Aging and Disease
www.aginganddisease.org
Volume 6, Number 2; 95-108, April 2015
http://dx.doi.org/10.14336/AD.2014.0228

Review Article

Modelling the p53/p66Shc Aging Pathway in the Shortest Living Vertebrate *Nothobranchius Furzeri*

Chiara Priami[1, 3], Giulia De Michele[1], Franco Cotelli[3], Alessandro Cellerino[4], Marco Giorgio[1], Pier Giuseppe Pelicci[1,2*], Enrica Migliaccio[1*]

[1]European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy.
[2]Dipartimento di Medicina, Chirurgia e Odontoiatria, University of Milan, Italy
[3]Dipartimento di Bioscienze, University of Milan, Italy.
[4]Scuola Normale Superiore, Pisa, Italy

[Received January 3, 2014; Revised February 28, 2014; Accepted February 28, 2014]

ABSTRACT: Oxidative stress induced by reactive oxygen species (ROS) increases during lifespan and is involved in aging processes. The p66Shc adaptor protein is a master regulator of oxidative stress response in mammals. Ablation of p66Shc enhances oxidative stress resistance both in vitro and in vivo. Most importantly, it has been demonstrated that its deletion retards aging in mice. Recently, new insights in the molecular mechanisms involving p66Shc and the p53 tumor suppressor genes were given: a specific p66Shc/p53 transcriptional regulation pathway was uncovered as determinant in oxidative stress response and, likely, in aging. p53, in a p66Shc-dependent manner, negatively downregulates the expression of 200 genes which are involved in the G2/M transition of mitotic cell cycle and are downregulated during physiological aging. p66Shc modulates the response of p53 by activating a p53 isoform (p44/p53, also named Delta40p53). Based on these latest results, several developments are expected in the future, as the generation of animal models to study aging and the evaluation of the use of the p53/p66Shc target genes as biomarkers in aging related diseases. The aim of this review is to investigate the conservation of the p66Shc and p53 role in oxidative stress between fish and mammals. We propose to approach this study trough a new model organism, the annual fish Nothobranchius furzeri, that has been demonstrated to develop typical signs of aging, like in mammals, including senescence, neurodegeneration, metabolic disorders and cancer.

Key words: stress response, p53, cell cycle checkpoint G2/M, senescence, aging, *nothobranchius furzeri*, animal models

Aging results in over-time increasing susceptibility to aging-related diseases and death. The free radical theory of aging proposes that aging is strictly correlated to the rate of oxidative damage (oxidative stress). Indeed, aging-related diseases such as diabetes, neurodegenerative and cardiovascular diseases are often associated with increased oxidative stress, whereas resistance to oxidative challenges is associated with retarded aging and longevity in different models [1, 2, 3], including the p66Shc-/- mouse [4, 5, 6, 7]. At molecular levels oxidative stress is caused by the accumulation of reactive oxygen species (ROS, e.g. hydrogen peroxide, superoxide anions and hydroxyl radicals) generated by aerobic metabolism [8]. Cells that accumulate excessive damage to DNA, proteins or lipids, arrest proliferation (transiently or definitively, entering the so called senescence state) or eventually undergo apoptosis. All these processes reduce tissue functionality and are crucial in physiological aging in mammals [9, 10]. On this basis, key regulators of intracellular ROS levels and oxidative stress response

***Correspondence should be addressed to:** Pier Giuseppe Pelicci and Enrica Migliaccio, European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy. Email: enrica.migliaccio@ieo.eu, piergiuseppe.pelicci@ieo.eu.

ISSN: 2152-5250

play a role in aging and are potential targets of anti-aging strategies.

p66Shc is the longest isoform encoded by the ShcA locus. The other two Shc isoforms discovered, p52/p46Shc, are involved in the transduction of signal from tyrosine kinases to Ras [11,12]. The third isoform, p66Shc, is encoded by the human and mouse shc loci through alternative splicing and contains the entire p52/46Shc sequence plus an additional amino-terminal region. Although p66Shc is phosphorylated, like p52/46Shc, by active tyrosine kinase receptors, p66Shc is not involved in Ras signalling [13, 14] but it is serine-phosphorylated upon oxidative stress (H2O2 in vitro treatment) or UV light and participates in the p53-dependent apoptosis [4,15]. In fact, p66Shc deficient mice are resistant to paraquat, a potent ROS inducer, and show a delayed onset of aging phenotype [see for review: 16, 17] and controversial effects on longevity [18]. Biochemical studies on the role of p66Shc in oxidative stress response revealed that it localizes within the mitochondrial intermembrane space where it functions as a redox enzyme, oxidizing reduced cytochrome c of the mitochondrial electron transfer chain (ETC) to catalyze the partial reduction of molecular oxygen to hydrogen peroxide and finally triggering mitochondrial swelling and apoptosis [19,20].

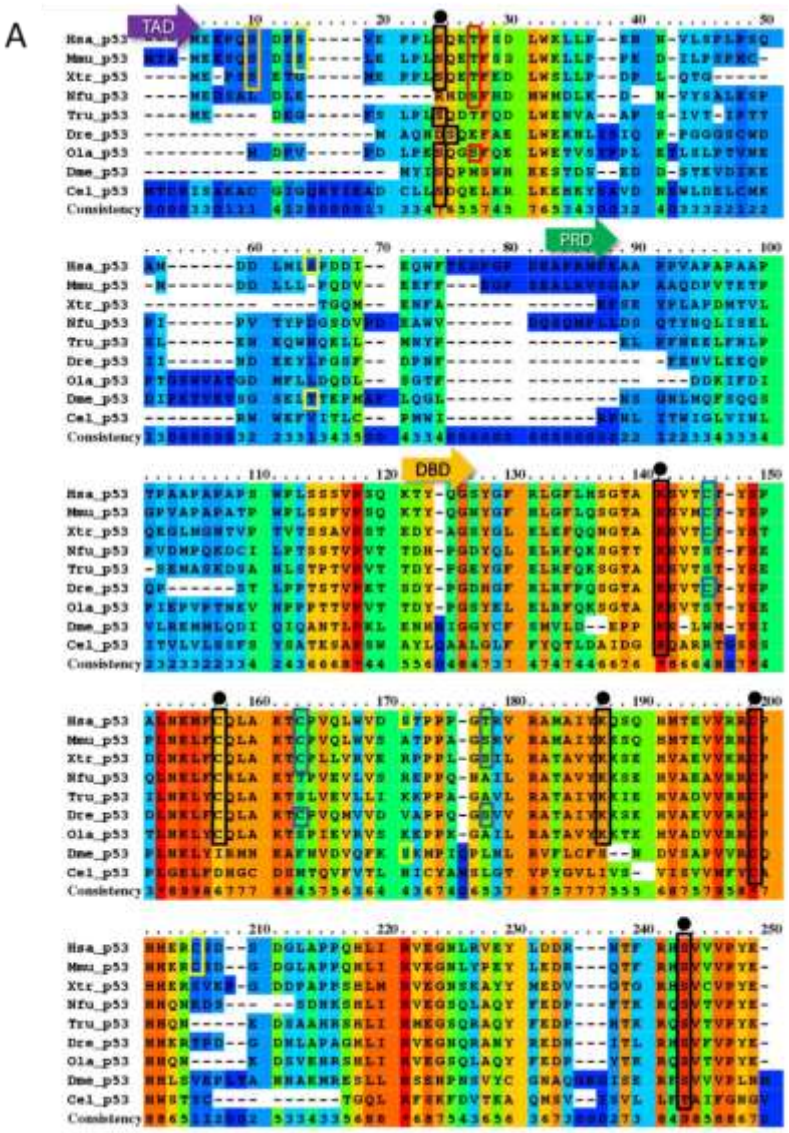
p53 (TRP53) is universally known for its role in tumor suppression, but its role in aging is still unclear. Analyzing the physiological functions of p53, it seems that this protein is both the major defense against cancer and the road that leads to aging. In this view, aging is considered an unavoidable phenomenon, the results of a complex mechanism that promotes health during early-life at the cost of a progressive decay that occurs after reproductive phase [21]. Interestingly, mice overexpressing a N-terminally truncated isoform of p53 (Deltap44) are resistant to cancer at the cost of an accelerated aging and reduced lifespan [22]. Recently we have established the existence of a p53/p66Shc transcriptional regulation network that is activated by oxidative stress and leads to cell cycle arrest at G2/M transition point [23]. The link between p53 and p66Shc indicates that p53 could participate in two different signalling pathways to exert its double role in tumor suppression and aging. In fact, two transcriptional networks, that start upon oxidative stress and specific DNA damage induced by mutagens, are both led by p44/p53 but are fundamentally different in terms of genes involved. In the mouse, oxidative stress regulates a specific transcriptional network which is dependent on p66Shc expression and involves the downregulation of approximately 200 genes critical for cell-cycle progression, suppression of senescence and, for few of them, aging. However, to validate in vivo the role of such a large gene network on aging and lifespan, adopting the reverse genetic approach in mammals will be highly problematic. The aim of this review is to propose to approach this study using the annual fish Nothobranchius furzeri as a model organism. It has been demonstrated that this fish show typical mammalian signs of aging, including senescence, neurodegeneration, metabolic disorders and cancer [24, 25] and it is now considered an outstanding model to study the molecular mechanisms of aging.

Nothobranchius furzeri as new model for aging research

Caenorabditis [26], Drosophila [27] and laboratory mice [28] have been traditionally utilized to model aging for basic and applied research. Indeed, most of the research on lifespan and aging is produced in the worm Caenorhabditis elegans, since the established vertebrate models are too long-living to fit common research programs. Nowadays, a new model for aging research is available: the annual fish Nothobranchius furzeri, known as “turquoise-killifish”, native of middle-east Africa. The advantage of N. furzeri resides in the lifespan: it is the shortest-living vertebrate that can be kept in captivity [25]. The median lifespan of this fish is strictly linked to the ephemeral habitat (pools of water that become dry during dry season) and consists of 3-to-6 months depending on the strain [29]. Two laboratory strains with different lifespan are now available: a wild-type strain with a median lifespan of 6 months named MZM and an extremely inbred strain with a median lifespan of 3 months. Fish of both strains reach sexual maturity at one month old [30]. A substantial body of evidence shows that N. furzeri shares a large variety of molecular, histopathological and behavioural aging-related signatures with mammals. At a first sight, it is very easy to distinguish the old fish from the young: old fishes appear emaciated, with their spine curved and less colored. At a behavioural level, N. furzeri shows cognitive and locomotor age-dependent decay. Histological markers of aging such as lipofuscin in the liver and senescence-associated β-galactosidase in the skin accumulate during aging in N. furzeri of both laboratory strains, suggesting that the shorter-living strain undergoes accelerated onset of aging phenotypes [31]. Moreover, the brain of N. furzeri replicates two typical hallmarks of mammalian aging: gliosis and reduced adult neurogenesis, demonstrating that an age-dependent neurological decay is detectable in this model. Notably, lifespan, aging-related histological markers and cognitive decay in N. furzeri are improved by caloric restriction [30]. Then, the effect on lifespan and aging of resveratrol, known for its anti-cancer and anti-inflammatory in vitro

action in mammalian cells, was evaluated for the first time in a vertebrate model using N. furzeri: resveratrol prolongs lifespan and healthspan in N. furzeri, preserving fertility, learning capacity and locomotor activity, delaying neurodegeneration and onset of aging-related histological markers [32].

The availability of two laboratory strains, which significantly differ in their median lifespan, led to two studies carried out to highlight molecular mechanisms and genetic determinants involved in aging and lifespan determination. The quantitative trait loci analysis performed by Kirschner et al. [33] shows that genes involved in metabolism likely play a role also in lifespan determination. The second study of Baumgart et al. [34] reveals the key role of microRNAs in aging, showing how miRNAs with similar functions in cell cycle regulation are similarly regulated during aging: a set of well-known and conserved tumor suppressor and cell cycle inhibitors miRNAs, with positive interactions with p53, is upregulated during aging not only in N. furzeri, but also in zebrafish and mouse; accordingly, a set of well-known and conserved proto-oncomiRNA, with negative interactions with p53 and a role in promoting cell proliferation, is downregulated during aging in N. furzeri, zebrafish and mouse. Recently, the complete and annotated transcript catalogue of N. furzeri has been published. 85 genes show significantly changes in transcription levels over N. furzeri lifetime: genes upregulated in aging are involved in apoptosis whereas genes downregulated in aging are involved in cell cycle control, cell division and proliferation [35].



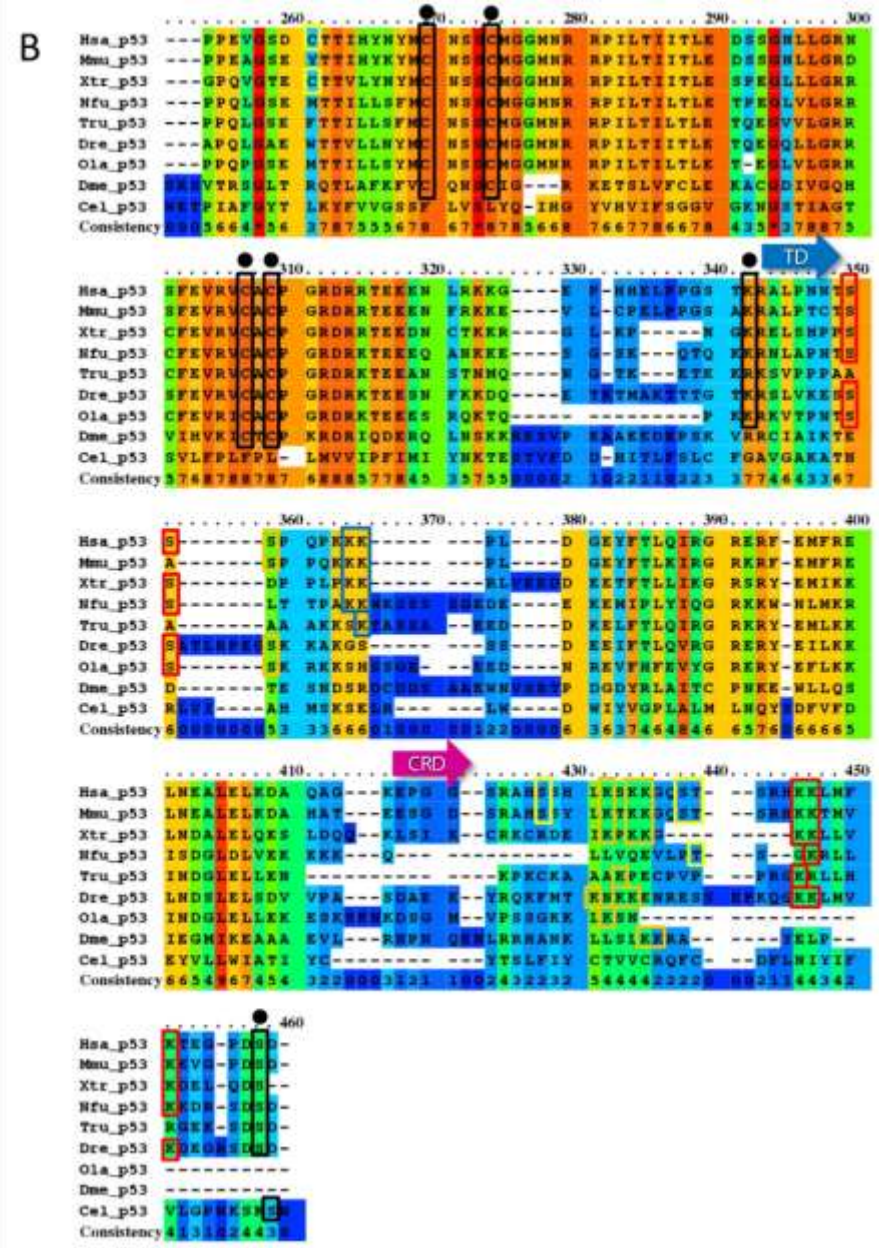


Figure 1. Multiple sequence alignment of p53 of different species. Predicted p53 orthologs were searched using online resources (UCSC Genome Browser, Ensembl Genome Browser and Nothobranchius furzeri transcriptome browser found at https://gen100.imb-jena.de/EST2UNI/nfintb/) and aligned using the online Praline program. Post-translational modified amino acid residues conserved in 7 or more out of 9 species are boxed in black and highlighted with a black spot: note that S15, the phosphorylation site of ATM, is conserved in all the species with the exception of N. furzeri; post-translational modified amino acid residues conserved in 6 or 5 out of 9 species are boxed in red; post-translational modified amino acid residues conserved in 3 or 4 out of 9 are boxed in orange or blue; post-translational modified amino acid residues conserved in 2 out of 9 species are boxed in yellow; post-translational modified amino acid residues present only in human sequence are not indicated. Starting site of Transactivating Domain (TAD), Proline rich Domain (PRD), DNA Binding Domain (DBD), Tetramerization Domain (TD) and C-terminal Regulating Domain (CRD) are indicated. Hsa: Homo sapiens, Mmu: Mus musculus, Xtr: Xenopus tropicalis, Ola: Oryzias latipes (medaka), Tru: Takifugu rubripes (fugu), Dre: Danio rerio (zebrafish), Nfu: Nothobranchius furzeri, Dme: Drosophila melanogaster, Cel: Caenorhabditis elegans.

The body of evidence collected through this last decade and the recent availability of transcriptomics resources make N. furzeri the most promising vertebrate model for research on aging. Nevertheless, since we suggest to investigate the biological role of p53/p66Shc dependent genes in cell cycle progression and senescence before assessing their role in aging, we propose to couple the use of N. furzeri with zebrafish. Zebrafish embryos, with their fast development, transparency, easy handling and the potential to easily perform highly powerful statistical studies, are the ideal alternative to in vitro cell proliferation and senescence assays [36,37]. Although also zebrafish has been successfully used as a model in aging studies [38,39], we suggest to concentrate efforts in definitively establish N. furzeri as the new and widespread model for aging research to make the most of its potential.

p53 conservation between fish and mammals

p53 is well known for its tumor suppression function and less clearly for its role in aging. The expression of dominant active p53 isoforms, that induce overall p53 stabilization and the consequent activation of p53 dependent senescence and apoptotic pathways, is associated with impairment of tissue renewal and premature aging [40,41]. However, the enhanced wild-type p53 expression, as it was induced in "super-p53" mice carrying one or two extra copies of wild-type p53 [42] improves cancer resistance but not accelerated aging or premature aging. Further studies and experimental models are indeed required to clear the role of p53 in aging.

A common ancestor of p53 family genes, more closely related to the two p53-related proteins, p63 and p73 than p53, is found in the nematode Caenorhabditis elegans and the insect Drosophila. The first splitting in p53 family appears with cartilaginous fishes: the elephant shark has two paralogs: one is related to p53 and the other is likely a p63/p73 hybrid. It is with bony fish (zebrafish, fugu, tetraodon, medaka, etc.) that we can clearly find three orthologs of p53, p63 and p73 (p53 evolution is extensively reviewed in [43]). The functional conservation of p53 role between zebrafish and mammals is well established [44,45] , as well as its conserved role in other fish models such as fugu [46] and medaka [47].

A p53 ortholog is present in the N. furzeri transcriptome browser. We cloned the N. furzeri p53 cDNA from the skin of an adult individual, confirming the p53 sequence already present in the database. We compared p53 sequences of C. elegans, D. melanogaster, Danio rerio (zebrafish), N. furzeri, Xenopus tropicalis, Mus musculus and Homo sapiens through protein alignment (Figure 1a and 1b) and analysis of conservation of amino acid residues known to be involved in post-translational modifications with a role in modulating p53 activity and stabilization (Figure 2).

Several post-translational modifications of p53 (reviewed in [48]) can be abolished without any significant phenotype [49], suggesting that functional redundancy evolved to ensure p53 regulation maintenance and each specific combination of different modifications trigger the exact p53 functions including specific localization, degradation rate and transcriptional activity. To unravel this complex scenario, it is useful to evaluate the conservation of these amino acid residues and reconstruct the evolution of the p53 locus.

From the comparison of p53 sequences from C. elegans to H. sapiens the following conclusions can be drawn: (i) the division in functional domains of p53 (amino-terminal domain with transactivation activity, DNA-binding domain, tetramerization domain and carboxy-terminal regulatory domain) is conserved among vertebrates; (ii) the amino-terminal domain is the less conserved not only between vertebrates and invertebrates but also among vertebrates: most of the transactivation domain (TAD) amino acid residues with a proven role in regulating p53 activity are progressively lost from H. sapiens to C. elegans, as well as the proline rich domain (PRD) results progressively depleted of proline residues; (iii) the DNA binding domain is the most conserved, as expected, among all species; (iv) the C-terminal regulatory domain is substantially conserved among vertebrates, from fish to mammals, but not conserved between vertebrate and invertebrates.

The analysis of conserved amino acids (Figure 2) reveals that one of the most relevant post-translational modified residues, S15, which is phosphorylated by ATM (Ataxia-Telangiectasia Mutated) kinase upon DNA damage, increasing p53 stability and reducing its affinity for its best known negative regulator (Double Minute 2 proteins, MDM2 in the mouse; [50]) is conserved in invertebrates. Therefore, the link of p53 with DNA damage sensors appeared early in the evolution of p53. Interestingly, the short-living vertebrate N. furzeri misses the S15 residue as well as S20, which is functionally redundant with S15 [51].

The K120 residue, which is acetylated by TIP60 (Tat-interactive Protein of 60kDa) and MOF (Males absent On the First) proteins and essential for the p53-dependent transcription of genes involved in apoptosis but not cell cycle arrest [52], is present in all the considered species, including N. furzeri.

Other conserved residues are: (i) K164, which is acetylated by CBP (CREB Binding Protein)/p300; like K120, K164 is important for p53 activation and transcription of downstream targets [53] and it is conserved among all the considered vertebrates; (ii)

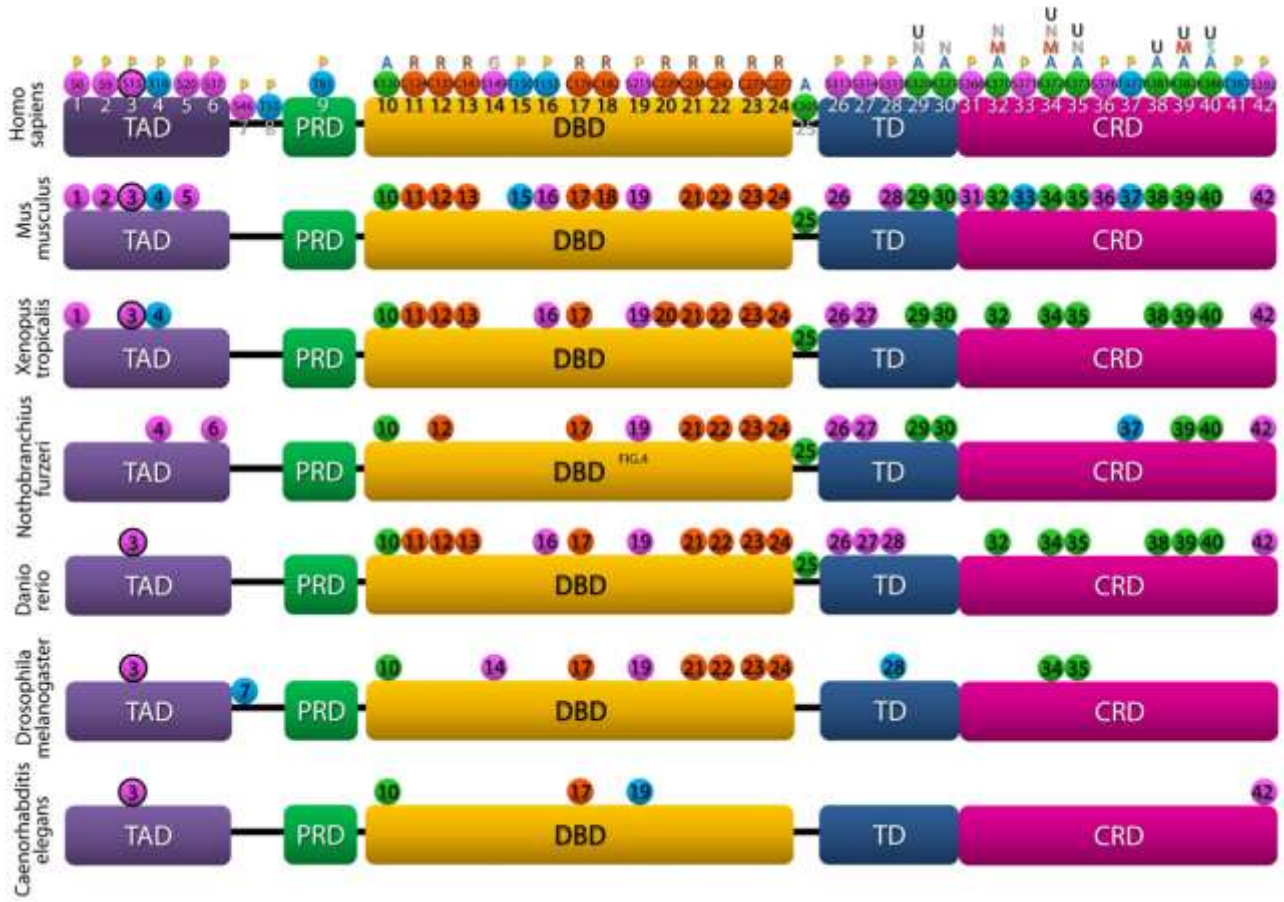


Figure 2. Modular organization of p53 orthologs of different vertebrate and invertebrate species. TAD, violet: Transactivation Domain; PRD, green: Proline Rich Domain; DBD, yellow: DNA Binding Domain, TD, dark blue: Tetramerization domain; CRD, fuchsia: C-terminal Regulatory Domain. Post-translational modified amino acid residues are indicated: serines with a pink spot, threonines with a blue spot, and lysines with a green spot and cysteines with an orange spot. For Homo sapiens, it is also indicated the position in the sequence and the type of post-translational modification (P=phosphorylation, A=acetylation, R=changing of the redox status, G=glycosylation, N=neddylation, S=sumoylation, M=methylation and U=ubiquitination). Amino acids of the human sequence are also numbered from 1 to 43: in other sequences than human, conserved amino acid residues are indicated only with the corresponding number and not with the position in their sequence. Amino acids are considered conserved even if they are substituted with a functionally similar one: for example, T18 is not found in N. furzeri, but this amino acid is here substituted with a serine, thus the number (4) is the same, but the color of the spot changes from blue to pink. S15 is indicated with a pink spot surrounded by a black circle: we want to focus the attention on this residue because it is the best known target site of ATM (Ataxia-Telangiectasia Mutated) kinase and it is one of the few amino acids conserved among all the considered species, with the interesting exception of N. furzeri.

C176 is the most conserved among Zn-coordinating residues, which show a general high conservation, in the DNA binding domain: redox state of cysteines and their capacity of bind zinc ions are important for p53 to exert its role of transcription factor by modulating its ability to bind DNA [54]; (iii) S215 is one of the most conserved serine residue: Aurora B, a mitotic checkpoint kinase, phosphorylates p53 at S215 to accelerate the degradation of p53, functionally suppressing the expression of p53 target genes involved in cell cycle inhibition and apoptosis [55]; phosphorylation of S215 by Aurora A kinase is also a major mechanism of p53 inactivation [56]. Another high conserved residue is S392, which is present in all the considered species with the exception of medaka and D. melanogaster: this residue is phosphorylated in response to UV light induced DNA damage and it is involved in the stabilization of p53 tetramers, but it has a controversial role in cancer development [57].

Two others high conserved residues, not well characterized yet, are K305, that could be acetylated by p300 [58], and S313. The six lysine residues (K370, K372, K373, K381, K382 and K386) at the CRD, acetylated by CBP/p300 or ubiquitinated by HDM2, promoting p53 stabilization and activation or triggering

its degradation [59], are not present (with the exception of K372 and K373 in Drosophila) in invertebrates.

In conclusion, the comparison of p53 sequences indicates that p53 is conserved between fish and mammals. The N-terminal domain (TAD and PRD) is the most variable region between fish and mammals and the highly conserved serine residue which is phosphorylated by ATM upon DNA damage (S15 in human) is not present only in N. furzeri, even if in this species an ATM ortholog is present (see Nothobranchius transcriptome browser assembly 2013/06), suggesting that an atypical p53-dependent DNA damage response may contribute to the high incidence of tumors and early onset of aging typical of N. furzeri.

p66Shc is a vertebrate protein conserved in fish

p66Shc is one of the three isoform encoded by the mammalian shcA locus. The other three isoforms, p52Shc and p46Shc, were identified [11] as two different protein products of the first found shc transcript, which has two in-frame start codons. These two isoforms share an amino-terminal phosphotyrosine binding domain (PTB) followed by a collagen homology 1 domain (CH1) and a carboxy-terminal Src homology 2 domain (SH2) (Figure 3).

p52/p46Shc were found to be involved in Ras signaling pathway: they are phosphorylated on at least three tyrosine residues (discussed later) by Tyrosine Kinases (TKs) and, upon phosphorylation, they function as adaptors between TK receptors and GRB2 (Growth factor Receptor-Bound protein 2), an adaptor protein that, in turn, localize the SOS (Son Of Sevenless) guanine nucleotide exchange factor to the membrane, triggering Ras activation [12]. Therefore, p53/p46Shc are involved in positively regulating cell proliferation [60].

p66Shc was identified as the protein product of a second alternative spliced shc transcript. p66Shc contains the entire p52/46Shc sequence plus an additional amino-terminal region: the proline-rich collagen homology 2 domain (CH2) (Figure 3). Even it can be phosphorylated by active tyrosine kinase receptors, like p52/46Shc, p66Shc is not involved in growth factor-dependent Ras signaling [13]. p66Shc was shown instead to have a role in environmental stress response. p66Shc is phosphorylated following several stresses, including oxidative stress, on a specific serine residue within the CH2 domain, triggering p53-dependent apoptosis, which results abrogated in p66Shc null mice and cells [4, 15]. Research attention was focused on p66Shc because of the evidences that p66Shc null mice not only live the 30% longer [4] but they are resistant to a wide spectrum of age related pathologies including obesity [61,62], diabetes [63,64], atherosclerosis [65,66], and ischemic injury [67,68]. Even if the molecular mechanisms underlying p66Shc role in aging are, to date, only partially unrevealed [18], we demonstrated that p66Shc cooperates with p53 to negatively regulate the expression of a set of genes involved in cell cycle progression and physiologically downregulated in aging [23]

Shc are the only class of proteins with a N-terminal PTB domain and a C-terminal SH2 domain and these proteins have likely a common ancestor (11): among Shc proteins we find at least eight proteins: p66/p52/p46Shc (ShcA), the brain-specific ShcB and ShcC, with two isoforms each [69,70], and ShcD, expressed, among adult tissues, only in melanomas [71].

Shc proteins are poorly studied in amphibians and fishes: we know that a 58kDa Shc-related protein is present in Xenopus eggs and may act upstream of the calcium-dependent pathway of egg activation [72]; interestingly, this protein is phosphorylated upon H2O2 treatment of Xenopus eggs [73]. Always in Xenopus, a 60kDa Shc protein, which is likely the ortholog of mammalian p52Shc, was identified and found involved in Ras dependent oocyte maturation induced by insulin/IGF-1 [74]. Among fishes, we know that a neuronal Shc, named N-Shc, is present in fugu and that it is likely the ortholog of mammalian ShcC [75] ; p52/p46Shc orthologs have been identified in zebrafish, where they are essential for embryonic angiogenesis, as in mammals [76].

We compared the predicted p66Shc sequences of Danio rerio (zebrafish), Takifugu rubripes (fugu), Oryzias latipes (medaka), Nothobranchius furzeri, Xenopus tropicalis, Mus musculus and Homo sapiens through protein alignment (Figure 3) and analysis of the conservation of post-translational modified amino acid residues involved in p66Shc activity (Figure 4). We also considered Drosophila melanogaster Shc ortholog and the two predicted Shc-like proteins identified in Caenorhabditis elegans, F54A5.3a and T27F7.2 [14]. In all the considered species, a predicted p66Shc ortholog is present and distinguished from p52Shc by virtue of a ~110 amino acids CH2 domain. At a genomic level, the CH2 domain should be entirely encoded by an alternative spliced intron found in all the species. As far for N. furzeri, this is the only species among the considered ones for which a sequenced genomic DNA is not available yet. We cloned the genomic sequence corresponding to the CH2 domain and we confirmed that, even in this fish, the CH2 domain of the predicted p66Shc ortholog should be encoded by a single alternative spliced intron. On the contrary, we did not find a CH2-like domain in the predicted invertebrate Shc orthologs with the exception of long T27F7.2 Shc-like protein of C. elegans, indicating that the radiation of Shc isoforms occurred in vertebrates.

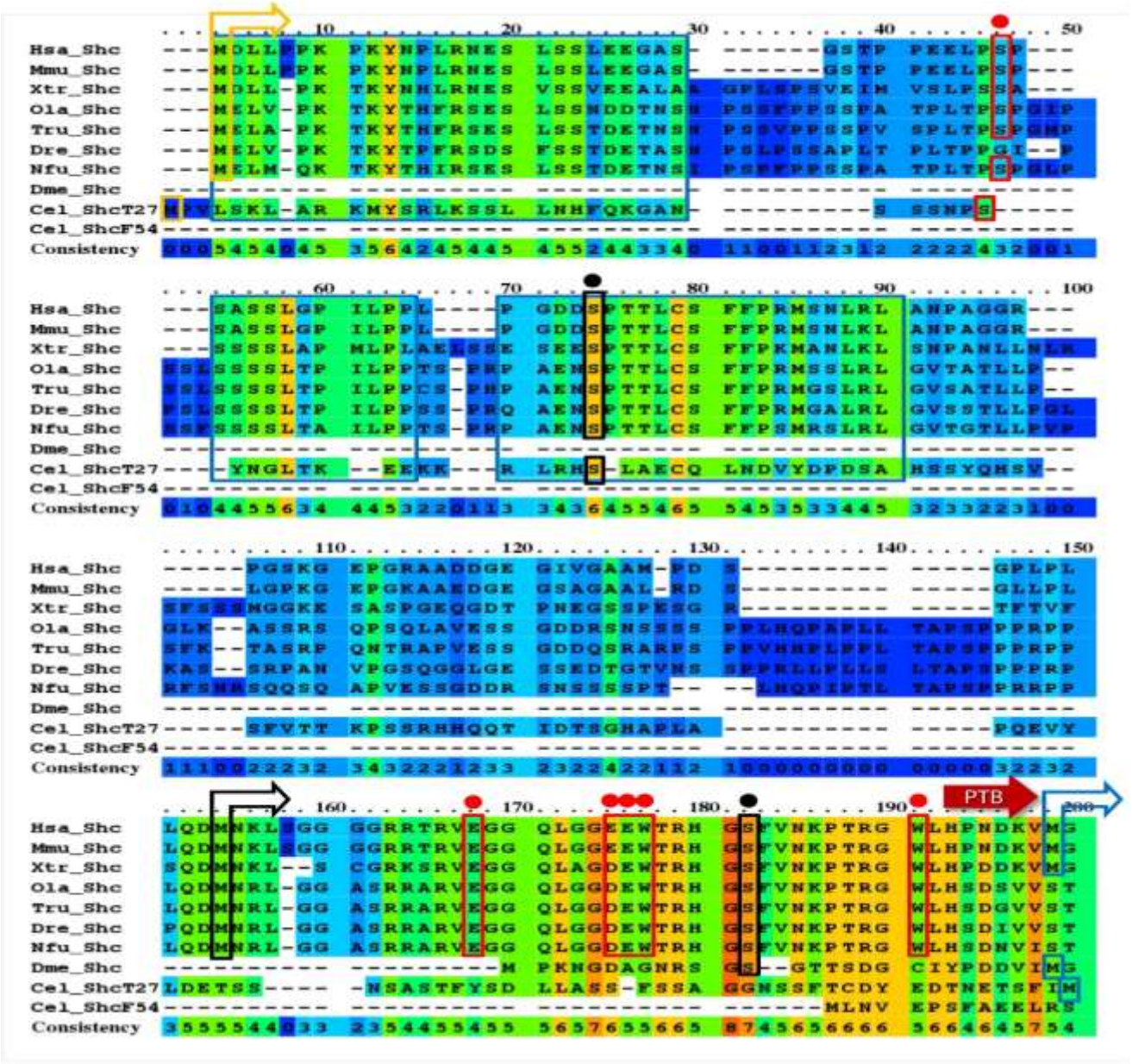
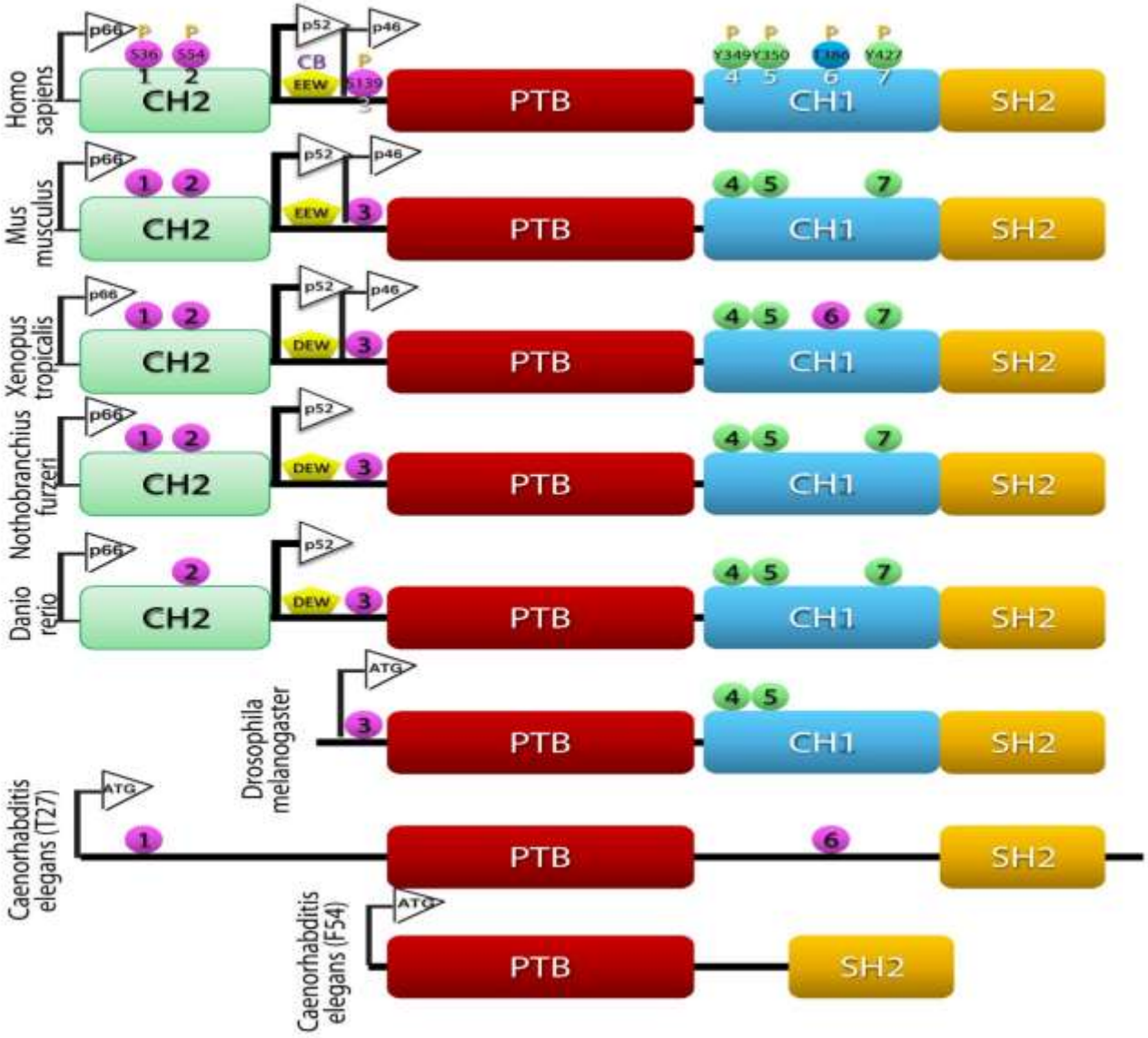


Figure 3. Multiple sequence alignment of p66Shc CH2 domain of different species. Predicted p66Shc orthologs were searched using online resources (UCSC Genome Browser, Ensembl Genome Browser and Nothobranchius furzeri transcriptome browser found at https://gen100.imb-jena.de/EST2UNI/nfintb/) and aligned using the online Praline program. High homology regions within the CH2 domains are boxed in blue. Post-translational modified amino acid residues conserved in 8 or more out of 10 sequences are boxed in black and highlighted with a black spot: S54 is conserved among vertebrates and C. elegans T27F7.2; S139 is conserved among vertebrates and Drosophila. Post-translational modified amino acid residues conserved in 7 out of 10 sequences are boxed in red and highlighted with a red spot: S36 is conserved in vertebrates (with the exception of D. rerio) and C. elegans T27F7.2; amino acids constituting the Cytochrome c binding domain (E125, E132, E133, W134 and W148) of p66Shc are conserved in vertebrates. Starting methionine of the predicted p66Shc is boxed in orange. Starting methionine of the predicted p52Shc is boxed in black: it is strictly conserved among vertebrates. Starting methionine of the predicted p46Shc is boxed in blue: it is conserved in mammals, X. tropicalis, D. melanogaster and C. elegans T27F7.2. Starting site of Phosphotyrosine Binding Domain (PTB) is indicated. Hsa: Homo sapiens, Mmu: Mus musculus, Xtr: Xenopus tropicalis, Ola: Oryzias latipes (medaka), Tru: Takifugu rubripes (fugu), Dre: Danio rerio (zebrafish), Nfu: Nothobranchius furzeri, Dme: Drosophila melanogaster, Cel: Caenorhabditis elegans T27F7.2 and F54A5.3a.



Figuer 4. Modular organization of p66Shc orthologs of different vertebrate species. CH2, light green: Collagen Homology 2 domain; PTB, red: Phosphotyrosine Binding Domain; CH1, light blue: Collagen Homology 1 domain; SH2, yellow: Src homology 2 domain. Note that a CH2 region is present in all the vertebrate species, whereas it is absent or not recognizable in invertebrates. Post-translational modified amino acid residues are indicated: serines with a pink spot, threonines with a blue spot and tyrosines with a light green spot. For Homo sapiens, it is also indicated the position in the sequence and the type of post-translational modification (P=phosphorylation). Amino acids of the human sequence are also numbered from 1 to 7: in other sequences than human, conserved amino acid residues are indicated only with the corresponding number and not with the position in their sequence. Amino acids are considered conserved even if they are substituted with a functionally similar one: T386 is not found in X. tropicalis, but this amino acid is here substituted with a serine, thus the number (6) is the same, but the color of the spot changes from blue to pink. The Cytochrome c Binding (CB) domain is indicated with a yellow pentagon with the amino acids of the core: EEW in H. sapiens and M. musculus, DEW in X. tropicalis, N. furzeri and D. rerio. Starting site of the p52Shc isoform is also indicated with an arrow.

The PTB and SH2 domains are highly conserved among all the considered species, whereas the CH1 domain is conserved only among vertebrates, but it is still recognizable in Drospophila; moreover, the short F54A5.3a Shc-like protein of C. elegans does not show a CH1-like sequence, suggesting that the ancestor of Shc proteins probably consisted of only a N-terminal PTB domain and a C-terminal SH2 domain. As expected, the CH2 domain is the less conserved among vertebrates; nevertheless, we found three regions of high conservation.

P66Shc-S36 in human and mouse, is strictly conserved among vertebrates. S36 seems not to be conserved only in zebrafish, whereas it is likely present in the long T27F7.2 Shc-like protein of C. elegans. The functional role of S36 has been well characterized: this residue is rapidly and persistently phosphorylated, in vitro, upon pro-apoptotic stresses and is necessary for the p66Shc mitochondrial translocation [77] and apoptosis. In fact, when this amino acid is substituted with an alanine residue, p66Shc-mediated cell death is abrogated [4]. Moreover, it has been demonstrated that Endothelin-1 (ET-1), a vasoconstrictor peptide known to be a potent mitogen for glomerular mesangial cells (GMC), can trigger the phosphorylation of p66Shc on S36: p66Shc serine phosphorylation results in its association with the sequestering protein 14-3-3 [78]. Human and mouse S36 are found within the minimal S/TP (serine/threonine followed by proline) target motif that is recognized by MAPK (Mitogen Activated Protein Kinases), comprising ERKs (Extracellular signal Regulated Kinases), p38MAPK and JNKs (c-Jun N-terminal Kinases) [79, 80]. As expected, p66Shc-S36 is the target of ERKs [81], p38MAPLK and JNKs [82]. Notably, S36 is followed by a proline residue even in fish (but not in X. leavis and C. elegans), leading to the hypothesis that p66Shc could be the substrate of MAPK even in these species (Figure 3).

The other group of amino acids with a proven function is located in the Cytochrome c-binding region (CB) of p66Shc. In particular, this region includes the three acidic (human E125, E132, and E133) and two tryptophan residues (human W134 and W148) involved respectively in the ionic and electron transfer interactions with the cytochrome c. The same motif is found in the cytochrome c pocket region of COX IV (Cytochrome c Oxidase subunit IV) and cytochrome c peroxidase of yeast, which represent the two known redox enzymes that use cytochrome c as a substrate [83,16]. Substitution of the acidic and tryptophan residues within the CB region of p66Shc impairs its ability in producing ROS [20, 84]. According to this, the cytochrome c binding motif is strictly conserved among vertebrates: E125, E133, W134 and W148 are present among all the considered vertebrate species, with the only exception of the glutamic acid 132 substituted, in Xenopus and fishes, with an aspartic acid residue, thus maintaining the negative charge essential for cytochrome c binding. Another crucial residue conserved in fishes is the human cysteine 59 (Figure 4) whose oxidation-reduction cycle regulates structural rearrangements in p66Shc CH2-CB, the oligomerization state and the reaction rate of the mammalian p66Shc within mitochondria [85].

Therefore, the redox activity of p66Shc on cytochrome c appears to be conserved from fish to mammals.

There are few other post-translational modified residues that are proven to be functionally important. (i) S139 is phosphorylated in vitro after TPA (12-O-tetradecanoylphorbol-13-acetate, a potent tumor inducer and an activator of Protein Kinase C – PKC) treatment and the consequence of this modification is the binding of p66Shc with protein-tyrosine phosphatase PTP-PEST, which is responsible for the de-phosphorylation and down-regulation of ShcA [86]. S139 is conserved among vertebrates and it is also present in Drosophila Shc ortholog. (ii) S54 and T386 are both targets of phosphorylation and this modifications likely affect p66Shc stability. These two residues are found within the minimal target motif that is recognized by MAPK: p38MAPK phosphorylates S54 and T386 after Rac1 activation. The hypothetical mechanism is that phosphorylation of p66Shc on S54 and T386 serves to mask p66Shc PEST sequences: indeed, S54 and T386 are found within PEST motifs that can be recognized by the ubiquitin-26S proteasome degradation pathway [87]. T386 is present only in human p66Shc, whereas S54, which lies within the CH2 domain, is conserved, together with the following proline residue, among vertebrates. (iii) We show, finally, that also the three tyrosine residues found in the CH1 domain (Y349, Y350 and Y427) are strictly conserved among vertebrates; two of them, Y349 and Y350, are conserved even in Drosophila. These residues are not proven to be relevant for p66Shc though they may be involved in the recruitment of the Grb2-SOS complex on the plasma membrane and Ras activation [88, 89].

In conclusion, our analyses suggest that: (i) a Shc isoform involved in oxidative stress regulation is appeared in vertebrates evolution (ii) a p66Shc ortholog is present in N. furzeri with, potentially, all the effects of mammalian p66Shc on intracellular ROS production and apoptosis.

The short isoform of p53 is present in fish and the p53-p66 target genes are present in Nothobranchius furzeri

In normal adult tissues, the basal level of p53 protein is low. Cellular stresses induce post-translational

modifications and p53 stabilization, leading to activation of distinct transcriptional programmes through which p53 can orchestrate different cellular outcomes such as DNA repair, apoptosis and senescence [90]. How p53 can react specifically to the different stresses is unclear. Emerging findings suggest that p53 activity on specific targets is regulated by specific p53 isoforms, in the context of specific activating-signals [91].

The structure of the human p53 gene is very complex, alternative promoter usage and/or splicing of p53 mRNA gives rise to at least nine mammalian p53 proteins with distinct N- and C-termini which are differentially expressed in normal and cancer cells. The three known N-terminal p53 variants contain either the full-length (FL), or truncated (DN/D47) or no transactivation domain (D113/D133) altogether. The mouse p53 gene expresses six different p53 isoforms, the zebrafish gene expresses three p53 isoforms, as well the Drosphila p53 gene, whereas in C.elegans only one p53 isoform is encoded, corresponding to the full-length human p53 protein [92]. Like the homo sapiens, all the analyzed species express a full-length p53 protein and a truncated DeltaN (DN) p53 or Delta40p53 isoforms, which are similar to the human p47 and the mouse p44/p53. The mammalian DNp53 forms homo-oligomers and hetero-oligomers with p53 and induces G2/M cell-cycle arrest in response to serum deprivation or endoplasmic reticulum (ER) stress [93, 94, 95, 96]. Analysis of p53 sequences of N. furzeri shows that downstream of ATG that encodes full length protein, another in-frame ATG is present. However, in zebrafish the Delta Np53 isoform is generated from an ATG located within an intronic sequence retained by alternative splicing [94].

In Mammals, the p66Shc redox activity regulates the pro-aging function of p53 by modulating the expression of the Delta40p53 short isoform. Furthermore, protein damage rather than DNA damage induced by chronic oxidative stress regulates the expression of a set of approximately 200 genes in a p53 and p66Shc dependent manner (p53-p66Shc signalling pathway) [23]. These sets of genes is conserved in N. furzeri and around 60% of them were found down-regulated during the life of the fish (unpublished results). In particular, all the genes of this set, which are involved in mitosis entry and progression, execution of mitotic processes and spindle checkpoint, resulted to be regulated with aging as it was observed in mice.

Conclusions and Future Perspectives

Intensive efforts have focused on understanding the role of the tumour suppressor p53 in both cancer and aging, the manner in which it is activated and the effect of its isofoms on its function. Many studies have in turn led to the development and identification of several novel molecules that promote and restore p53 activity for implementation of therapy in cancer treatment. Recently, we have found that p66Shc protein is one of the crucial effectors of p53 function in aging. We demonstrated that the p53 transcriptional-response to oxidative stress largely depends on p66shc protein and involves a large number of G2/M-mitosis genes, suggesting that p66Shc is critical for the inhibitory effects of p53 on cell cycle after oxidative stress (p53-p66 G2/M genes).

The aim of this review is to investigate the conservation of the p66Shc and p53 role in oxidative stress between fish and mammal in order to propose to approach this study trough a new model organism, the annual fish Nothobranchius furzeri, characterized by an exceptionally short life span (3–9 months). It has been demonstrated that this fish develops typical signs of aging, like mammals, including senescence, neurodegeneration, metabolic disorders and cancer.

However, despite important questions remain on the role of stress dependent mitotic checkpoint regulation in cancer and aging, the preliminary study of N. furzeri transcriptomic resources confirms the importance of p53 and p66Shc genes for aging phenotypes and leaves open the possibility to evaluate the impact of p53/p44-p66 dependent G2/M pathways on age-dependent degenerations and cancer incidence, in a wide array of organs, that could lead to identify new genes affecting aging or cancer in vertebrates.

Acknowledgements

We would like to thank Lucilla Luzi for helpful discussion and Gianluca Deflorian for exceptional technical support and critical discussion. The Associazione Italiana per la Ricerca sul Cancro (AIRC) supported this work.

References

[1] Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P, Le Bouc Y (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature, 421: 182-187

[2] Bartke A, Brown-Borg H (2004). Life extension in the dwarf mouse. Curr Top Dev Biol, 63:189-225

[3] Yamamoto M, Clark JD, Pastor JV, Gurnani P, Nandi A, Kurosu H, Miyoshi M, Ogawa Y, Castrillon DH, Rosenblatt KP and Kuro-o M (2005). Regulation of oxidative stress by the anti-aging hormone klotho. J Biol Chem, 280: 38029-38034

[4] Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, Lanfrancone L, Pelicci PG (1999). The p66shc adaptor protein controls oxidative stress

response and life span in mammals. Nature, 402:309-313

[5] Carpi A, Menabo R, Kaludercic N, Pelicci P, Di Lisa F, Giorgio M (2009). The cardioprotective effects elicited by p66(Shc) ablation demonstrate the crucial role of mitochondrial ROS formation in ischemia/reperfusion injury. Biochim Biophys Acta, 1787:774-780

[6] Fadini GP, Albiero M, Menegazzo L, Boscaro E, Pagnin E, Iori E, Cosma C, Lapolla A, Pengo V, Stendardo M, Agostini C, Pelicci PG, Giorgio M, Avogaro A (2010). The redox enzyme p66Shc contributes to diabetes and ischemia-induced delay in cutaneous wound healing.Diabetes, 59:2306-2314

[7] Savino C, Pelicci P, Giorgio M (2013). The P66Shc/mitochondrial permeability transition pore pathway determines neurodegeneration. Oxid Med Cell Longev, 2013:719407.

[8] Harman D (1981). The aging process. Proc Natl Acad Sci USA,78:7124-7128

[9] Kujoth GC, Hiona A, Pugh TD, et al (2005). Mitochondrial DNA mutations, oxidative stress and apoptosis in mammalian aging. Science 309:481-484

[10] Naylor RM, Baker DJ, van Deursen JM (2013). Senescent cells: a novel therapeutic target for aging and age-related diseases.Clin Pharmacol Ther, 93:105-116

[11] Pelicci G, Lanfrancone L, Grignani F, et al (1992). A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell 70:93-104

[12] Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L, Pelicci PG (1996). Not all Shc's roads lead to Ras. Trends Biochem Sci, 21:257-261

[13] Migliaccio E, Mele S, Salcini AE, et al (1997).Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. EMBO J, 16:706-716

[14] Luzi L,Confalonieri S,Di Fiore PP, Pelicci PG (2000).Evolution of Shc functions from nematode to human.Curr Opin Genet Dev,10:668-674

[15] Trinei M, Giorgio M, Cicalese A, et al (2002). A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. Oncogene,21:3872-3878

[16] Migliaccio E, Giorgio M, Pelicci PG (2006). Apoptosis and aging: role of p66Shc redox protein. Antioxid Redox Signal, 8:600-608

[17] Trinei M, Berniakovich I, Beltrami E, Migliaccio E, Fassina A, Pelicci P, Giorgio M (2009). P66Shc signals to age. Aging, 1(6): 503-510

[18] Ramsey JJ, Tran D, Giorgio M, et al (2013). The Influence of Shc Proteins on Life Span in Mice. J Gerontol A Biol Sci Med Sci, in press.

[19] Orsini F, Migliaccio E, Moroni M, et al (2004). The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. J Biol Chem, 279:25689-25695

[20] Giorgio M, Migliaccio E,Orsini F, et al (2005). Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. Cell, 122:221-233

[21] Feng Z, Lin M, Wu R (2011). The Regulation of Aging and Longevity: A New and Complex Role of p53. Genes Cancer, 2:443-452

[22] Maier B, Gluba W, Bernier B, et al (2004). Modulation of mammalian life span by the short isoform of p53. Genes Dev, 18:306-319

[23] Gambino V, De Michele G, Venezia O, et al (2013). Oxidative stress activates a specific p53 transcriptional-response that regulates cellular senescence and aging. Aging Cell, 1 2:435-45.

[24] Di Cicco E, Tozzini ET, Rossi G, Cellerino A (2011). The short-lived annual fish Nothobranchius furzeri shows a typical teleost aging process reinforced by high incidence of age-dependent neoplasias. Exp Gerontol, 46:249-256

[25] Valdesalici S, Cellerino A (2003). Extremely short lifespan in the annual fish Nothobranchius furzeri. Proc Biol Sci, 270 Suppl 2S189-191

[26] Kenyon C (1996). Ponce d'elegans: genetic quest for the fountain of youth. Cell, 84:501-504

[27] Guarente L, Kenyon C (2000). Genetic pathways that regulate ageing in model organisms. Nature, 408:255-262

[28] Ladiges W, Van Remmen H, Strong R, Ikeno Y, Treuting P, Rabinovitch P, Richardson A (2009). Lifespan extension in genetically modified mice. Aging Cell, 8:346-352

[29] Dorn A, Ng'oma E, Janko K, Reichwald K, Polacik M, Platzer M, Cellerino A, Reichard M (2011). Phylogeny, genetic variability and colour polymorphism of an emerging animal model: the short-lived annual Nothobranchius fishes from southern Mozambique. Mol Phylogenet Evol, 61:739-749

[30] Terzibasi E, Valenzano DR, Benedetti M, Roncaglia P, Cattaneo A, Domenici L, Cellerino A (2008). Large differences in aging phenotype between strains of the short-lived annual fish Nothobranchius furzeri. PLoS One, 3:e3866

[31] Genade T, Benedetti M, Terzibasi E, et al (2005). Annual fishes of the genus Nothobranchius as a model system for aging research. Aging Cell, 4:223-233

[32] Valenzano DR, Terzibasi E, Genade T, et al (2006). Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr Biol, 16:296-300

[33] Kirschner J, Weber D, Neuschl C, et al (2012). Mapping of quantitative trait loci controlling lifespan in the short-lived fish Nothobranchius furzeri--a new vertebrate model for age research. Aging Cell, 11:252-61.

[34] Baumgart M, Groth M, Priebe S, et al (2012) Age-dependent regulation of tumor-related microRNAs in the brain of the annual fish Nothobranchius furzeri Mech Ageing Dev, 133:226-33.

[35] Petzold A, Reichwald K, Groth M, et al (2013). The transcript catalogue of the short-lived fish Nothobranchius furzeri provides insights into age-dependent changes of mRNA levels. BMC Genomics, 14: 185-201

[36] Li IC, Chan CT, Lu YF, et al. (2011) Zebrafish kruppel-like factor 4a represses intestinal cell proliferation and promotes differentiation of intestinal cell lineages. PLoS One, 6:e20974.
[37] Donnini S, Giachetti A, Ziche M (2013) Assessing vascular senescence in zebrafish. Methods Mol Biol, 965:517-31.
[38] Santoriello C, Deflorian G, Pezzimenti F, et al (2009) Expression of H-RASV12 in a zebrafish model of Costello syndrome causes cellular senescence in adult proliferating cells. Dis Model Mech, 2:56-67.
[39] Anchelin M, Alcaraz-Pérez F, Martínez CM, et al (2013) Premature aging in telomerase-deficient zebrafish. Dis Model Mech. 6:1101-12.
[40] Maier B, Gluba W, Bernier B, et al (2004). Modulation of mammalian life span by the short isoform of p53. Genes Dev, 18:306-319
[41] Tyner SD, Venkatachalam S, Choi J, et al (2002). p53 mutant mice that display early ageing-associated phenotypes. Nature, 415:45-53
[42] Garcia-Cao I, Garcia-Cao M, Martin-Caballero J, et al (2002). "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. EMBO J, 21:6225-6235
[43] Belyi VA, Ak P, Markert E, et al (2010). The origins and evolution of the p53 family of genes. Cold Spring Harb Perspect Biol, 2: a001198
[44] Jeon HY and Lee H (2013). Depletion of Aurora-A in zebrafish causes growth retardation due to mitotic delay and p53-dependent cell death. FEBS J 280:1518-1530
[45] Gunther EJ, Moody SE, Belka GK, et al (2003). Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. Genes Dev, 17: 488-501
[46] Le Bras M, Bensaad K, Soussi T (2003). Data mining the p53 pathway in the Fugu genome: evidence for strong conservation of the apoptotic pathway. Oncogene, 22:5082-5090
[47] Yasuda T, Oda S, Li Z, Kimori Y, Kamei Y, Ishikawa T, Todo T, Mitani H (2012). Gamma-ray irradiation promotes premature meiosis of spontaneously differentiating testis-ova in the testis of p53-deficient medaka (Oryzias latipes). Cell Death Dis, 3e395
[48] Dai C, Gu W (2010). p53 post-translational modification: deregulated in tumorigenesis. Trends Mol Med, 16:528-536
[49] Iwakuma T, Lozano G (2007). Crippling p53 activities via knock-in mutations in mouse models. Oncogene, 26; 2177-2184.
[50] Tang Y, Luo J, Zhang W, Gu W (2006). Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. Mol Cell, 24:827-839
[51] Chao C, Herr D, Chun J, Xu Y (2006). Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. EMBO J, 25:2615-2622
[52] Tang Y, Zhao W, Chen Y, Zhao Y, Gu W (2008). Acetylation is indispensable for p53 activation. Cell, 133: 612-626.
[53] Rainwater R, Parks D, Anderson ME, Tegtmeyer P, Mann K (1995). Role of cysteine residues in regulation of p53 function. Mol Cell Biol, 15:3892-3903
[54] Stoner CS, Pearson GD, Koc A, Merwin JR, Lopez NI, Merrill GF (2009). Effect of thioredoxin deletion and p53 cysteine replacement on human p53 activity in wild-type and thioredoxin reductase null yeast. Biochemistry, 48: 9156-9169
[55] Gully CP, Velazquez-Torres G, Shin JH, et al (2012). Aurora B kinase phosphorylates and instigates degradation of p53. Proc Natl Acad Sci U S A, 109:E1513-1522
[56] Liu Q, Kaneko S, Yang L, et al (2004). Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. J Biol Chem, 279: 52175-52182
[57] Matsumoto M, Furihata M, Ohtsuki Y (2006) Posttranslational phosphorylation of mutant p53 protein in tumor development. Med Mol Morphol 39:79-87
[58] Wang YH, Tsay YG, Tan BC, Lo WY, Lee SC (2003) Identification and characterization of a novel p300-mediated p53 acetylation site, lysine 305. J Biol Chem, 278:25568-25576
[59] Kruse JP, Gu W (2008). SnapShot: p53 posttranslational modifications. Cell 133, 930-930 e931.
[60] Lanfrancone L, Pelicci G, Brizzi MF, et al (1995). Overexpression of Shc proteins potentiates the proliferative response to the granulocyte-macrophage colony-stimulating factor and recruitment of Grb2/SoS and Grb2/p140 complexes to the beta receptor subunit. Oncogene, 10:907-917
[61] Berniakovich I, Trinei M, Stendardo M, et al (2008). p66Shc-generated oxidative signal promotes fat accumulation. J Biol Chem 283:34283-34293
[62] Ranieri SC, Fusco S, Panieri E, et al (2010). Mammalian life-span determinant p66shcA mediates obesity-induced insulin resistance. Proc Natl Acad Sci U S A, 107:13420-13425
[63] Menini S, Amadio L, Oddi G, et al (2006). Deletion of p66Shc longevity gene protects against experimental diabetic glomerulopathy by preventing diabetes-induced oxidative stress. Diabetes 55:1642-1650
[64] Fadini GP, Albiero M, Menegazzo L, et al (2010). The redox enzyme p66Shc contributes to diabetes and ischemia-induced delay in cutaneous wound healing. Diabetes, 59: 2306-2314
[65] Napoli C, Martin-Padura I, de Nigris F, et al (2003). Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high fat diet. Proc Natl Acad Sci U S A, 100:2112-2116
[66] Graiani G, Lagrasta C, Migliaccio E, et al (2005). Genetic deletion of the p66Shc adaptor protein protects from angiotensin II-induced myocardial damage. Hypertension, 46:433-440
[67] Zaccagnini G, Martelli F, Fasanaro P, et al (2004). p66ShcA modulates tissue response to hindlimb ischemia. Circulation, 109: 2917-2923
[68] Carpi A, Menabo R, Kaludercic N, et al (2009). The cardioprotective effects elicited by p66 (Shc) ablation

demonstrate the crucial role of mitochondrial ROS formation in ischemia/reperfusion injury. Biochim Biophys Acta, 1787: 774-780

[69] Cattaneo E, Pelicci PG (1998). Emerging roles for SH2/PTB-containing Shc adaptor proteins in the developing mammalian brain. Trends Neurosci, 21:476-481

[70] Kojima T, Yoshikawa Y, Takada S, et al (2001). Genomic organization of the Shc-related phosphotyrosine adapters and characterization of the full-length Sck/ShcB: specific association of p68-Sck/ShcB with pp135. Biochem Biophys Res Commun, 284:1039-1047

[71] Fagiani E, Giardina G, Luzi L, et al (2007). RaLP, a new member of the Src homology and collagen family, regulates cell migration and tumor growth of metastatic melanomas. Cancer Res, 67:3064-3073

[72] Aoto M, Sato K, Takeba S, et al (1999). "A 58-kDa Shc protein is present in Xenopus eggs and is phosphorylated on tyrosine residues upon egg activation." Biochem Biophys Res Commun, 258:265-70.

[73] Sato K, Ogawa K, Tokmakov AA, Iwasaki T, Fukami Y (2001). Hydrogen peroxide induces Src family tyrosine kinase-dependent activation of Xenopus eggs. Dev Growth Differ, 43:55-72

[74] Chesnel F, Heligon C, Richard-Parpaillon L, Boujard D (2003). Molecular cloning and characterization of an adaptor protein Shc isoform from Xenopus laevis oocytes. Biol Cell, 95:311-320

[75] Yamaguchi F, Yamaguchi K, Tokuda M, Brenner S (1999). Molecular cloning of EDG-3 and N-Shc genes from the puffer fish, Fugu rubripes, and conservation of synteny with the human genome. FEBS Lett, 459:105-110

[76] Sweet DT, Chen Z, Wiley DM, Bautch VL, Tzima E (2011). The adaptor protein Shc integrates growth factor and ECM signaling during postnatal angiogenesis. Blood 119:1946-1955

[77] Pinton P, Rimessi A, Marchi S, et al (2007). Protein kinase C beta and prolyl isomerase 1 regulates mitochondrial effects of the life-span determinant p66Shc. Science, 315: 659-663

[78] Foschi M, Franchi F, Han J, La Villa G, Sorokin A (2001). Endothelin-1 induces serine phosphorylation of the adaptor protein p66Shc and its association with 14-3-3 protein in glomerular mesangial cells. J Biol Chem, 276:26640-26647

[79] Kyriakis JM, Avruch J (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. J Biol Chem, 4:24313-6.

[80] Karandikar M, Cobb MH (1999) Scaffolding and protein interactions in MAP kinase modules." Cell Calcium, 26:219-26

[81] Hu Y, Wang X, Zeng L, Cai DY, Sabapathy K, Goff SP, Firpo EJ, Li B (2005). ERK phosphorylates p66shcA on Ser36 and subsequently regulates p27kip1 expression via the Akt-FOXO3a pathway: implication of p27kip1 in cell response to oxidative stress. Mol Biol Cell, 16:3705-18.

[82] Le S, Connors TJ, Maroney AC (2001). c-Jun N-terminal kinase specifically phosphorylates p66ShcA at serine 36 in response to ultraviolet irradiation. J Biol Chem, 276:48332-6.

[83] Zhen Y, Hoganson CW, Babcock GT, Ferguson-Miller S (1999). Definition of the interaction domain for cytochrome c on cytochrome c oxidase. I. Biochemical, spectral, and kinetic characterization of surface mutants in subunit ii of Rhodobacter sphaeroides cytochrome aa. J Biol Chem, 274:38032-38041

[84] Trinei M, Migliaccio E, Bernardi P, Paolucci F, Pelicci P, Giorgio M (2013). p66Shc, mitochondria, and the generation of reactive oxygen species. Methods Enzymol, 528: 99-11

[85] Gertz M, Fischer F, Wolters D, Steegborn C (2008). Activation of the lifespan regulator p66Shc through reversible disulfide bond formation. Proc Natl Acad Sci U S A., 105: 5705-9.

[86] Faisal A, el-Shemerly M, Hess D, Nagamine Y (2002). Serine/threonine phosphorylation of ShcA.Regulation of protein-tyrosine phosphatase-pest binding and involvement in insulin signaling. J Biol Chem, 277:30144-30152

[87] Khanday FA, Yamamori T, Mattagajasingh I, et al (2006). Rac1 leads to phosphorylation-dependent increase in stability of the p66shc adaptor protein: role in Rac1-induced oxidative stress. Mol Biol Cell, 17:122-129

[88] Salcini AE, McGlade J, Pelicci G, et al (1994). Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. Oncogene, 9:2827-2836

[89] Gotoh T, Niino Y, Tokuda M, Hatase O, Nakamura S, Matsuda M, Hattori S (1997). Activation of R-Ras by Ras-guanine nucleotide-releasing factor. J Biol Chem, 272:18602-18607

[90] Aylon Y, Oren M (2007). Living with p53, dying of p53. Cell, 130:597-600.

[91] Olivares-Illana V, Fahraeus R (2010). p53 isoforms gain functions Oncogene, 29:5113-5119

[92] Marcel V, Dichtel-Danjoy ML, Sagne C, et al (2011). Biological functions of p53 isoforms through evolution: lessons from animal and cellular models. Cell Death Differ 18:1815-1824

[93] Bourougaa K, Naski N, Boularan C, et al (2012). Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47. Mol Cell, 38:78-88

[94] Davidson WR, Kari C, Ren Q, et al (2010). Differential regulation of p53 function by the N-terminal DeltaNp53 and Delta113p53 isoforms in zebrafish embryos. BMC Dev Biol, 10:102-113

[95] Bauer JH, Poon PC, Glatt-Deeley H, Abrams JM, Helfand SL (2005). Neuronal expression of p53 dominant-negative proteins in adult Drosophila melanogaster extends life span. Curr Biol, 15:2063-2068

[96] Ventura N, Rea SL, Schiavi A, Torgovnick A, Testi R, Johnson TE (2009). p53/CEP-1 increases or decreases lifespan, depending on level of mitochondrial bioenergetic stress. Aging Cell, 8:380-393