Chapter 2

Sestrins Link Tumor Suppressors with the AMPK-TOR Signaling Network

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Additional information is available at the end of the chapter

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1. Introduction

The strength of the mechanisms involved in the control of health and lifespan determines the rate of aging in any organism. Aging is fueled by the accumulation of damage in a multitude of tissues, causing many age-related diseases including cardio-vascular diseases, neuro-degenerating diseases, metabolic syndrome and cancer[1]. We know that a healthy life-style, good habits, exercise and positive attitude are all factors that support a healthy, long life while the consumption of unhealthy, low nutritional and high caloric diet, bad ecology, bad habits and constitutive stress are known to shorten life and lead to the accumulation of a number of pathologies. However, exposure to low level stresses, for example induced by exercise, increases our resistance to detrimental stress insults, this process is referred to as hermesis[2]. With respect to what is beneficial and what is detrimental for health, we still do not fully understand the underlying processes that support our health and long life nor what allows us to become more resistant to a constantly changing, and sometimes unfriendly, environment.

The causative link between aging and age-related diseases emphasizes how understanding the mechanisms that control aging could aid in the development of approaches for the prevention and treatment of many human diseases. The involvement of similar signaling pathways in the control of aging and defense against different diseases supports this concept. Two protein kinases, Target of Rapamycine (TOR) and AMP-activated protein kinase (AMPK), are central regulators of aging that are often found to be malfunctioned in many human diseases and, according to different animal models, play a role in cancer, diabetes, neurodegeneration and other syndromes[3, 4]. Strikingly, AMPK directly regulates the TOR activity, indicating that these proteins have overlapping functions and are involved in the same pathways[3]. The proteins involved in

the AMPK activation and the TOR suppression are potential regulators of longevity and aging. Among the modulators of AMPK and TOR, tumor suppressors p53 and members of the Forkhead Box O (FoxO) family play a central role in defense from stress, determination of lifespan and protection from age-related pathologies via activation of the stress-responsive Sestrin genes.

2. The function of the AMPK –TOR pathway and its role in lifespan regulation

2.1. TOR

The kinase TOR was originally identified as protein that can be inhibited by rapamycin, a macrolid found in the soil of Easter island, named after local name for the island - Rapa Nui[5]. Rapamycin is produced by the bacteria *Streptomyces hygroscopicus* and acts as an antifungal metabolite. Mutagenesis analysis in *Saccharomyces cerevisiae* led to the isolation of strains resistant to rapamycin and the identification of the two genes *TOR1* and *TOR2* that are responsible for this effect[5]. *TOR* genes encode proteins belonging to phosphatidylinositol kinase-related kinase (PIKK) family of Ser/Thr protein kinases[5]. Protein products of *TOR* genes form two complexes called TOR complex-1 (TORC1) and TOR complex-2 (TORC2)[5]. Later TOR orthologs were identified in all studied eukaryotic organisms including mammals, although the mammalian genome contains only one functional *TOR* gene (mammalian *TOR* or *mTOR*)[5]. The mammalian TORC1 (mTORC1) and TORC2 (mTORC2) complexes share several subunits: mTOR itself, lethal with Sec13-protein 8 (mLST8, also known as GβL), DEP-domain containing mTOR-interacting protein (DEPTOR) and Tti1/Tel2[3]. TORC1 contains unique regulatory-associated protein of mTOR (raptor) and proline-rich Akt-substrate 40 kDa (PRAS40)[3]. TORC2 includes the rapamycin-insensitive mTOR companion (rictor), mammalian stress-activated MAP kinase-interacting protein-1 (mSIN1) and protein observed with rictor-1 and -2 (protor1/2)[3, 6]. Raptor and Rictor determine the specificity of mTORC1 and mTORC2 toward their substrates that are responsible for mTOR-dependent processes[5, 6]. Among the two complexes, mTORC1 is sensitive to inhibition by rapamycin, although prolonged rapamycin treatment can also inhibit mTORC2[6]. Rapamycin inhibits mTORC1 via interaction with 12 kDa FK506-binding protein (FKBP12), which in complex with rapamycin, binds and inhibits mTORC1[5] (Fig.1).

mTORC1 and mTORC2 have different substrate specificity, dictated by different subunit composition. Two well-established substrates of mTORC1 are S6 kinase-1 (S6K1) and the eukaryotic translation initiation factor (eIF) 4E (eIF4E) binding protein-1 (4EBP1), involved in regulation of protein synthesis [3] (Fig.1). S6K was originally identified as a kinase phosphorylating ribosomal S6 protein although other substrates involved in the regulation of protein translation were discovered later. Among the targets of S6K1 that support translation initiation and elongation, are eukaryotic elongation factor 2 kinase,

(eEF2K), S6K Aly/REF-like target (SKAR), and 80 kDa nuclear cap-binding protein (NCBP1) and eIF4B. eIF4B, activated via S6K-dependent phosphorylation, stimulates the activation of eukaryotic translation initiation factor 4A (eIF4A), an RNA helicase that enhances translation, unwinding structured 5'-untranslated regions of many RNA[6]. 4EBP1 is another regulator of translation which, in its hypo-phosphorylated form, inhibits initiation of cap-dependent translation via interaction with cap-binding protein eIF4E, a component of eIF4F translation initiation complex. When phosphorylated by mTORC1, 4EBP1 dissociates from eIF4E allowing recruitment of translation initiation factor eIF4G and the subsequent stimulation of protein synthesis[3]. mTORC1 also phosphorylates unc-51-like kinase-1 (ULK1/ATG1) and mammalian autophagy-related gene-13 (ATG13), the components of ULK1/ATG13/FLP200(focal adhesion kinase family-interacting protein of 200 kDa) required for the activation of autophagic proteolysis (Fig.1). mTORC1 also regulates other proteins involved in autophagy such as suppressor of autophagy death-associated protein-1 (DAP1) and regulator of early autophagosome formation WIPI2, a mammalian ortholog of yeast Atg18. Additionally, mTORC1 is involved in lysosomal biogenesis through phosphorylation and inhibition of transcription factor EB (TFEB)[3].

The mTORC2 substrates appear to be members of AGC kinase family which includes AKT, serum- and glucocorticoid-induced protein kinase-1 (SGK1), and protein kinase C-α (PKCα), involved in the regulation of metabolism and viability. AKT, phosphorylated by mTORC2 in hydrophobic motif on Ser473, is an important regulator of metabolism and cell viability, while SGK1 controls growth and ion transport, and PKCα is involved in actin cytoskeleton regulation via paxilin and Rho GTPases[3, 7].

The mTOR-containing complexes have different, although in some aspects overlapping, functions[5, 6]. mTORC1 supports many anabolic processes in the cells such as protein and lipid biosynthesis, and ribosomal biogenesis[5]. mTORC1 also influences energy metabolism through the stimulation of mitochondrial respiration and glycolysis[8-11](Fig.1). The importance of mTOR in mitochondrial function is supported at several levels of control including: (i) regulation of mitochondrial biogenesis via the stimulatory effect on Ying-Yang-1 (YY1) - PPAR-γ coactivator-1α (PGC1α) transcriptional complex, involved in transactivation of mitochondrial genes and intensification of mitochondrial respiration[8]; (ii) direct effects on mitochondrial function potentially through interaction with regulatory mitochondrial proteins such as VDAC1 and Bcl-xL, which regulate mitochondrial substrate permeability and mitochondrial integrity[9]; (iii) activation of Hypoxia-Inducible Factor-1 (HIF1), composed of stable HIF1β and inducible HIF1α subunit, the activator of the genes involved in glycolysis, glucose transport and mitochondrial respiration[12]. mTORC1 activates HIF1 via the upregulation of HIF1α translation[13].

Another important function of TOR, critical for the control of metabolism and cell integrity, is the negative regulation of macroautophagy (therein called autophagy)[3, 5] (Fig.1).

Autophagy is the process of double membrane encapsulation and lysosomal degradation of cellular constituents such as organelles, protein aggregates, lipid droplets and portions of cytoplasm[14]. The process of autophagy involves a nucleation step via the formation of a preautophagosomal structure (PAT), continuing to the formation of phagophore and autophagosome vesicles. Finally, the autophagosome fuses with a lysosome allowing lysosomal degradation of the autophagosome content[14]. Autophagy plays three major roles in cells. Firstly, it provides energy through digestion of cellular constituents during starvation and other conditions affecting nutrient availability. This allows cells to survive nutrient limitations and autophagy might be vital for cell survival during stress such as ischemia. Secondly, autophagy regulates cell integrity by removing deposits and aggregates that affect normal cell physiology as well as damaged and malfunctioning organelles, which are the major source of oxidative stress[1]. In agreement, reactive oxygen species (ROS) produced by damaged mitochondria or through other mechanisms stimulate autophagy, results in suppression of oxidative stress[15]. Thirdly, completed autophagy might cause cell death, referred to as type II cell death, although the physiological relevance of this is unclear and disputable[16]. Autophagy is often associated with apoptosis and can be activated by many pro-apoptotic proteins such as p53 upregulated modulator of apoptosis (Puma) and Bcl-2–associated X (Bax) and inhibited by antiapoptotic proteins of the Bcl2 family[17-19]. In some cases, autophagy can be activated in response to pro-apoptotic stimuli as a potential pro-survival control mechanism or as a by-stander of the cell death associated with energy decline. In the other experimental settings, it was shown that autophagy mediated apoptosis in response to genotoxic stress[20]. Nevertheless, autophagy can modulate many effects of stress on cell viability and consequently, can be an important factor in anticancer treatment, which often involves extensive stress response[21].

Hyperactivity of TORC1, which leads to dysregulation of metabolism and inhibition of autophagy, is associated with extensive ROS production[1, 15, 22]. ROS, not being properly decomposed, induces oxidative stress, the major source of cell damage such as DNA-oxidation, lipid peroxidation and protein carbonylation. The accumulation of damage associated with oxidative stress is the driving force of aging, and, accordingly, TORC1 is the critical controller of aging in all eukaryotes from yeast to mammals[5, 6]. This data demonstrates that inhibition of TORC1 via mutagenesis or rapamycin treatment extends lifespan of yeast, worms, flies and mice [3, 23, 24]. Interestingly, two well-established mechanisms of lifespan extension such as caloric restriction and suppression of Insulin/Insulin growth factor-1(IGF1) -dependent signaling pathway control TORC1 activity, indicating that TORC1 inhibition may be critical for lifespan extension and suppression of age-associated pathologies imposed by low-calorie diet and inhibition of Insulin/IGF1-regulated pathway[3].

Stimulation of metabolism, cell growth and ROS by mTORC1 contributes to different pathologies including carcinogenesis, and mTORC1 is often activated in human cancers[3]. Many tumor suppressors found inactivated in cancers such as Tuberoses Sclerosis -1 and -2 (TSC1 and TSC2), liver kinase-B (LKB1), phosphatase and tensin homolog (PTEN), p53 and

neurofibromatosis type 1 (NF1) are inhibitors of mTORC1, while proto-oncogenes Ras, PI3K and AKT are mTORC1 activators[3, 5]. Obesity and type II diabetes are associated with chronic mTORC1 activation in metabolically active tissue, and mTORC1 impairs insulin sensitivity, stimulates hyperinsulemia and hyperglycemia[3, 5]. Obesity-associated mTORC1 activation also is a major risk factor for the development of nonalcoholic fatty liver disease (NAFLD), a risk factor of cirrhosis and hepatocellular carcinoma[3]. mTORC1 can also be involved in the pathogenesis of neurodegenerative disorders such as Parkinson-, Alzheimer-, Huntington- diseases, amyotrophic lateral sclerosis and frontotemporal dementia[3, 6]. All of these pathologies share similar etiology defined by the accumulation of toxic protein aggregates, which generate cellular damage, oxidative stress and cause cell death. These inclusions are cleared by autophagy, with mTORC1 potentially contributing to these syndromes via regulation of autophagy and protein synthesis, two processes affecting deposit accumulation[1, 3].

mTORC1 activity is regulated by nutrients (the source of ATP), insulin and growth factors, and amino acids through small GTPases Ras homolog enriched in brain (Rheb) and the members of Ras-related GTP-binding (Rag) protein family RagA, RagB, RagC and RagD (Fig.1). Active GTP-bound Rheb directly interacts with mTORC1 and stimulates its activity through undefined mechanism. The members of Rag family control mTORC1 in a sophisticated manner, forming heterodimers between either RagA or RagB with either RagC and RagD. Interestingly, the RagA and RagB are active in GTP-bound form, whilst RagC and RagD are functional when loaded with GDP. [6]. According to a contemporary model, active Rag complexes directly interact with the mTORC1 subunit raptor directing mTORC1 into the surface of endosomes and late lysosomes, enabling its interaction with activated Rheb[6]. As Rags do not have any membrane-targeting signals, they are delivered to lysosomal surface by Ragulator protein complex, composed of p14, MAPK scaffold protein 1(MP1) and p18[6]. The activity or Rag proteins is regulated by amino acids with Rags being critical transducers of the activating signal from amino acids to mTORC1[3]. As described recently, Rag activation in response to amino acids is mediated by leucyl-tRNA synthetase, which binds Rag proteins and acts as GTPase-activating protein (GAP) for Rags[25]. The other proposed mechanisms of mTORC1 activation by amino acids involve mitogen-activated protein kinase kinase kinase (MAP4K3), inositol polyphosphate monokinase (IPMK) and mammalian vacuolar protein sorting 34 homolog (hVps34), belonging to class 3 PI3K[3, 26]. hVps34 is required for activation of phospholipase-D (PLD) in response to availability of amino acids. Amino acids induce interaction between phosphatidylinositol 3-phosphate and the Phox homology (PX) domain of PLD1, which causes PLD translocation to the lysosomal compartment, required for mTORC1 activation[27].

Rheb GTPase is a critical regulator of mTORC1 in response to insulin, growth factors, energy and stress[1, 6]. Rheb is negatively controlled by the TSC1:TSC2 protein complex where TSC2 is GAP for Rheb, while TSC1 plays a supporting role stabilizing TSC2[28]. The TSC1:TSC2 complex integrates signals from different signaling pathways that positively or negatively modulate the TSC1:TSC2 activity [1]. Insulin and IGF1, the activators of cell

growth, stimulate mTORC1 via inhibitory phosphorylation of TSC2 by AKT[3]. Insulin/IGF1 stimulate Insulin/IGF1 receptor (In/IGF1R), which through the engagement of insulin receptor substrate-1 (IRS1) tethers and activates phosphatidylinositol-3-kinase (PI3K), which then converts phosphatidylinositol-4,5-biphosphates (PIP2) into phosphatidylinositol-3,4,5-triphosphates (PIP3). PIP3 induce AKT phosphorylation on Thr308 by phosphoinositide-dependent kinase-1 (PDK1) kinase recruiting both kinases to cytoplasmic membrane via their pleckstrin homology (PH) domains. PI3K also stimulates phosphorylation of AKT on Ser473 by mTORC2 which is required for full AKT activation[3, 7, 15]. The PI3K activity is counteracted by the tumor suppressor PTEN, which is a PIP3 phosphatase[3]. Activated AKT directly phosphorylates TSC2 on multiple sites, causing mTORC1 activation. In parallel AKT also stimulates mTORC1 via phosphorylation of mTORC1 interacting protein PRAS40, an inhibitor of mTORC1, and inhibitory phosphorylation of both TSC2 and PRAS40 cooperates in mTORC1 activation[3]. IGF1 also activates Ras GTPases, activating extracellular-signal-regulated kinase (ERK) and ribosomal S6 kinase (RSK1)[3]. Both ERK and RSK1 directly phosphorylate TSC2, which cause inhibition of its activity and TORC1 activation[3]. Inflammation, accompanied by production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), stimulates mTORC1 via IκB kinase (IKKβ)-mediated phosphorylation of TSC1[3]. The canonical Wnt signaling pathway, the regulator of embriogeneis, provides another important mechanism of mTORC1 regulation. The Wnt pathway inhibits glycogen synthase kinase-3β (GSK3β), which under normal conditions phosphorylates and activates TSC2[3] (Fig.1).

2.2. AMPK

While Insulin, growth factors and nutrients inhibit the TSC1:TSC2 complex and stimulate mTORC1, many stress insults have the opposite effect, activating TSC2 and inhibiting TORC1. Inhibition of mTORC1 under stress conditions allows cells to stop cell growth and proliferation in the unfavorable conditions switching to high-economy mode and supporting stress-relieving measures. Nutrient deficiency and many metabolic derangements cause an accumulation of AMP and ADP, which, in turn, activate AMPK. AMPK inhibits mTORC1 through phosphorylation of TSC2 and raptor[4]. Besides AMP accumulation, AMPK can be activated by different insults such as oxidative stress, DNA-damage or the accumulation of Ca^{2+} potentially through an AMP-independent mechanism. Also some hormones such as leptin and adiponectin are able stimulate AMPK through mechanisms that are yet to be defined[4].

AMPK is a protein complex composed of 3 subunits catalytical AMPKα, (encoded by AMPK*α*1 and *α*2 genes scaffold AMPKβ subunit, (encoded by *AMPKβ1* and *β2* genes) and regulatory AMPKγ subunit, (encoded by *AMPKγ1*, *γ2* and *γ3* genes) in mammals. AMPK is activated via phosphorylation of AMPKa subunit on Thr172, although phosphorylation of other sites on AMPK*α*, β and γ subunits are also involved in AMPK regulation[4]. The upstream AMPK kinase critical for AMPK activation in response to energy deficiency is

LKB1, which constantly phosphorylates AMPKα subunit on Thr172. The most established mechanism of AMPK activation involved binding AMP by AMPKg subunit which stimulates conformational changes in AMPKα subunit making it less accessible for AMPK phosphatases[4, 29]. Several protein phosphatases (PP) have been shown to be involved in the regulation of AMPK phosphorylation including PP2C[30], PP2A[31, 32], PPM1E[33], PP1[33, 34]. LKB activity can be stimulated by oxidative stress via the phosphorylation by ataxia telangiectasia mutated (ATM) kinase, leading to AMPK activation[35]. Other AMPK kinases shown to directly phosphorylate AMPK are Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII), activated by accumulation of Ca^{2+} ions, and TAK kinase[36], activated in response to treatment with TNF-related apoptosis-inducing ligand (TRAIL) [37](Fig.1). Some proteins such as kinase repressor of Ras-2 (KSR2) can also regulate AMPK phosphorylation working as scaffold protein through regulation of access of either protein kinases or phosphatases to AMPKα subunit[38].

Activated AMPK phosphorylates many targets involved in the regulation of glucose metabolism: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), glycogen synthase-1 (GYS1), glutamine:fructose-6-phosphate amidotransferase (GFAT1), TBC1D1; lipid metabolism: acetyl- CoA Carboxylase-1 and -2 (ACC1 and ACC2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), PLD1; polarity: cytoplasmic linker protein-170 (CLIP170), golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1), kinesin light chain-2 (KLC2); transcription: (PGC1α, sterol regulatory element-binding protein-1 (SREBP1), FoxOs, Histone-2B, p300 and HDAC4,5,6; and mitosis: protein phosphatase-1 regulatory subunit 12C (PPP1R12C), p21-activated protein kinase (PAK2)[4, 39]. AMPK also contributes to mitochondrial function stimulating mitochondrial biogenesis via phosphorylation of PGC1α co-activator and regulating expression of mitochondrial genes[4]. AMPK regulates cell growth and autophagy in part, through TORC1 inhibition, although as shown recently AMPK can stimulate autophagy through direct phosphorylation of autophagy ULK1 protein. AMPK is also involved in the regulation of cell death in response to genotoxic stress, although the mechanisms are yet to be described[40-42].

Interestingly, lipid biosynthesis and autophagy controlled by mTORC1 via SREBP1 and ULK1, are under direct control by AMPK. AMPK directly phosphorylates and inhibit SREBP1 protein, suppressing lipogenesis[43]. AMPK also phosphorylates and activates ULK1 stimulating autophagy [44]. The redundancy of the mechanisms of regulation of lipid biosynthesis and autophagy though direct and indirect effects of AMPK demonstrates the critical role of AMPK in these processes and the importance of accurate regulation of these processes by mTORC1 and AMPK.

The regulation of metabolism and autophagy by AMPK potentially contributes to lifespan regulation. Accordingly, the inactivation of one of the AMPK subunits (AMP-activated kinase-2 (AAK-2)) in *Caenorhabditis elegans* shortened lifespan by 12%, while animals with increased AAK-2 expression lived 13% longer than their wild type (WT) counterparts[45].

The mechanism of life extension by AMPK involves CRTC-1 and FoxO transcriptional factors[46, 47]. Interestingly, the activity of AAK-2 is also required for protection against oxidative stress [48]. Similar results were obtained in *Drosophila Melanogaster* model, where the inhibition of drosophila AMPK shortened lifespan and increased susceptibility to oxidative stress and starvation[49] In accordance, gain-of-function LKB1 mutant extended lifespan in flies[50]. Although there is no such evidence for mammals, mammalian AMPK presumably plays a similar role in lifespan regulation. This idea is supported by the observation that AMPK activity is decreased in old animals potentiating the aging process[51].

Although the effects of AMPK on the lifespan regulation in mammals are not known, AMPK is involved in protection from different diseases[4, 52]. AMPKα2 subunit controls of glucose metabolism and AMPKα2 mice have pro-diabetic phenotype associated with diminished insulin secretion and glucose intolerance[53]. AMPK can also affect lifespan via the suppression of inflammation, a process associated with aging and many age-related diseases, including cancer[54]. Metformin, the most commonly prescribed anti-diabetic drug, activates AMPK and its activation is required for effect of metformin on glucose production in hepatocytes and, potentially, in glucose uptake in muscle[55]. Resveratrol, the plant-derived polifenol, which prolongs lifespan and improves health conditions of mice kept on high-calorie diet, also activates AMPK, which can potentiate the effects of resveratrol on health and aging [56].

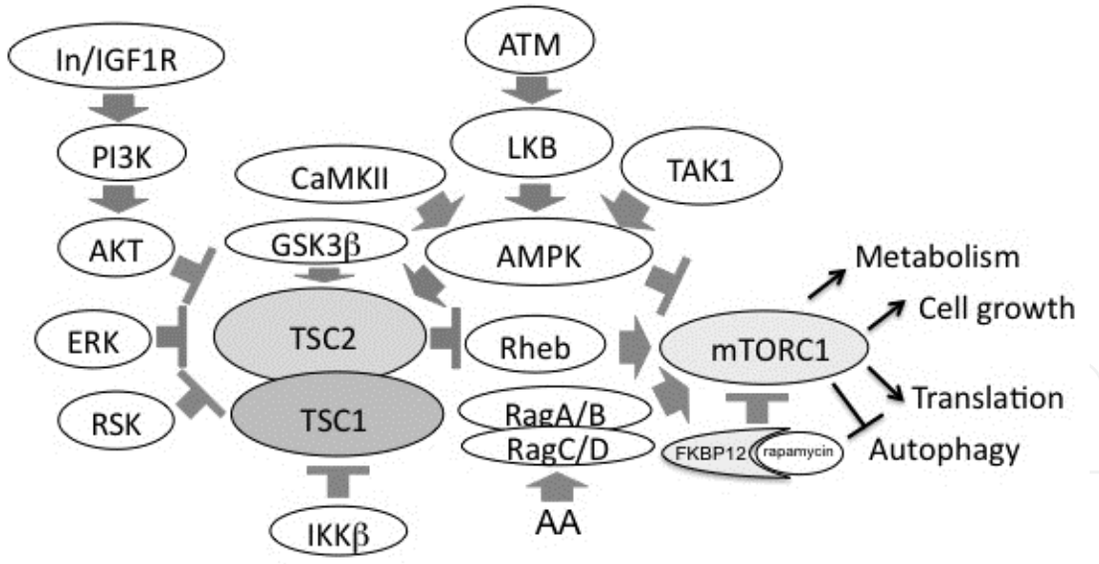

Figure 1. Regulation of the AMPK-mTORC1 axis and the role of mTORC1 in cellular processes. mTORC1 is activated by insulin and IGF1 via stimulation of the PI3K-AKT pathway, followed by inhibitory phosphorylation of TSC2 by AKT. The other signaling pathways stimulate mTORC1 through activation of ERK, RSK and IKKβ kinases in a TSC1:TSC2-dependent manner. TSC1:TSC2 inhibition leads to stimulation of Rheb, an activator of mTORC1. mTORC1 activation also required amino acids (AA) which stimulate mTORC1 via Rag complexes. Many stress insults activate AMPK which inhibits mTORC1 modulating many mTORC1-dependent processes such as metabolism, protein synthesis, cell growth and autophagy.

3. p53 and its role in aging and diseases

Among the proteins involved in the lifespan regulation, the tumor suppressor p53 plays a central role. Originally identified as interactor of human polioma SV40 virus large T antigen, accumulated in transformed cells[57], it was proposed to be involved in cell transformation. Studies further demonstrated that p53 suppressed cell transformation and carcinogenesis[58]. Furthermore, p53 gene is mutated in more than 50% of human tumors, supporting the idea that p53 inactivation is a critical step in carcinogenesis[58]. The patients with Li-Fraumeni syndrome, characterized by predisposition to different form of cancer at early age, often carry a mutant p53 allele[58]. In the following study the tumor suppressive function of p53 was confirmed in mouse knockout model where *p53*-deficeint mice developed cancers and chronic inflammation and the median of survival of *p53*-null mice is six months [59]. The extensive efforts to understand the function of p53 led to characterization of many p53 activities such as the regulation of genomic stability, control of cell cycle, induction cell death and senescence, and suppression of angiogenesis. All these mechanisms potentially contribute to tumor suppressive function of p53, although the mechanisms most critical for tumor suppression are yet to be defined[58].

The vast majority of processes controlled by p53 are exhibited through the regulation of gene expression. Being a transcription factor, p53 directly activates many genes involved in DNA repair such as growth arrest and DNA damage-45 (GADD45) and p53R2; cell cycle arrest and senescence: p21, 14-3-3σ and plasminogen activator inhibitor-1 (PAI-1); cell death: Bax, Puma, apoptosis inducing factor (AIF), Noxa; and inhibition of angiogenesis: trombospondin-1 (TSP-1)[60] (Fig.2). p53 activity is regulated through several mechanisms, many involving protein stabilization. p53 trancriptionally activates its negative regulators murine double minute 2 (MDM2), p53-induced protein with a RING-H2 domain (Pirh2) and constitutively photomorphogenic 1 (COP1), which bind and stimulate p53 degradation working as p53 E3-ubiquitine ligases[61].

The elimination of damaged and modified cells, imposing the threat to the organism, is not the only strategy to fight cancer. It seems reasonable that many functions of p53 in the prevention of carcinogenesis operate via the regulation of metabolism and stress response. Not being properly controlled the derangements of these processes can lead to the accumulation of damage, the major source of mutations[15]. We have described that one of the mechanism involves the regulation of ROS, which being accumulated, might fuel mutagenesis and genomic instability[15]. ROS can also stimulate the cell cycle, migration and angiogenesis, so they can support initiation, promotion and progression of carcinogenesis[62]. Accordingly, our studies showed that inactivation of p53 via different mechanisms such as knockout, knockdown with shRNA, over-expression of Mdm2 or dominant-negative form of p53 led to ROS accumulation causing oxidative DNA-damage, increased rate of mutagenesis and genomic instability[62]. A xenograft study with lung adenocarcinoma A549 cells showed that p53 silencing accelerated tumor growth, and it was reverted by treatment with an antioxidant N-acetylcysteine (NAC)[62]. Moreover, p53-

knockout mice, predisposed to carcinogenesis, were protected from the disease by NAC treatment and had significantly extended lifespan as compared to vehicle-treated controls[62]. A number of p53 targets with antioxidant activities including Sestrins, glutathione peroxidase 1 (GPx1), manganese superoxide dismutase (MnSOD), catalase, aldehyde dehydrogenase 2 (ALDH1), gutaminase 2 (GLT2) and p53-induced nuclear protein 1(INP1)[1, 15] were identified (Fig.2).

Some of the p53-inducible proteins such as TP53-induced glycolysis and apoptosis regulator (TIGAR), an inhibitor of glycolysis, regulate ROS via control of metabolism. TIGAR lowers the fructose-2,6-bisphosphate levels and suppresses activity of phosphofructokinase-1 (PFK1) [63]. It can lead to the re-direction of glycolytic intermediates into the pentose phosphate pathway providing cells with NADPH, the important reducing equivalent for antioxidant reactions in the cell[64]. p53 also coordinates bioenergetic processes such as mitochondrial function regulating mitochondrial respiration via activation of cytochrome C oxidase 2 (SCO2), regulator of complex IV, subunit I of complex IV and apoptosis inducing factor 1 (AIF)[64]. In addition Parkin (PARK2), a gene associated with Parkinson disease, is a p53 target, inactivation of which leads to enhancement of glycolysis, suppression of mitochondrial respiration and ROS accumulation[65]. As a result, p53 may control different metabolic pathway involved in ROS production. On the contrary when stress is too high and damages are irreparable, imposing the threat of mutations and genomic instability, p53 induces ROS production through up-regulation of proapoptotic genes Puma, Bax and PIG3, facilitating cell disorganisation during apoptosis[62, 66] (Fig.2).

The protective effects of p53 can also be mediated by regulation of the AMPK-mTORC1 axis[15]. Elevated activity of mTORC1 is associated with the accumulation of damage and oxidative stress[15, 67], resulting in exacerbated aging and imposing health risks. Overactivation of mTORC1 might be even more detrimental under stress conditions such as genotoxic stress, when the anabolic and catabolic processes should be coordinated to enable the repair and elimination of the damage. Accordingly, it was shown that p53, activated by DNA-damage and via other mechanisms, inhibited mTORC1 and mTORC1-dependent translation[68, 69]. p53 inhibits mTORC1 through activation of genes involved in negative regulation of mTORC1, such as a suppressor of insulin signaling insulin-like growth factor binding protein 3 (IGF-BP3), PTEN, TSC2, AMPKb1, Sestrin1 (Sesn1) and Sestrin2 (Sesn2)[15]. mTORC1 inhibition by p53 is also mediated by stimulation of AMPK phosphorylation on Thr172[70]. The other potential mechanism involves the clearance of epithelial growth factor receptor, the activator of PI3K-AKT-mTORC1 pathway from the surface, disabling the signaling from the receptor[71, 72].

Autophagy is another mechanism imposed by p53 to protect cells from the accumulation of damage [71] (Fig.2). p53 activates autophagy via transcriptional activation of genes involved in the regulation of the AMPK-mTORC1 pathway[70, 73], although other p53 targets are directly involved in the autophagic process or operate through other mechanisms. The list of pro-autophagic genes regulated by p53 includes lysosomal protein

damage-regulated autophagy modulator (DRAM), ULK1[74], p53INP1 (which interacts with ATG8 and promote autophagic cell death)[75], Bax, Puma[18] and a new gene with unknown function ISG20L1[76]. Interestingly that autophagy also contributes to p53-induced cell death, potentially providing the ground for elimination of damaged cells, if they are irreparable. The cytoplasmic form of p53 can negatively regulate autophagy, counteracting the effects of the nuclear form of p53[77]. This potential mechanism involves interaction with components of autophagic machinery, such as family kinase interacting protein 200 (FIP200/ATG17)[78].

The effects of p53 on the control of stress response, metabolism, ROS and autophagy connect p53 with the lifespan regulation and longevity. p53 knockout mice develop cancer and die by the age of 6 months, indicating a critical role of p53 in the lifespan regulation through suppression of carcinogenesis[59]. Nevertheless, this model precluded characterization of the role of p53 in aging and longevity. To study the effects of p53 on aging several models were established and these models show different, sometimes contradicting, phenotypes.

In the first model, mice expressed a truncated p53 lacking exons 1-6 (called m allele in $p53^{+/m}$ mice). The $p53^{m}$ locus produced a 24K C-terminal part of the p53 protein[79]. Interestingly, these mice demonstrated 23% reduction in median of longevity. Although young (3-12 months old) mice did not show any difference compared with WT mice, after 18 months $p53^{+/m}$ mice revealed exacerbated aging-related phenotype exhibiting weight loss, lordokyphosis, and an absence of vigor[79]. Tissue analysis demonstrated a decrease in muscle and adipose tissue as well as reduced kidney, liver, spleen and testes mass, typical features of aging. Also, mice showed thickness in both bone density and dermal thickness, and defects in wound healing. Moreover, the mice had reduced survival in response to stress[79]. Surprisingly the frequency of many other pathologies associated with aging including liver diseases, brain atrophy, hair graying and alopecia, skin ulceration, amyloidal deposits and cataract were not increased in this model[79]. The feature reported in the $p53^{+/m}$ mice was a decreased predisposition to cancer[79].

As reported in another study, mice with increased levels of expression of natural N-terminally truncated p53 (p44) had an accelerated aging phenotype, deteriorated health and decreased lifespan, characterized by defects in fertility linked with testicular degeneration, lordokyphosis and decrease in bone density, cognitive decline and synaptic deficit early in life[80, 81]. These mice demonstrated overactivated IGF1R signaling and many phenotypes were rescued by IGF1R heterozygosity. p44 also exacerbated a neurodegenerative phenotype in the mouse model of Alzheimer's disease based on the overexpression of human amyloid precursor protein (APP), where p44 facilitated degeneration of memory-forming and memory retrieving areas of the brain[81].

A common phenotype observed in these two models is accelerated aging and decreased susceptibility to carcinogenesis[79, 80]. As it was suggested, p53 is overactivated by short

form and stimulated an aging phenotype, it also provided better protection from cancer[79, 80]. An alternative explanation of the effects of truncated p53 on lifespan and aging involves suppression of many p53 activities and downregulation of some critical genes involved in the stress response and metabolism, resulting in an effect on viability, although the expression of some p53 targets was still retained and even increased. Both artificial 24K, and natural p44 forms of p53 lack transactivation domains, which are required for the most of p53 activities including tumor suppression[82]. Both truncated forms bind full-size p53 and counteract many of its activities similar to the effects of N-terminally truncated forms of the other p53 family members p63 and p73 on full size proteins[83]. Accordingly, overexpression of p44 causes accumulation of p53 and disability of full-size p53 to transactivate most of its targets[80, 84, 85]. The truncated p53 retains a full form in cytoplasm, inhibiting the p53 nuclear function [86]. Also, an inhibitory effect of truncated p53 is evident as p44 may be selected in human cancers, supporting carcinogenesis[87]. Thus truncated p53 might compromise p53 activities rather than enhance them. Simultaneously, some p53 functions, for example those involved in ROS production and senescence, might be intact or even increased causing accelerated aging phenotype. A more widespread analysis of p53 targets is required in these models to better understand the impact of truncated p53 in this phenotype.

Other models indicate that tumor suppressive and aging-regulatory functions of p53-dependent might be separated. In one model, a genomic segment containing whole p53 gene was integrated in the mouse genome (p53Tg mice). These mice exhibited increased p53 response to DNA-damage and were able to rescue the cancer-prone phenotype of *p53*-deficient mice. The p53Tg mice were resistant to carcinogenesis, but in contrast to previous models, they did not reveal any signs of accelerated aging. Interestingly, a similar phenotype was observed with *Mdm2* hipomorphic (haploinsufficient) mice, characterized by increased p53 activity, which were cancer resistant but aged normally[88, 89].

MDM2 binds p53 and stimulates protein degradation, inhibiting p53 activity. The Arf tumor suppressor, a product of the INK4A locus, stimulates p53 activity antagonizing MDM2-dependent degradation. Transgenic mice with an extra copy of the Arf gene, along with an extra-copy of p53 gene (super-Arf/p53 (s-Arf/p53) mice) demonstrated increased activity of p53 and enhanced expression of p53-dependent p21 and antioxidant Sesn1 and Sesn2 genes. These mice were resistant to cancer, similar to the p53Tg mice, and fibroblasts from s-Arf/p53 mice were resistant to immortalization and transformation, implying low susceptibility of these mice to carcinogenesis. In contrast to previous models, these mice had an extended average lifespan of 16% and show signs of delayed aging as evident by the test on neuromuscular coordination (tightrope success test) and hair re-growth test. Aging is associated with the accumulation of DNA-damage and oxidative stress, and s-Arf/p53 mice exhibited decreased levels of DNA damage as illustrated by H2AX staining and decreased oxidative stress evident from analysis of ROS

in splenocytes, decreased lipid peroxidation and low abundance of carbonylated proteins in liver. The s-Arf/p53 mice were also resistant to oxidative stress, emphasizing the potential role of the mechanisms of stress response in suppression of aging and carcinogenesis[90]. This data is in concordance with previous observations that showed that inactivation of p53 or its upstream regulator ATM decreased lifespan and caused accumulation of oxidative damage in mice[62, 91]. Both *p53*- and *ATM*-null mouse strains were predisposed to cancer, and these phenotypes were prevented by antioxidant NAC treatment[62, 92]. Interestingly, the activity of p53 is decreased with age supporting a concept that p53 might be an important anti-aging factor[93].

Suppression of carcinogenesis was not the primary function of p53 in evolution as p53 is found in the organisms, such as *Caenorhabditis elegans* and *Drosophila Melanogaster,* which do not develop cancer. In the *Drosophila* model, overexpression of p53 in adult flies increased the lifespan of males, but limited lifespan in female flies. Also, the moderate overexpression of p53 during larvae stage extended lifespan in both male and female flies. *p53*-deficient flies were sick and had a decreased lifespan[94]. It is possible that p53 controls the accumulation of damages as well as other functions. Interestingly, the overexpression of dominant-negative form of p53 in flies brain extended their lifespan [94]. This data suggests that p53 can play an opposite role in lifespan regulation and aging in the peripheral organs and in the brain, where it can potentiate hormone production or other processes affecting aging. p53 can also regulate hermesis in response to γ-irradiation[95] and, moreover, p53 also potentiates life extension in response to the mitochondrial stress associated with downregulation of mitochondrial genes[96]. Accordingly, it was reported that the positive and negative effects of p53 on lifespan was dependent on the level of mitochondrial bioenergetic stress[97].

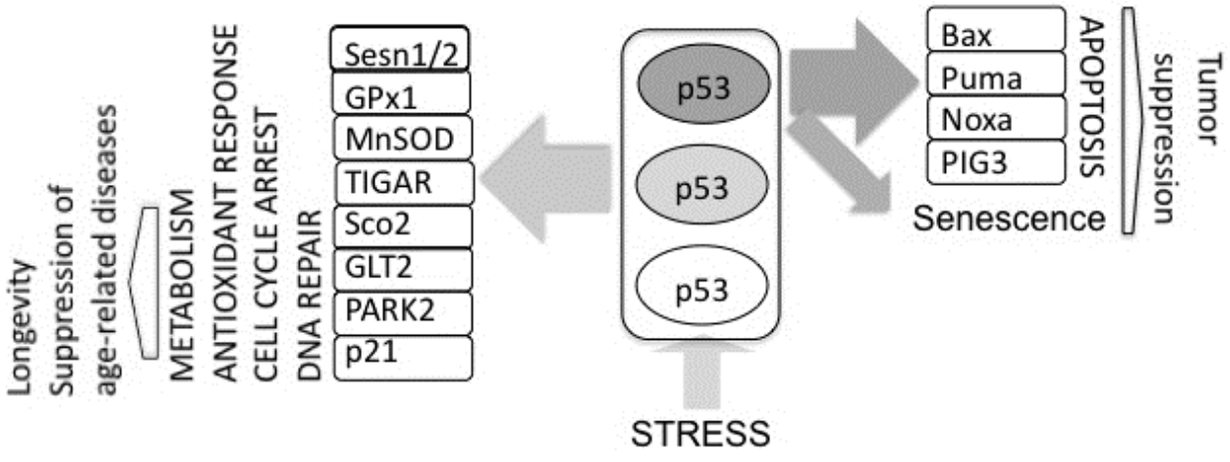

Figure 2. Tumor-suppressor p53 regulates lifespan and suppresses age-related diseases. p53 is activated in response to different stress insults and dependent on the stress intensity triggers different sets of genes either supporting pro-survival or cell death programs. p53 can support longevity and suppress age-related diseases via regulation of DNA-repair, cell cycle, metabolism and antioxidant response. Otherwise p53 eliminates damaged and potentially dangerous cells through activation of apoptosis or senescence, suppressing carcinogenesis.

4. Role of FoxO family in cellular processes and longevity

Another group of proteins playing a critical role in the lifespan regulation and aging are the transcription factors of the FoxO family. Analysis of *Caenorhabditis elegans* mutants, which extended lifespan of the worms, led to the identification of DAF2 and AGE1 genes, the orthologues of the mammalian In/IGF1R and PI3K genes respectively. Simultaneous inactivation of DAF16 suppressed extended lifespan phenotype of the *DAF2-* and *AGE1-* mutants providing a link between DAF2, AGE1 and DAF16. There is only one FoxO gene in invertebrate genome (DAF16 in *Caenorhabditis elegans*; dFoxO in *Drosophila Melanogaster*) while a mammalian genome is composed of four members FoxO1, FoxO3A, FoxO4 and FoxO6[98]. In spite of high sequence similarity and recognition similar sequences in the promoters of target genes, the mammalian FoxO family members have different tissue-specific expression and potentially different, although overlapping, functions in the organism. According to knockout studies, inactivation of FoxO1 is embryonically lethal due to insufficient vascularisation of the embryo. *FoxO3A*-deficient animals are viable, although demonstrate defects in ovarian follicle activation, while *FoxO4*-deficient animals are viable with no significant abnormalities[98].

Insulin/IGF1 signaling inhibits FoxO via activation of the PI3K-AKT pathway(Fig.3), followed by phosphorylation the FoxO proteins by AKT on highly conserved residues. AKT phosphorylation makes FoxOs susceptible for interaction with 14-3-3 proteins, which bind and retain FoxOs in the cytoplasm masking their nuclear localization signal[98]. Thus, AKT phosphorylation invalidates the transcriptional activity of FoxOs, inhibiting FoxO-dependent processes. On the contrary, FoxO can be phosphorylated and activated by Jun N-terminus kinase (JNK) and STE20-like protein kinase 1 (MST1). JNK and MST1 can be activated by different stress insults, most evidently oxidative stress, with activating phosphorylation of FoxOs by JNK and MST1 having predominate inhibitory effects of AKT[98, 99]. Strikingly, prolonged activation of In/IGF1R stimulates ROS production, which leads to JNK activation followed by FoxO phosphorylation and nuclear translocation, counteracting effects of AKT on FoxO suppression. Similar to FoxOs, JNK is an important regulator of longevity[100], extending lifespan in worms and flies by suppressing Insulin/IGF1 signaling pathway [101, 102].

FoxOs impinge on the control of lifespan potentially via transcriptional regulation of genes involved in the stress-response, although the critical targets involved in this process are unknown. Potential candidates are ROS scavengers, such as: MnSOD, Catalase and peroxiredoxin3 (Prx3); regulators of protein synthesis, such as: 4EBP1 and regulators of autophagy, including BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), LC3 and Garabl12[98]. Interestingly, FoxO can also stimulate autophagy through a mechanism independent of its transcriptional activity[103]. Besides the activation of stress-relieving pathways, FoxOs also inhibit cell cycle though several mechanisms, such as activation of cell cycle inhibitors p27 and p21, and suppression of cell cycle regulators c-Myc and cyclin D[98, 99]. Accordingly, overexpression of FoxO3A causes G2-M delay in

fibroblasts[98]. Regulation of the cell cycle by FoxOs might be important for stress-relieving mechanisms, as cells need to restrain proliferation in order to restore homeostasis and avoid accumulation of damages[98].

The activities of FoxOs are not limited to the activation of stress-relieving and pro-survival pathways. FoxOs also activate the expression of proapoptotic proteins Bim, Puma, Fas ligand (FasL) and TRAIL and stimulate cell death under certain conditions[98]. The exact mechanism that discriminates between pro-survival and pro-death programs regulated by FoxOs are unknown, the outcome of FoxOs activation might be dependent on severity of stress and cell type.

The regulation of pro-survival stress-relieving genes and pro-apoptotic genes by FoxOs is reminiscent of the effects p53 on cell homeostasis, which protects under low stress conditions and induces cell death when stress is too strong and causes accumulation of damages within the cell[15]. Strikingly, FoxOs and p53 are able to activate the same targets including MnSOD, catalase, Sesn1, p21 and Puma[15, 98]. It is possible that there is some functional redundancy in the regulation of stress response by p53 and FoxO proteins, ensuring the proper outcome should one of the pathways be disabled. Interestingly, p53 and FoxOs are under mutual regulation. p53 positively regulate FoxO3A via transcriptional activation of FoxO3A gene[104], although p53 also stimulates expression of MDM2, which is involved in FoxO ubiquitination and degradation[105]. Surprisingly, in response to DNA-damage p53 can inhibit FoxO3A via SGK1 kinase activation and subsequent FoxO3A retention in cytoplasm[106]. FoxOs can also regulate p53 in a positive or negative manner. FoxOs stimulate activity of AKT, which negatively regulates p53 via phosphorylation of MDM2[99]. FoxO3A can also impair p53 transactivational function, although can positively regulate p53-dependent cell death in serum starved cells[107]. Otherwise, FoxO3A directly interacts with the ATM kinase, upstream p53 activator, and stimulates ATM phosphorylation on Ser1981, regulating DNA-damage response which might be involved in p53 activation[108]. FoxOs can also upregulate p53 via activation of upstream p53 regulator Arf[109]. This complicated picture illustrates that there is a communication between p53 and FoxOs providing the mutual control of activity determining cell fate (Fig.3).

The antioxidant function of FoxOs is especially important for control of stem cell maintenance. The mice with simultaneous inactivation of FoxO1, FoxO3A and FoxO4 in hematopoietic system had diminished number of hematopoietic stem cells (HSC) in bone marrow, paralleling expansion of myeloid progenitor cells in the blood. *FoxO*-deficient HSC had elevated levels of ROS in comparison to WT control and have strong defects in the ability to restore hematopoietic system of recipient mice, indicating the critical role of FoxO in self-renewal of stem cells. These defects were rescued by treatment with antioxidant NAC, confirming the critical role of FoxO in the regulation of HSC ROS, which is important for regulation of HSC quiescence and regenerating ability[98].

Another function of FoxOs is the control of metabolism. FoxO1 is highly expressed in insulin-responsive tissues and regulates glucose and lipid metabolism, ensuing adaptation to different feeding conditions. In response to the decrease of insulin levels, FoxO1

intensified gluconeogenesis in the liver through regulation of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPK). FoxO1 also stimulated transcriptional co-activator PGC1α and synergizes with PGC1α for G6Pase transactivation. The activation of G6Pase and PEPK ensures stable blood glucose levels in fast conditions. FoxO1 also regulates lipid metabolism via activation of an inhibitor of lipoprotein lipase apoliprotein (ApoCIII), which is involved in hypertrigyceridemia development in diabetic patients[98].

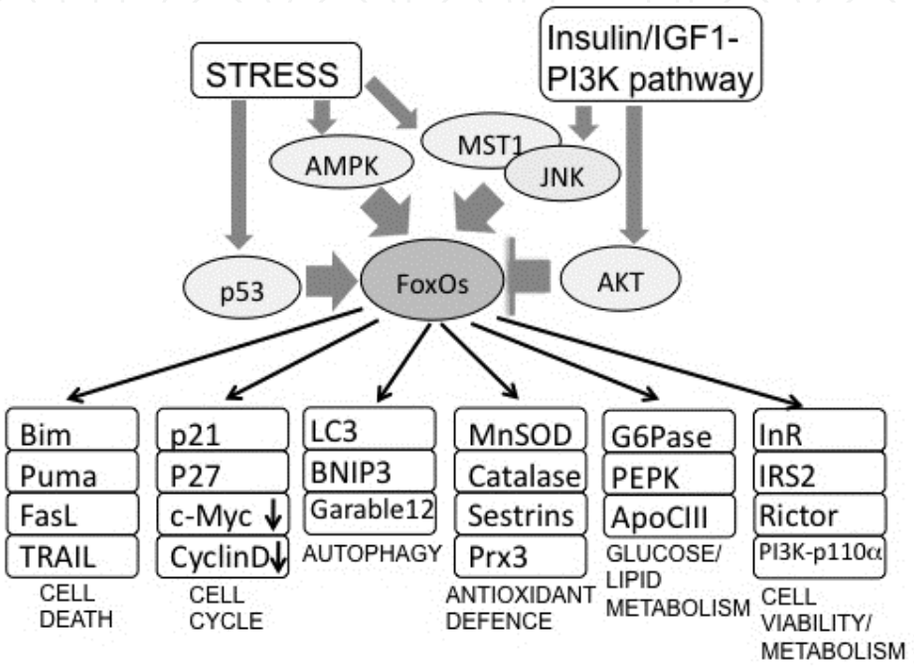

Figure 3. Stress and the insulin/IGF1-PI3K-AKT pathway control cellular processes through regulation of FoxOs. While many stress insults activate FoxOs, the Insulin/IGF1-PI3K-AKT inhibits their activity. Thus FoxO factors integrate the information from different signaling pathways and control the expression of genes involved in regulation of cell proliferation and viability, autophagy, metabolism and cell death. As a result they contribute to regulation of longevity by FoxOs.

The regulation of genes involved in glucose and lipid metabolism underlines the potential role of FoxOs in diabetes which might work in a protective fashion or exacerbate the diabetic phenotype. Insulin resistance and glucose intolerance, the hallmark of type II diabetes, are characterized by the suppression of the PI3K-AKT signaling pathway [110]. This might be due to the elevated activity of mTORC1 which phosphorylates and regulates IRS1 and Grb10 proteins[3]. IRS1 transduces signals from In/IGF1R toward PI3K and its phosphorylation by mTORC1-dependent p70S6K causes its degradation. Grb10 negatively regulates growth factor signaling through binding of IGF1R and its phosphorylation by mTORC1 stabilizes and activates Grb10[111, 112]. FoxOs can modulate insulin signaling through AMPK activation and TORC1 inhibition as well as via transcriptional activation of IRS2, PI3K (p110a) and InR [99] and the activation of these proteins potentially support insulin sensitivity. FoxOs also activate rictor, the critical component of TORC2 complex required for phosphorylation and full activation of AKT [7, 99]. In contrast, the diabetic

phenotype caused by InR deficiency was rescued by FoxO1 heterozygosity, where deletion of one FoxO1 allele restored insulin sensitivity[98], indicating that tight control of FoxO activity was required for proper protective function of FoxO factors.

5. Identification and characterization of Sestrins

Sestrins are highly conserved gene gamily found in all multicellular organisms of the animal kingdom. The invertebrate genome contains one Sestrin (Sesn) gene while there are three genes in vertebrates Sesn1, Sesn2 and Sesn3[15]. Sesn1, originally named p53-activated gene #26 (PA26), was identified as a p53-inducibe gene in a screening where p53 was induced in a tetracycline–dependent manner[113]. The human *SESN1* gene is found in 6q21 position[114] and is transcribed into three different mRNA translated into proteins with Mw~46, 55 and 68kD[114]. The Sesn1 mRNAs are transcribed from 3 promoters using an alternative first exon (Exon 1,2 or 3), which is spliced with the common exon 4[114]. All protein products of *SESN1* share the same C-terminal part encoded by exons 4-10. Among the three transcripts only short transcripts 2 and 3 are induced by p53, while transcript 1 is constantly expressed regardless of p53 status[114]. The gene is ubiquitously expressed in all tissues, although at different levels and predominantly in the pancreas, kidney, skeletal muscle, lung, placenta, brain, ovary and testis[114]. Sesn1 is a stress-responsive gene activated in response to genotoxic stress imposed by γ-irradiation, UV-light and doxorubicin treatment in a p53-dependent manner[114]. Sesn1 is induced with kinetics similar to the "classical" p53-inducible genes MDM2 and p21, indicating that this is a direct p53 target[114]. p53-responsive elements were identified within intron 1[115] and intron 2[114] of the Sesn1 gene, although the exact role of either of these elements in Sesn1 activation by p53 *in vivo* requires additional analysis. Besides genotoxic stress, Sesn1 is also activated in response to serum withdrawal[114], indicating the regulation by a transcription factor(s), which are under control by growth factors. Among them, FoxOs are the most prominent candidates, which expression is negatively regulated by insulin and IGF1 through activation of the PI3K-AKT signaling pathway followed by inhibitory phosphorylation of FoxOs by AKT[98, 99]. Accordingly, Sesn1 was identified by microarray analysis and real time PCR as a gene activated by FoxO3A[116] and FoxO1[117, 118] (Fig.4).

Sesn2 was identified by microarray analysis as a gene activated by severe hypoxia in glioblastoma A172 cells [119]. The human *SESN2* gene is located in position of 1p35.3[119] and is transcribed into one mRNA that encodes a polypeptide with Mw~60 kDa[119]. Similar to Sesn1, Sesn2 is expressed in all tissues, but predominantly in the placenta, lung, liver, kidney, pancreas, testis and leucocytes (AVB and Chumakov PM, unpublished). Besides hypoxia, Sesn2 is activated in response to many stress insults including oxidative stress, DNA-damage and metabolic derangements[119]. In spite of the important role of HIF1 in the regulation of hypoxia-inducible genes, activation of Sesn2 by hypoxia was not dependent on HIF1[119]. This is supported by several observations: (i) the kinetics of Sesn2 induction is significantly delayed compared to typical HIF1-dependent genes such as VEGF

and RTP801, but similar to induction of HIF1-independent GADD153 gene; (ii) no HIF1-binding sites were found in the promoter or introns of the Sesn2 gene[119]; (iii) Sesn2 was induced in response to hypoxia mimetic deferoxamine mesylate in *HIF1a*-proficient but not *HIF1α*-deficient immortalized astrocytes (ABV, unpublished). Nevertheless, HIF1 can contribute to Sesn2 expression under some conditions. Thus it was showed that HIF1 protected airway epithelium against oxidative stress potentially via the activation of Sesn2[120]. Sesn2 is also activated by NO and hypoxia in a HIF1-dependent manner in macrophages[121], so it is possible that HIF1 plays a role in Sesn2 regulation in tissue-specific manner. Genotoxic stress imposed by γ-irradiation, UV light, doxorubicin, etoposide and camptothecin induces Sesn2 expression in a p53-dependent manner[119, 122]. The activation of two of the three members of Sestrin family by p53 indicates the importance of Sestrins in p53-dependent processes. The p53-binding site was identified by chromatin immunoprecipitation (ChIP) with the paired-end ditag (PET) sequencing strategy in the region 9.6 kb downstream of *SESN2* gene[115]. Oxidative stress activates Sesn2 in a p53-independent manner, although p53, which is also activated by oxidative stress, contributes to transactivation of Sesn2[123]. The mechanism of Sesn2 activation in response to oxidative burst induced by NMDA receptor is described in neurons, where Sesn2 induction is mediated by C/EBPβ transcription factor via –378 to –249 and –249 to –107 regions in *SESN2* promoter[124] (Fig.4A).

Another mechanism of Sesn2 regulation involves nerve growth factor-induced-B member Nur77 (NGFI-Bα/TR3), an orphan nuclear receptor expressed in multiple tissues[125]. Two Nur77 activators 1,1-Bis(3'-indolyl)-1-(*p*-methoxyphenyl)-methane (DIM-C-pPhOCH3) and 1,1-bis(3'-indolyl)-1-(*p*-phenyl)methane (DIM-C-pPhOH) induce Sesn2 and activation of Sesn2 in response to these compounds is inhibited by shRNA against Nur77[125]. Stimulated Nur77 inhibits cell proliferation and induces cell death. Moreover treatment with Nur77 activator DIM-C-pPhOCH3 suppressed growth of human bladder cancer cell line KU7, suggesting the impact of Sesn2 and several other genes co-activated with Sesn2 in suppression of tumor growth and tumor cells' viability [125]. Accordingly we showed that Sesn2 suppressed colony-formation in different cancer cell lines originated from lung, colon, breast and kidney tumors[119] (Fig.4A).

Other stimuli activate Sesn2 via a yet to be defined mechanisms. Sesn2 is activated in response to expression of HIV Tat protein in the brain potentially through induction of inflammation and oxidative stress[126]. Accordingly Sesn2 is also activated in response to β-amyloid peptides associated with oxidative stress in neurons[127]. Impact inflammation in Sesn2 expression might be mediated by NO-production, and accordingly NO is the Sesn2 activator[128]. Sesn2 is also activated in the brain of *Securin*-deficient mice[129]. Securin is a protein, which in complex with Separase, regulates chromosomal separation and metaphase-anaphase transition[129]. Securin deficiency causes genomic instability and might regulate Sesn2 via p53 activation. Sesn2 transcriptional activity is also regulated by the mechanisms involved acetylation/deacetylation of histones, which can be

controlled by oxidative stress and other stimuli[130]. In accordance, treatment with the histone deacetylase inhibitor trichostatin A (TSA) induces Sesn2 expression in neurons[130].

Sesn2 modulates cell viability in response to stress, aggravating cell death in response to DNA-damage, induced by UV light and doxorubicin, and serum starvation but supporting cell viability in conditions of H2O2 treatment and hypoxia[119]. It might play an important role in tissue protection in response to ischemia/hypoxia and some other stress insults. According to our data, Sesn2 is activated in the brain in a model of acute ischemia induced by acute hypoxia in a model of stroke, created in rats by permanent middle cerebral artery occlusion (MCAO)[119].

Sesn3 was originally identified *in silico* via search of databases for the sequences sharing homology with the Sesn1 and Sesn2 genes[119, 131]. The human *SESN3* gene is located in position 11q21[119] and is transcribed into 2 mRNA which translated into proteins with Mw~53kDa and ~44kDa, lacking 72 AA at N-terminal part[118]. Similar to other Sestrins, Sesn3 is expressed in all tissues, and increased levels of expression are observed in skeletal muscle, placenta, small intestine, leucocytes, kidney, colon and brain[131]. Sesn3 was identified as a direct target of FoxO3A and FoxO1 transcription factors [117, 118]. FoxO1 directly activates Sesn3 via binding of a 250bp region within 1st intron of human and mouse *SESN3* gene[118] (Fig.4A).

Protein products of Sesrin genes from different organisms display the highest similarity with Sesn1. According to prediction by GLOBE, (http://cubic.bioc.columbia.edu/predictprotein) Sestrins are compact globular domain proteins composed of α-helical regions. Three of these α-helical regions which include helices α3-α8, α9-α10, and α11-α16 are highly conserved among Sestrins and are separated by less conserved hinge regions. According to ProSite analysis (http://www.expasy.ch/prosite) Sestrins contain several Ser/Thr and Tyr phosphorylation sites, mainly located in α-helical regions, including CK2 phosphorylation sites in α8 and α10, three PKC sites in α11, α15 and α16, one cAMP/cGMP-dependent protein kinase phosphorylation site in α10 and one tyrosine phosphorylation site within helix α14. There are also 13 potential tyrosine residues within helices α11-α16, which can be phosphorylated by Tyr kinases[119]. Although these predictions require verification, phosphorylation of several residues was demonstrated in high-throughput screenings, thus S352 of Sesn1 is phosphorylated by ATM kinase, implying the role of Sesn1 in DNA-damage response and metabolism[132]. According to phosphopeptyde database (http://www.phosphosite.org) Sesn2 phosphorylations on Tyr342 and Tyr356 were found in acute myelogenous leukemia indicating involvement of Sesn2 in tyrosine-kinase signaling.

Efforts to characterize Sestrins in the other organisms led to the identification of Sesn1 gene in *Xenopus Laevis*[133]. Although no function was assigned, it was showed that Sesn1 was accumulated in notochord at the onset of neurolation[133]. The reciprocal translocation (6;18)(q21;q21) observed in heterotaxia (abnormal organs arrangement in the body) patients

revealed breakpoint region within 1st intron of *Sesn1* gene, which led to propose that Sesn1 might be responsible for the heterotaxia phenotype[131]. These observations were followed by analysis of zebrafish model. Knockdown of Sesn1 in the zebrafish caused lateral disturbances in the heart and gut, providing evidence for a potential role of Sesn1 in the regulation of left-right asymmetry via Nodal signaling pathway[134]. Interestingly, Nodal auto-activation is mediated by a forkhead transcription factor FoxH1 (known as Sur in zebrafish) and Sesn1 is able to interact with FoxH1 *in vitro*[134].

To gain insight into the physiological function of Sestrins we studied the functions of drosophila (d) Sesn (dSesn) in fly model[135]. The analysis of expression has been shown that the dSesn expression is increased in adult flies in comparison to larvae stage, and dSesn is highly expressed in thoracic muscle (analog of skeletal muscle in mammals)[135]. Interestingly, dSesn expression in fly muscles recapitulates the high expression level of the members of the Sestrin family in skeletal muscles in mammals[114, 119, 131]. Overexpression of dSesn in the dorsal wing region produces a bent up wing phenotype and Sesn2 activation in the eyes diminishes the eye size. Both phenotypes were evoked by decreased cell size, while dSesn overexpression did not affect cell number[135]. Knockout of dSesn do not seem to cause any developmental problems indicating no role of dSesn in morphogenesis. Nevertheless, the adult flies accumulated many age related defects, suggesting a role of Sentrins in the regulation of aging (see below)[135]. Interestingly, similar to mammalian Sestrins, dSesn is also under control of p53 and FoxO, although dFoxO appeared to be the predominant transcriptional activator of dSesn[135] (Fig.4B).

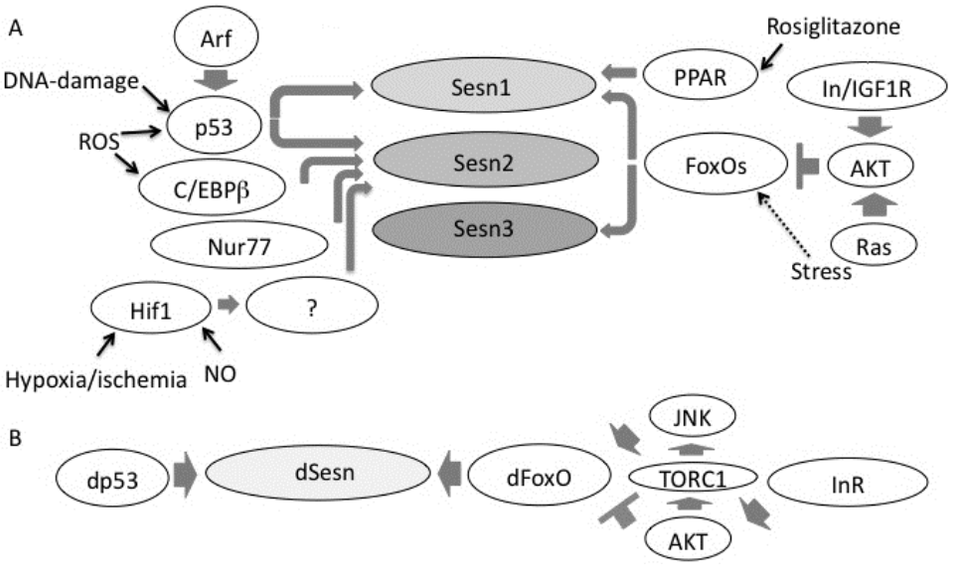

Figure 4. Regulation of Sestrins' expression. (A) Different stress insults regulate Sestrin genes via activation of p53, FoxOs, C/EBPβ and some other transcriptional factors in mammals. On the contrary AKT activated in response to insulin/IGF1 pathway or Ras can suppress activity of FoxOs. **(B)** Similar to mammals, drosophila (d) Sesn is activated by dp53 and dFoxO. Prolonged stimulation of InR stimulates dSesn expression through the TORC1-JNK-dFoxO axis, counteracting an inhibitory effect of AKT activation.

6. Antioxidant function of Sestrins

Identification of Sestrin gene family did not provide any clues toward their functions due to low similarity with any other proteins [1]. To better understand Sestrins' function, an iterative analysis of Sesn2 protein sequence using PSI-BLAST and structural analysis using 3D-PSSM programs were performed. We have observed that a fragment of protein around 75 amino acids in length (corresponding to region amino acids 100-175 of Sesn2) shares sequence and structural homology with *Mycobacterial Tuberculosis* AhpD protein and other related proteins[123]. The homology spans 5 a-helices of conserved region of Sestrins and C-terminal a-helical portion of AhpD[123]. Interestingly, some AhpD family members, such as caroboxymuconolactone decarboxylases, consist of this domain only. AhpD is a critical component *Mycobacterium tuberculosis* hidroperoxide reductase responsible for regeneration of bacterial peroxiredoxin AhpC, which is oxidized during reduction and decomposition of ROS or reactive nitrogen species (RNS). Peroxiredoxins are thiol-containing peroxidases conserved among prokaryotes and eukaryotes which catalytical center contains 2 conserved cysteines, one is a peroxidatic cysteine oxidized to SOH group during reaction with peroxides, and the other is resolving cysteine which forms disulfide bridge with catalytical cysteine. Oxidized cysteines are regenerated by AhpD in *Mycobacterium tuberculosis* or the thioredoxin/thioredoxin-reductase (Trx/TrxR) system in eukaryotes[15, 136]. AhpD contains two critical cysteines, whereas only one of them is conserved in Sestrins[123]. The major difference between prokaryotic and eukaryotic peroxiredoxins is that reactive cysteine of eukaryotic peroxiredoxins can be easily overoxidized to cysteine sulphinic acid ($-SO_2H$) or sulphonic acid ($-SO_3H$) forms and special enzymatic system is required for the regeneration of the overoxidized cysteine[136].

The homology between Sestrins and AhpD indicates that Sestrins might be antioxidant proteins regulating mammalian peroxiredoxins. Accordingly, Sesn1- or Sesn2-silenced cells have elevated ROS levels and exhibit oxidative stress as supported by elevated levels of DNA-oxidation and increased mutagenesis[62, 123]. Sesn1- and Sesn2-silenced cells also have higher RNS levels comparatively to control implying the role of Sestrins in RNS metabolism as well. Complementary experiments have been shown that ectopic expression of either Sesn1 or Sesn2 downregulates ROS in different cell lines, indicating that Sestrins are stress-responsive antioxidant proteins[123]. The important role of the conserved cysteine was supported mutation analysis, demonstrating that substitution of the conserved cysteine impairs antioxidant activity of Sestrins. Sestrins co-localize and interact with peroxiredoxin1 (Prx1) and peroxiredoxin2 (Prx2) proteins within the cell and support regeneration of overoxidized peroxiredoxins[123]. However, the following studies showed that sulfiredoxin protein, unrelated to Sestrin family, plays a major role in regeneration of peroxiredoxins in most eukaryotic species and Sestrins do not have intrinsic sulfinil reductase activity[137]. Thus, Sestrins play an indirect role in peroxiredoxin signaling working as auxiliary or regulatory proteins[1]. According to other observations, the important impact of Sestrins on

peroxiredoxin-mediated antioxidant response were demonstrated in macrophages and NMDA-stimulated neurons[121, 124].

Being p53-activated proteins, Sesn1 and Sesn2 mediate antioxidant activities of p53, and ectopic expression of Sesn1 and Sesn2 partially restores normal ROS levels, elevated in p53-deficient cells[62]. Sesn1 and Sesn2 also suppress DNA-oxidation and mutagenesis in p53-silenced lung carcinoma A549 cells [62]. FoxO-inducible gene Sesn3 also inhibits ROS accumulation, and AKT stimulates ROS production via FoxO-dependent downregulation of Sesn3[117]. As mentioned, FoxOs, similar to p53, regulate ROS in a pro-oxidant and an anti-oxidant fashion, dependent upon conditions[15, 138]. In response to detrimental genotoxic stress induced by etoposide and doxorubicin, FoxO3A stimulates expression of the pro-apoptotic protein Bim and its induction is associated with oxidative burst and induction of cell death. Simultaneously, FoxO3A stimulates expression of the antioxidant Sesn3 protein, and silencing of Sesn3 accelerates the levels of FoxO3A-induced cell death[138]. It is possible that Sestrins set up a protective mode against misfired induction of cell death by FoxOs and p53 preventing undesirable cell death (Fig.5).

The antioxidant activities of Sestrins play a potential role in carcinogenesis, and inactivation of Sestrins might be desirable for cancer cells, which can exploit the effect of Sestrin deficiency on mild ROS production. ROS are involved in mutagenesis and genomic instability, associated with selection of more malignant cells, stimulation of cell cycle, angiogenesis and epithelial-mesenchymal transition. At the same time, high ROS levels can be detrimental for viability of cancer cells, this feature is exploited by some anticancer treatments[139]. Mutant Ras proteins induce ROS, which are important for cell transformation by the Ras oncogene[140]. Accordingly, expression of Sesn1 and Sesn3 genes are inhibited by Ras, supporting ROS accumulation in response to Ras expression[141]. The expression of both FoxO-dependent genes Sesn1 and Sesn3 were decreased in response to Ras, while Sesn2 was not affected [141]. Ras activates AKT through stimulation of PI3K and potentially inhibit Sesn1 and Sesn3 via suppression of FoxOs[1].

7. Regulation of AMPK-TOR signaling by Sestrins

As antioxidant proteins, Sestrins are potential regulators of many cell signaling pathways which are sensitive to redox status in the cell. One of them is the mTORC1-dependent pathway, which is regulated by ROS on different levels. ROS directly affect activity of mTOR[15] or works upstream via inhibition of different phosphatases, such as PTEN or members of the tyrosine phosphatase family, which catalitical cysteine is sensitive to inhibitory oxidation [142]. Inactivation of these phosphatases can enhance the signaling pathways activated by receptor tyrosine kinases leading to the PI3K-AKT-mTORC1 activation[15]. Ectopic expression of either member of the Sestrin family suppresses mTORC1 activity in different human and mouse cells (Fig.5), as indicated by inhibition of

phosphorylation of p70S6K, S6 and 4E-BP proteins[122], similar to effects of rapamycin[122]. In complementary experiments knockdown of either Sesn1 or Sesn2 stimulated mTORC1 activation[122]. Surprisingly, ectopic expression of a redox-deficient mutant of Sesn2 inhibited mTORC1 with the same efficiency as WT Sesn2 protein, indicating that Sestrins regulate mTORC1 in a ROS-independent manner[122].

To gain insight into the mechanism of mTORC1 regulation, Sesn2 was co-expressed with different upstream mTORC1 activators including H-Ras, AKT or Rheb. Sesn2 was able to suppress mTORC1 activity in the cells transfected with H-Ras or AKT constructs, but all effects of Sesn2 were eliminated by co-expression of Rheb. This data indicates that Sestrins regulate mTORC1 downstream of H-Ras and AKT but upstream of Rheb[122]. Rheb-GTP analysis in breast carcinoma MCF7 cells demonstrated that induction of Sesn2 strongly inhibited Rheb-GTP loading, indicating an inhibitory effect of Sestrins on Rheb[122].

Rheb activity is regulated by the TSC1:TSC2 protein complex and Sestrins inhibit Rheb in a TSC2-dependent manner. The following experiments showed that Sestrins regulated TSC1:TSC2 activity not trough regulation of upstream TSC2 kinases AKT and ERK [122]. AMPK is the kinase which directly phosphorylates and activates TSC2 in response to stress and we have shown that either Sesn1 or Sesn2 stimulate AMPK phosphorylation on Thr172 followed TSC2 phosphorylation and activation by AMPK [122] (Fig.5). Moreover, it has been demonstrated that Sesn2 stimulates expression of AMPK subunits in response to DNA-damage[143]. An inhibition of mTORC1 in an AMPK- and TSC2-dependent manner was demonstrated later for Sesn3[118].

To examine whether Sesn2 can be directly involved in TSC2 and AMPK activation, purification and analysis of Sesn2-containing protein complexes were performed in gel-filtration experiments. As shown, Sesn1 and Sesn2 were co-eluted in a high molecular weight fractions (411-1175 kDa), together with the TSC1, TSC2 and AMPK proteins[122]. Immunoprecipitation of protein complexes with anti-Sesn2 antibodies allowed us to co-purify AMPKα1 and AMPKα2 subunits and the TSC1:TSC2 complex with Sesn2, indicating an interaction of Sestrins with the AMPK, TSC1 and TSC2 proteins [122]. Moreover, we observed binding of GST-Sesn2 with AMPKα proteins, supporting the idea that Sestrins activate AMPK via direct protein-protein interactions (AVB, unpublished)[122].

The inhibition of mTORC1 by Sestrins has an impact on many mTORC1-dependent processes including translation, cell growth and proliferation. Accordingly, significant downregulation of CyclinD1 and c-Myc expression was observed in breast carcinoma MCF7 cells in response to Sesn2 induction[122]. The mechanism of inhibition of protein synthesis by Sestrins involves formation of the 4EBP1- eIF-4E complex, which suppresses initiation of translation of the Cap-dependent mRNAs[122]. Being stress-inducible proteins, Sestrins are potential regulators of protein synthesis in response to stress. Accordingly, it has been shown that Sesn1 and Sesn2 play a critical role in inhibition of protein synthesis in response to γ-irradiation in breast epithelial MCF10A cells [144].

Regulation of translation and metabolism via mTORC1 inhibition strongly affects cell growth, proliferation and cell viability. Accordingly, ectopic expression of Sestrins in different cell lines causes a decrease in cell size as compared to GFP control, supporting the inhibitory role of Sestrins on cell growth[122]. Cell growth is linked with cell proliferation, and we showed that Sestrins inhibited cell proliferation in many cell types such as human lung carcinoma H1299, human fibrosarcoma HT1080, human breast carcinoma MCF7 and human immortalized fibroblasts from Li-Fraumeni patient MDA041[119]. Using matched HCT116 cells with normal p53 and p21 status, p53-deficient or p21-deficient cells, we also showed that the inhibition of cell proliferation by Sestrins was p53- and p21- independent (AVB and Chumakov PM, unpublished). Sestrins inhibited cell proliferation as evident by accumulation of cells in the G1 phase of the cell cycle after Sesn2 overexpression[122]. To confirm the importance of mTORC1 in the regulation of cell growth and proliferation Sesn2 was ectopically expressed in *TSC2*-deficient cells, and no effects on cell growth and proliferation were observed in the absence of TSC2 protein[122].

AMPK activation and mTORC1 inhibition also regulates autophagy through phosphorylation of ULK1 kinase and we showed that Sestrins stimulated autophagy in H1299 cells (AVB and Karin M, unpublished). It has been demonstrated that Sesn2 regulates autophagy in response to rapamycin, nutrient-free medium, thapsigargin (an activator of endoplasmic stress), and lithium in human colon carcinoma HCT116 cells [145]. The activation of autophagy in response to these stimuli requires p53, and autophagy was significantly inhibited in HCT116 p53-null cells. The potential explanation for this effect is that expression of Sesn2 and other p53-dependent proteins involved in autophagy such as Sesn1, DRAM, LC3 and ULK1 is supported by p53[15, 20, 74, 146]. Accordingly, silencing of DRAM, another important regulator of autophagy, had similar inhibitory effects on autophagy as Sesn2 knockdown [145] (Fig.5).

The effects of Sestrins on cell physiology is not only limited to regulation of cell proliferation but also involves regulation of cell viability. Sesn2 overexpression in 293 cells stimulates cell death[119]. Sestrins also modulate cell viability under stress condition, protecting from oxidative stress, but supporting cell death in response to genotoxic stress[119, 123, 143, 147]. Cell death in response to γ-irradiation is regulated in an AMPK-dependent manner and Sesn2 plays a major role in regulation of AMPK phosphorylation in response to genotoxic stress[40, 41, 143].

The prominent role of p53 in the regulation of Sesn1 and Sesn2, and FoxOs in the regulation of Sesn3, make Sestrins potential mediators of p53 and FoxO-dependent processes. We showed that silencing of either Sesn1 or Sesn2 released the inhibitory effects of p53 on mTORC1 in different experimental contexts including overexpression of p53 in p53-deficient H1299 cells, stimulation p53 by Nutlin-3 in U2OS cells and activation by the genotoxic drug camptothecin in mouse embryonic fibroblasts[122]. Moreover, Sesn2 regulates mTORC1 activity *in vivo* in mouse liver in response to treatment with the alkylating liver-specific

poison diethylnitrosamine[122]. Another group also demonstrated that fangchinoline, bis-benzylisoquinoline alkaloid, which is considered as new antitumor agents, activated Sesn2 expression through p53 and regulates autophagy via activation of AMPK kinase[148]. Interestingly, fangchinoline is able to stimulate cell death through induction of autophagy, explaining the potential role of Sestrins in support of cell death in response to some stimuli[119]. p53 can be inactivated in many cancer cells through interaction with inhibitory proteins, which might be potential targets for anticancer treatment. In lung cancer cell lines p53 is bound to and inactivated by orphan nuclear receptor Nur77/TR3. Nur77 suppression by siRNA released p53, which in turn stimulated Sesn2. It led to AMPK activation and mTORC1 suppression, resulting in inhibition of growth and inducing apoptosis in lung carcinoma cells[149].

The regulation of the AMPK-TORC1 axis by Sestrins is highly conserved in evolution. We showed that similar to mammalian Sestrins, dSesn inhibited TORC1, as indicated by diminished levels of p70S6K phosphorylation, in an AMPK- and TSC2-dependent manner. Inactivation of dSesn led to many abnormalities associated with AMPK-TORC1 dysregulation, such as metabolic derangements and oxidative stress. Interestingly, the effects of dSesn inactivation can be normalized by reconstitution with mammalian Sestrins, while dSesn activates mammalian AMPK and inhibits mTORC1, indicating the highly conservative functions of Sestrins[135].

8. Regulation of TGFβ signaling by Sesn2

The AMPK-TOR axis is not the only signaling pathway modulated by Sestrins. A new role of Sestrins was described in the control of transforming growth factor-β (TGFβ) signaling. TGFβ signaling is regulated by binding of a dimer of TGFβ ligands (composed of TGFβ1,2 3) with TGFβ receptor (TGFβR 1 and 2), stimulating its heterodimerisation and activation of its intrinsic Ser/Thr kinase activity[150, 151]. Cells secrete TGFβ as large latent complex containing one of three latent TGFβ binding proteins LTBP1, LTBP2 and LTBP4 belonging to the fibrillin family of extracellular matrix (ECM) proteins[150]. LTBPs target TGFβ to ECM depositing them for mobilization when activation of TGF-β signaling is required. Activated TGF-βR transduces a signal to proteins of the Smad family, classified as receptor-associated Smads (R-Smads: Smad 1,2,3,5&8), cooperating Smads (Co-Smads: Smad4) and inhibitory Smads (i-Smads: Smad6&7). It was shown that R-Smads, Smad1-5, are substrates of TGFβ receptors, activated by TGFβ, while others are activated by other members of the TGFβ family via interaction with relevant receptors. Phosphorylated Smad2 and Smad3 form heteromeric complexes with Smad4, which translocate to the nucleus where they bind Smad-dependent promoters and activate expression of a number of genes such as α-smooth muscle actin (⟨SMA), connective tissue growth factor (CTGF) and matrix metalloproteinase 2 (MMP2) involved in regulation of cell growth, differentiation and migration[147, 152]. TGFβ also controls other signaling pathways through RhoA, Cdc42, Rac1, Ras, PI3K, PP2A, MEKK1, TAK1 and DAXX proteins. Dysregulation in TGFb-dependent signaling contributes to fibrosis, cancer, cardiovascular and congenital diseases[150]. Sesn2-deficiency leads to

activation of TGFβ signaling pathways in lung as well as in mouse lung fibroblasts (MLF) as indicated by increased phosphorylation of Smad2 and Smad3, and elevated expression of TGFb targets such as α-SMA, connective tissue growth factor (CTGF) and MMP2[147]. It has been also shown that inactivation of Sesn2 in MLF activates mTORC1, and TGFβ played a role in this process potentially stimulating the PI3K-AKT pathway[147] (Fig.5). TGFβ pathway is not the only receptor-activated cascade is regulated by Sestins. As reported recently, inactivation of Sesn2 also caused accumulation of platelet-derived growth factor receptor-β (PDGFRβ) in glioblastoma U87 cells. PDGFRβ was accumulated in Sesn2-silenced cell due to impaired ubiquitination and degradation. These cells had increased ROS production and higher rate of autophagy, which can indicate a compromised metabolism[153].

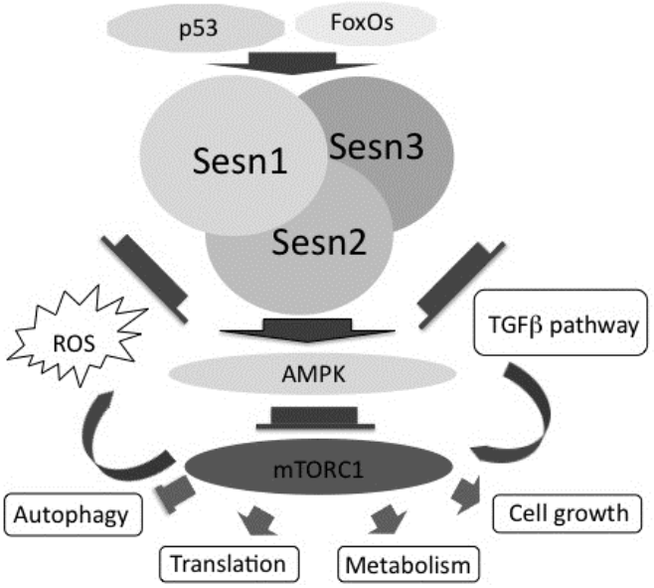

Figure 5. Functions of Sestrins. Sestrins suppress ROS accumulation, inhibit the TGFβ pathway and activate AMPK causing suppression of mTORC1. As a result, Sestrins control many mTORC1-dependent processes such as translation, metabolism, cell growth and autophagy. Inhibition of ROS by Sestrins prevents mutagenesis and genetic instability, the hallmarks of carcinogenesis. Being targets of p53 and FoxOs, Sestrins mediate many p53- and FoxO-regulated processes including regulation of ROS and metabolism potentially contributing to regulation of longevity by these transcriptional factors.

9. Role of Sestrins in aging and diseases

Aging and age-related diseases can be caused by deterioration of the mechanisms controlling stress responses which prevent accumulation of damaged organelles, macromolecular aggregates and ROS in cells through control of anabolic and catabolic processes. Two important functions of mTORC1, such as control of protein synthesis and autophagy are critical for lifespan regulation and fitness, and the pathways, which enhance or suppress mTORC1 activity, may contribute to longevity and health[6]. The members of the Sestrin family are antioxidant proteins involved in suppression of TORC1, so they are potential regulators of aging and longevity.

9.1. Role of Sestrins in aging

The redundancy of the Sestrin family members in mammals complicates the analysis of function of Sestrins in aging and diseases. To gain insight into the role of Sestrins in the regulation of aging, *Drosophila Melanogaster* containing only one Sesn gene (dSesn), was chosen as a convenient model. TORC1 is a critical regulator of aging and lifespan in Drosophila, and activity of TORC1 is elevated in *dSesn*-null flies[135]. Also, we have shown dSesn is induced in response to activation of InR, which stimulate activity of dTOR. dTOR is involved in activation of dSesn through ROS production, which induces JNK followed by activation of dFoxO. AKT, stimulated in response to InR in the same system, is a negative regulator of dFoxO. Thus the signals from AKT and JNK compete for regulation of this transcription factor, with JNK providing the dominant signal. dSesn is induced in response to InR-JNK-dFoxO activation and inhibits TORC1, providing a negative feedback loop toward regulation of InR-AKT-dTOR signaling[135]. Overactivated InR signaling contributes to many age-related pathologies in flies including metabolic derangement and heart and muscle deterioration, so dSesn might have a protective role against these diseases[1, 135].

Accordingly, the *dSesn*-deficient flies have many health-related problems. First, the flies have impaired lipid metabolism and accumulate lipids (Fig.6). The mechanisms of lipid regulation involve two processes: lipogenesis and lipolysis. TOR controls lipolysis through activation of transcriptional factor SREBP. We observed that expression of dSREBP and its targets dFAC, dFAS, dACC and dACS were up-regulated in dSesn-deficient animals, while the expression of the genes involved in lypolisis such as dPGC1α, lip3, CG5966, CG11055 were downregulated. Interestingly, the accumulation of lipids in dSesn-deficient flies was prevented by treatment of flies with either AMPK activators AICAR or metformin, or TOR inhibitor - rapamycin[135].

Second, *dSesn*-null flies demonstrated heart dysfunction manifested in arrhythmia and decreased heart rate due to expansion of diastolic period. This phenotype was largely prevented when flies were treated with AICAR and rapamycin, indicating the role of AMPK-TOR signaling in this process. The activated TOR signaling is associated with ROS accumulation. To examine whether ROS contribute to the phenotype, ROS were suppressed by antioxidant vitamin E or via expression of catalase in heart muscle. Strikingly, both conditions suppressed arrhythmia in *dSesn*-deficient flies but did not prevent the decrease in heart rate, indicating some ROS-independent effects of dSesn on heart protection. We also observed massive disorganization of myofibrils indicated by F-actin staining in *dSesn*-null flies. Thus, dSesn might be important for prevention of heart degeneration associated with activated TOR signaling[135] (Fig.6).

Third, inactivation of dSesn had a detrimental effect on thoracic (skeletal) muscle. The flies were characterized by muscle degeneration as evidenced by loss of sarcomeric structure and diffused sarcomeric boundaries. The muscles exhibited mitochondrial abnormalities such as rounded shape, enlargement and cristae disorganization. Oxidative stress is the typical

feature of mitochondrial malfunction and muscle from *dSesn*-null flies showed an increased accumulation of ROS. The detrimental muscle phenotype evoked by dSesn inactivation was prevented by treatment with antioxidant vitamin E, supporting the idea that ROS contribute to muscle degeneration. Deterioration of muscle structure, associated with mitochondrial abnormalities and oxidative stress might be linked to impaired autophagy, the important mechanism for control of muscle cell integrity and function. The defects in autophagy, the controller of muscle integrity, in *dSesn*-deficient flies was evident from accumulation of ubiquitinated protein aggregates, which are cleared via autophagic proteolysis. To examine the potential impact of autophagy on regulation of cardiac and muscle homeostasis we knocked down the ULK1(ATG1) gene, the critical component of the autophagic machinery, which is inhibited by TOR and activated by AMPK. Silencing of ULK1 had effects similar to dSesn inactivation such as cardiac deficiency, muscle degeneration, mitochondrial abnormalities and oxidative stress[135].

The phenotypes observed in young (2-3 weeks old) *dSesn*-deficient flies resembled those in old WT flies, indicating that *dSesn*-null flies in early age have many features of aging animals[135]. Thus dSesn controls processes important for homeostasis, which being improperly regulated can accelerate aging. These processes potentially involve the mechanisms of stress response, which act to repair or remove damaging consequences of stress. Aging and age-related diseases might be a result of unresolved stress which lead to accumulation of damage causing more intense stress, supporting a vicious cycle (Fig.6).

Although the data on the role of Sestrin in aging in vertebrates are scant, there is some evidence that Sestrins might play an important role in protection against aging and age-related diseases in mammals. Strong activation of Sesn1 and Sesn2 was found in s-Arf/p53 mice[90], which demonstrated delayed aging and were protected from carcinogenesis. The activities of AMPK and p53 decline with age as well as the levels of autophagic proteolysis[51, 93, 154]. Sestrins being a link between p53 and AMPK might be suppressed in aging animals, and as a result this dysregulation can weaken the mechanisms protecting their health.

9.2. Sestrins and neurodegenerative diseases

The pathogenesis of many neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's disease are associated with accumulation of protein deposits, which can affect cell physiology and induce oxidative stress and cell death[1]. Inhibition of mTORC1 has a protecting effect by suppressing accumulation of protein deposits, potentially via inhibition of protein translation and activation of autophagy[1, 3]. Sesn2 was activated in human neuroblastoma CHP134 cells in response to amyloid β-peptides, the toxic deposits found in the brain of Alzheimer patients[127]. In another study Sesn2 was found to co-localize with Tau, another protein forming deposits or tangles in neurons, in a subset of neurofibrillary lesions[155]. Pathogenesis of neurodegenerative diseases is associated with oxidative stress and accordingly Sesn2 was activated by ROS in neurons, where Sesn2 plays

an antioxidant role[124]. Thus, Sestrins can protect neuronal cells from the toxic effects of neuronal deposits and oxidative stress. The other member of the Sestrin family, Sesn1 was activated in response to the neuroprotective drug rosiglitazone, a member of the thiazolidinedione family of synthetic peroxisome proliferator-activated receptor (PPAR) agonists. Rosiglitazone protects retinal cells from cell death mitigating the effects of ROS and Ca^{2+}, so Sesn1 might be a critical target of this drug involved in regulation of cell viability[156] (Fig.6).

9.3. Sestrins and diabetes

Dysregulation of the AMPK-mTORC1 pathway and ROS metabolism contributes to type II diabetes and Sestrins and their regulator p53 might play a protective role against this disease. According to resent observations, knockin mice where p53 Ser18 (analog of Ser15 in human) was replaced by alanine, showed increased metabolic stress and develop insulin resistance and glucose intolerance, the hallmarks of type II diabetes[157]. Ser18 is the site of phosphorylation of ATM kinase. Inactivation of ATM induces metabolic derangements and development of diabetic phenotype in mice. According to a hyperinsulinemic-euglycemic clamp study, diabetic phenotype of $p53^{S18A}$ mice was evoked by reduction of insulin-stimulated whole-body glycogen synthesis and inefficient suppression of hepatic glucose production by insulin. These mice had decreased levels of expression of Sestrin family members in liver, muscle and white adipose tissue, suggesting that downregulation of Sestrins in the $p53^{S18A}$ mice contributed to the diabetic phenotype. Impressively, downregulation of Sesn2 and Sesn3 genes was also observed in $ATM^{+/-}$ mice where ATM activity was reduced[157]. The fibroblasts from $p53^{S18A}$ mice were characterized by increased ROS levels associated with reduced expression of all members of Sestrin family. Ectopic expression of Sesn2 in the $p53^{S18A}$ fibroblasts restored normal ROS levels, supporting the critical role of Sestrins in ROS regulation in $p53^{S18A}$ mice. To study whether the diabetic phenotype of the $p53^{S18A}$ mice was linked with oxidative stress, associated with a compromised p53 function, the mice were treated with butylated hydroxyanisole (BHA). BHA treatment suppressed diabetic phenotype in the $p53^{S18A}$ mice supporting the role of oxidative stress associated with p53 dysfunction in diabetes[157] (Fig.6).

9.4. Sestrins and respiratory diseases

Antioxidant effects of Sestrins might also be important in protection of respiratory epithelium via control of barrier function. Pollutant-induced inflammation compromises the barrier function, which leads to different respiratory diseases such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD). The barrier function of the respiratory epithelium is protected by trasncriptional factor HIF1 fortifying an antioxidant defense, and this function is associated with activation of Sesn2, which seems to be a critical HIF1 target involved in this process[120]. Interestingly, according to another work, Sesn2 activity can complicate some aspects of COPD via negative regulation of TGFβ signaling which mitigates emphysema phenotype in mice. Thus, inactivation of Sestrins has some

therapeutic potential under some COPD conditions, although more work has to be done to elucidate the exact role of Sestrins in COPD and other respiratory diseases[147].

9.5. Sestrins and cancer

Tumor suppressor p53, the master regulator of Sesn1 and Sesn2, is inactivated in most of human cancers, and p53 inactivation causes downregulation of Sesn1 and Sesn2, which can be responsible for tumor suppressive activities of p53 [62, 114, 119, 123]. Tumor suppressor activity was also recently assigned for the members of the FoxO family. Somatic inactivation of three members of the FoxO family: FoxO1, FoxO3A and FoxO4 in mice stimulated development of lymphomas and hemangiomas[158, 159]. Sesn1 and Sesn3 are FoxO targets, which can potentiate the tumor suppressive effects of FoxOs via regulation of ROS. The potential tumor suppressive activity of Sestrins and their impact on p53 and FoxO-mediated tumor suppression might involve antioxidant defense imposed by Sestrins, which can protect from mutagenesis, genomic instability and angiogenesis, as well as can regulate some other cancer-relevant processes associated with dysregulation of ROS metabolism[62, 141]. The role of Sestrins in many types of cancer is strengthened by the importance of Sestrins in regulation of the LKB1-AMPK-mTORC1 pathway[52, 122]. In agreement with this, it was observed that *Sesn2*-deficient cells are more susceptible to transformation than WT counterparts and Sesn2-silenced A549 tumor xenografts grow faster in nude mice, similar to p53-deficeint cells[62, 122] (Fig.6).

Analysis of human tumors demonstrates that loss of heterozygosity (LOH) in Sesn1 (6q21) found in non-Hodgkin lymphoma, acute lymphoblastic leukemia, bladder carcinoma, ovarian, mammary carcinomas, squamous cell carcinomas of the head and neck, T cell lymphomas[160] and Sesn2 (1p34) loci found in pancreatic adenocarcinoma[161], glioblastoma[162], T-cell lymphomas[160], ovarian cancers [163], thyroid cancers [164], and neuroblastomas[165]. LOH in the Sesn3 locus (11q21) is found in non-Hodgkin lymphoma[166], nasopharyngeal carcinoma[167, 168], pancreatic endocrin tumors[169], and melanomas[170]. Analysis of gene expression have shown that Sesn1 and Sesn2 are downregulated in lung cancers of different origin such as large cell carcinoma, adenocarcinoma, squamos cell carcinoma and small cell lung carcinoma[171-173]. Sesn1 is also found downregulated in breast cancers[174, 175], head and neck cancers[176], brain tumors[177] and T-cell leukemia/lymphoma[177]. Moreover, missense mutation of Sesn2 P87S was recently found in myeloproliferative neoplasm essential thrombocythemia (ET), characterized by increased proliferation of megakariocytes and accumulation of circulated platelets[178]. Another mechanism of inactivation of the members of the Sestrin family was described for endometrial cancers, where Sesn3 is methylated in 20% of cases, supporting the potential role of this gene in tumor suppression [179]. Although more extensive analysis is required to label Sestrins as tumor suppressors, these data clearly indicate the indispensable impact of Sestrins in control of carcinogenesis.

The regulation of the TGFβ pathway by Sestrins can also contribute to carcinogenesis. Although TGFβ can inhibit cell growth and suppress carcinogenesis at early stages, during the late stages of carcinogenesis cells often lose their growth-inhibitory response to TGFβ. Moreover, TGFβ stimulates invasion and metastasis of cancer cells supporting tumor progression. Accordingly, overexpression of TGFβ1 was found in breast, colon, esophageal, gastric, hepatocellular lung and pancreatic cancers. Moreover, high levels of TGFβ correlate with cancer progression, metastasis, angiogenesis and bad prognosis[152]. Inactivation of Sestrins can stimulate TGFβ signaling and potentiate TGFβ-dependent processes contributing to carcinogenesis. The link between Sestrins and TGFβ in human cancers and the impact of TGFβ-dependent pathway in regulation of carcinogenesis by Sestrins has to be addressed in the future studies.

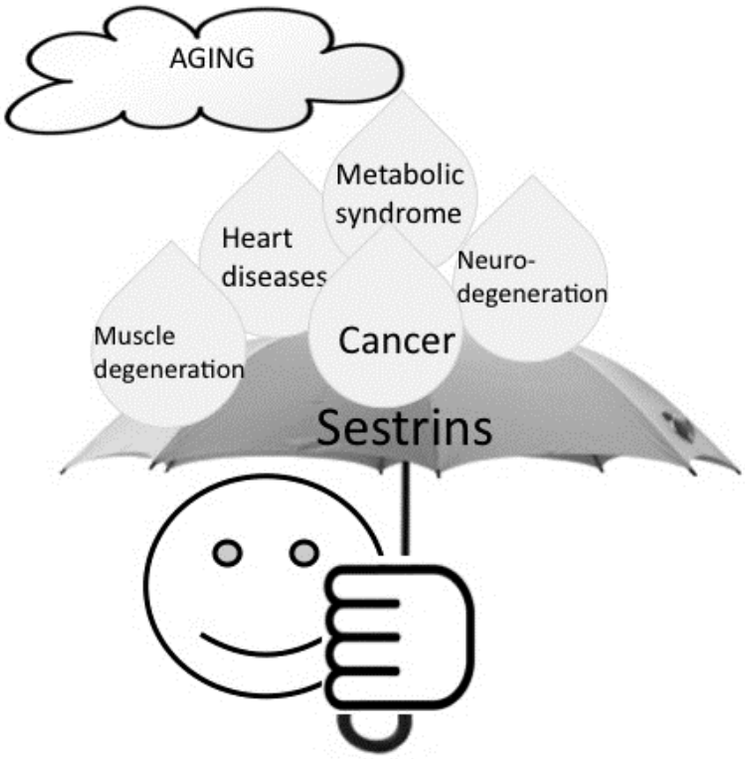

Figure 6. Sestrins protect from aging and age-related diseases. Drosophila Sestrin protect flies from aging, suppressing metabolic derangements, cardiac malfunction and muscle degeneration. In mammals, Sestrins are activated in the mice with delayed aging phenotype, while decreased expression of Sestrins is observed in diabetic mice. Moreover, Sestrins are found downregulated in many cancers. The conservatism of the mechanisms of regulation of longevity and aging between vertebrates and invertebrates via the AMPK-TORC1 pathway support the critical role of Sestrins in regulation of aging at mammals and their protecting activities against age-associated diseases.

Being stress-inducible genes, Sestrins are activated by many anticancer treatments, which involve stress response. Although the stress might be detrimental for cancer cells inducing cell death and senescence, which suppress tumor growth, cancer cells can eventually accumulate mutation in genes critical for the beneficial effects of anti-cancer therapy. Sestrins are important for cell death in response to genotoxic stress[119, 143], so they might

be targets for inactivation in response to therapy. Presumably reactivation of Sestrins might be beneficial for treatments involving genotoxic stress. On the contrary, Sestrins might protect against oxidative stress, which also contributes to death of cancer cells, so under some circumstances inactivation of Sestrins might be beneficial for the treatment efficiency. Thus, the detailed characterization of the role of Sestrins in the efficiency of anticancer treatment is very important to set up the best treatment strategy for different forms of cancer.

10. Conclusion

A decade ago we identified a novel Sestrin gene family which happens to be in the intersection of two vital roads controlled by two important guards, p53 and FoxOs, the grants of our well-being and longevity. Sestrins convey the message from them to the AMPK-TORC1 executive branch, responsible for integration of numerous signals from nutrient and energy sources, growth factors, hormones and stress insults to tune up many metabolic, biosynthetic and disposing facilities providing good conditions for the organism well-being. Unfortunately, some hereditary or environmental factors, including an unhealthy life style, can impair the guards and release the malfunctioning machine of TOR signaling leading to non-synchronized anabolic and catabolic process resulting in the accumulation of damage, the major source of our demise. The absence of careful control of these processes also lead to disturbances in different tissues laying the ground for many detrimental age-related diseases, the major threat of developed societies in the 21st century. The restoration of the guards' functions, in part, via enabling Sestrins that are important messengers of their orders, might prove to be a valuable approach to delay or prevent many undesirable manifestations of aging and unhealthy life style. Future experiments on mouse genetic models and detailed analysis of the role of Sestrins in human diseases let us establish the impact of Sestrins in the control of human health and longevity, understand the mechanisms of their action and exploit this knowledge for the development of efficient anti-aging therapies.

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