structural changes in switch regions of I and II components of Ras that exists in an active GTP-bound conformation, forms a GTP-dependent interface. In this conformation, Ras binds downstream effector molecules including Raf, which initiates a phosphorylation cascade via MEK and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK). Activated ERK phosphorylates multiple cytoplasmic and cytoskeletal proteins, including MAPK-activated protein kinases and ribosomal S6 kinase (280-281). Finally, ERK activated by Ras signaling translocates to the nucleus, phosphorylating and activating several transcription factors including members of the E-twenty-six (ETS) transcription factor family (282). Ras proteins are involved in cell division and differentiation to metabolism, senescence and

apoptosis (283). In yeast, the Ras proteins are part of the nutrient signaling pathway that includes cyclic AMP (cAMP) and protein kinase A (PKA) (284). Ras proteins are regulated by the activities of guanine nucleotide exchange factors (GEFs) that catalyze the replacement of GDP by GTP and GTPase activating proteins (GAPs) that increase the rate of GTP hydrolysis (285-286).

Given their broad actions, it is not surprising that Ras proteins are directly or in-directly involved in aging and in replicative life-span in different species from fungi, flies, and worms to mammals. For example, two Ras homologues, RAS1 and RAS2 in *Saccharomyces cerevisiae* influence both replicative and chronological life-span and deletion of RAS1 has been shown to extend the replicative life-span whereas deletion of RAS2 extends chronological life-span (284, 287-288). Such life-extension by deletion of Ras has been attributed to their effects by endowing stress resistance by Sch9 in yeast and by Msn2/Msn4 and Sod2m which are activated in response to RAS-cAMP-PKA signaling, in *Saccharomyces cerevisiae* (241, 281, 289-290). Both MSN2 and MSN4 are considered to be the link between calorie restriction and sirtuin-mediated life-span extension in *Saccharomyces cerevisiae* (291). Genetic inhibition of either Ras or ERK has been shown to extend the life-span in *Drosophila melanogaster* (292). Expression of an activated form of AOP, a transcriptional repressor that is inhibited by Ras activation, also has led to extension of life-span in this fly (293). Similarly, Trametinib, an inhibitor of the upstream kinase, MEK which also inhibits ERK, extended life-span in *Drosophila* (292). Similarly, in *C. elegans*, Ras Let-60 protein has been shown to modulate the effects of insulin/IGF-1 in aging (294). Mice that were deficient for *RasGrf1*, which acts downstream of insulin and IGF-1 receptors, were long-lived, and showed increased SIRT1 expression, lower circulating IGF-1 levels and resistance against oxidative stress, and development of cancer (295-296). However, since *RasGrf1* also has an affinity for other ligands, including Rac, Rho, it is not clear that the longevity induced by *RasGrf1* deficiency is merely through specific inhibition of Ras (277, 278, 297). The genetic variants of *HRAS1* and *APOE*, which



interact synergistically, are associated with extended health-span and life-span in humans (298-299). Costello syndrome, that arises in humans due to mutations in *HRAS*, is characterized by a short stature, failure to thrive, and oftentimes is associated with premature aging (300). Finally, HGPS that is associated with progeria and shortened life has been shown to be associated with up-regulation of mTOR, IGF1R, IP3, and ERK, showing that HRAS and activity of its downstream effector, ERK, are involved in human aging (301).

### 3.13. CRTC-1/CREB

CREB and its co-activators, cAMP-response element binding protein (CREB)-regulated transcription co-activators (CRTC)s, have emerged as sensors of hormonal and metabolic signals, energy homeostasis, and endoplasmic reticulum (ER) mediated stress (302-303). In mammals, CRTCs are co-activators of CREB-mediated gene expression (303). More importantly, after their activation, CREB and CRTCs are involved in mediating the effects of feeding as well as fasting signals on the expression of metabolic programs in insulin-sensitive tissues. Besides CRTC, many kinases, e.g. protein kinase A (PKA),  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases II/IV and p90 ribosomal S6 kinase, p90RSK, activate CREB-mediated gene transcription (303-304). It is notable that among these, the inhibition of PKA signaling, has been shown to enhance health-span while other studies have shown a link between the activation of calcineurin through dysregulation of  $\text{Ca}^{2+}$  and accelerated aging whereas calcineurin deficiency which increases autophagy, extends the life-span in *C. elegans* by altering the expression of *bec-1* and *atg-7* (263, 305-308).

To increase the transcription of target genes, the in-active, phosphorylated cytoplasmic CRTC gets activated by dephosphorylation by protein phosphatases such as calcineurin, and following this activation, migrates to the nucleus where it binds to CREB factors (309). The nuclear translocation of CRTC-1 is blocked by phosphorylation by AAK-2/AMPK, an effect that is associated with increase in life-span in *C. elegans* showing that factors that inhibit the CRTC-induced

CREB activation pathway are involved in the regulation of aging (303).

The CREB co-activator, TORC2, has been found to regulate fasting glucose metabolism, to stimulate the gluconeogenic program along with the forkhead factor FOXO1 and to endow stress resistance in *Drosophila* (310-311). TORC2 activation appears to underlie the effect of starvation in *Drosophila* (311). Moreover, the life-span extension in *C. elegans* is mediated by the CRTC-1 and CREB through AMPK and catecholamine by reprogramming the mitochondrial and metabolic signals (303, 312).

CRTCs modulate organismal aging in *C. elegans* and appear to be involved in age-related diseases in humans. CRTCs have been implicated in neurodegenerative diseases and their deregulation appears to increase the risk of age related pathologies including Alzheimer's disease, and Huntington's disease (313-316). Activation of the CRH-1/CREB axis by CMK-1/CaMKI in the AFD thermosensory neurons appears to regulate the life-span in *C. elegans* at warm temperatures (317).

### 3.14. NFkB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) is required for adaptive changes in gene expression and tissue homeostasis (318). NFkB is responsive to oxidative stress, DNA damage, immune activation and growth regulatory signals, and controls cell proliferation, innate and adaptive immunity, inflammation, and apoptosis. Interestingly, *NFKB1* gene in humans resides within a genetic locus on chromosome four that is associated with human longevity (319).

NFkB appears to be important in aging processes that are associated with inflammation during aging, the so-called inflammaging. Inflammatory response is initiated by activation of NFkB in macrophages which aggravates much of age related metabolic disturbances (320). Several age-related metabolic disorders, e.g. obesity, type 2 diabetes and atherosclerosis have been shown to lead to chronic inflammation due to increased NFkB signaling, a process that can be effectively

suppressed by AMPK leading to FoxO signaling and AMPK activators including some non-steroidal anti-inflammatory drugs, e.g. aspirin and flufenamic acid (26, 321-322). DNA-binding activity of NFkB complexes are significantly increased with aging leading to an increase in levels of p52 and p65 components of NFkB complex in several tissues of mice and rats (323-325). NFkB actively interacts with several regulators of aging. For example, FoxO factors, FoxO3a and FoxO4 are effective inhibitors of NFkB signaling and can prevent immune responses (326-327). FOXO3a, a homolog of the longevity gene *DAF-16* in *C. elegans*, which is also strongly associated with human longevity, represses NFkB nuclear translocation and transcriptional activity (326, 328). SIRT1, that deactivates NFkB by binding and deacetylating the p65 RELA, is known to underlie the life extension by calorie restriction (329).

The NFkB signaling is also subject to regulation by Nrf2 which reduces inflammatory response and conversely, the p65 component of NFkB complex binds to the Kelch-like ECH-associated protein 1 (Keap1) protein, and inhibits Nrf2 signaling. This, in turn, leads to increased localization of Keap1 into the nuclei and consequently reduces the binding of Nrf2 to its target sites (330). Decrease or deficiency in Nrf2 signaling during aging increases the inflammatory phenotype (331). Paradoxically, *in vitro* sustained pharmacological activation of Nrf2 in fibroblasts promotes the deposition of a matrix rich in plasminogen activator inhibitor-1 (PAI-1) that induces senescence (332). The *in vivo* sustained pharmacological activation of Nrf2 in fibroblasts promotes wound healing but also induces tumors in the skin. However, it can not be ruled out that such effects might be due to off-target effects of the drug.

An emerging concept is that aging is not a passive process, rather, age dependent disorders require active maintenance. Although it is not clear why NFkB gets activated during aging, diverse lines of studies show that NFkB appears to enforce and is required for the persistence of the global transcriptional program and tissue phenotypes that are hallmarks of aging. The analysis by using microarray of *cis*-regulatory motifs across nine tissue types in humans and mice revealed fourteen motifs

that predict age dependent gene expression. Among these, the role of NFkB was tested by its inducible blockade in the epidermis of aged mice. The results were consistent with the idea that continuous activation of NFkB, which controls cell cycle exit and gene expression, is required for the maintenance of tissue specific aging and that blockade of NFkB reverses the age related gene expression signature, leads to resumption of cell proliferation and reduces senescence (333). In support of adverse effects of NFkB in aging, there are also *in vitro* studies that show that NFkB regulates cellular senescence (334-337). Activation of NFkB can lead to age related complications ranging from insulin resistance, to muscle atrophy and amyloid-beta toxicity (338-340). Therefore, it follows that although aging results from a lifetime of sequential accumulation of damage that lead to senescence and halt the proliferation and cellular functions, such changes are reversible by inhibition of NFkB that maintains the aging phenotype. The effect of NFkB is likely not mediated through a small number of genes, rather, expression of many target genes must be controlled to impart a youthful phenotype. This has been shown for DAF-16 that targets hundreds of genes and extends life-span in *C. elegans*. Mere inhibition of single genes, that are controlled by DAF-16, had a significantly less effect, as compared to the RNA interference induced inhibition of a wide range of genes that participate in stress response and metabolism (55).

## 4. CONCLUSIONS

We have witnessed a great advance in our understanding of aging, the signaling pathways that are implicated and the downstream effectors that are required for cellular homeostasis. However, our knowledge regarding the main and proximal cause of cellular alterations that lead to the age related decline in cellular functions and the real cause of aging have, thus far, eluded us. It is greatly hoped, that we can prolong a healthy life-span, reduce the age related pathologies that place a significant economic burden on our societies, and to devise strategies to reverse aging.

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**Abbreviations:** Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA), 5' AMP-activated protein kinase (AMPK), FOX (forkhead box), Nicotinamide phosphoribosyltransferase (NAM-PRTase or Nampt), Nicotinamide mononucleotide (NMN), Nicotinamide mononucleotide (NMN), Nicotinamide adenine dinucleotide (NAD<sup>+</sup>), S-adenosylmethionine (SAM), Silent information regulator 1 (SIRT1), Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase  $\beta$  (CaMKK $\beta$ ), Transforming growth factor- $\beta$ -activated kinase 1 (TAK1), Deoxyribonucleic acid (DNA), Protein phosphatases (PP), Peroxisome Proliferator-activated

receptor Gamma Coactivator 1 $\alpha$  (PPGC-1 $\alpha$ ), Dietary restriction (DR), calorie restriction (CR), Nicotinic acid (vitamin B3), Soluble form (sKlotho), Fibroblast growth factors (FGF), Hydrogen sulfide (H<sub>2</sub>S), nitric oxide (NO), Carbon monoxide (CO), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), Sulfide quinone oxidoreductase (SQR), Glutathione persulfide (GSSH or GSS<sup>-</sup>), Cysteine (Cys), 3-mercaptopyruvate sulfur-transferase (3MPST or 3MST), cystathionine- $\gamma$ -lyase (CGL, also known as cystathionase, CTH, or CSE), Cystathionine  $\beta$ -synthase (CBS or CGL), D-amino-acid oxidase (DAO), Sulfide quinone oxidoreductase (SQR), Glutathione persulfide (GSSH or GSS<sup>-</sup>), Electron transport chain (ETC), DNA-damage regulated autophagy modulator 1 (DRAM1), Insulin growth factor (IGF), Growth hormone (GH), Insulin receptor substrate proteins (IRS1-4), Protein Kinase A (PKA), Dual-specificity A-kinase anchoring protein 1 (D-AKAP1), Casein kinase 2 (CK2), Cyclic AMP (cAMP, adaptable target of rapamycin (TOR), Mitogen-activated protein kinase (MAPK), E-twenty-six (ETS), Adenylyl cyclase (AC), Extracellular signal-regulated kinase (ERK), GTPase activating proteins (GAPs), cAMP-response element binding protein (CREB), AMP-activated kinase-2 (AAK-2), cAMP-response element binding protein (CREB)-regulated transcription co-activators (CRTC), Kelch-like ECH-associated protein 1 (Keap1), Nuclear factor erythroid 2-related factor 2 (Nrf2), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF $\kappa$ B)

**Key Words:** Aging, Life-span, Life extension, Signaling pathways, Dietary restriction, Calorie restriction, Review

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