UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Biologia Molecolare e Cellulare

XXIX Ciclo

The role of the p53/p66^{Shc} pathway in development and aging: Danio rerio *(zebrafish) and* Nothobranchius *as model organisms*

Chiara Priami

PhD Thesis

Scientific tutors:

Prof. Franco Cotelli
Dr. Enrica Migliaccio
Prof. Marco Muzi-Falconi

Academic year: 2016/2017

SSD: Biologia dello sviluppo (BIO/06), Biologia molecolare (BIO/11)

Thesis performed at:

Department of Biosciences, University of Milan

Department of Experimental Oncology, European Institute of Oncology

Table of contents

PART I

Abstract

The transcriptional response to oxidative stress (OS) is involved in aging. As ROS-induced damages accumulate, cell senescence or apoptosis are triggered: these two mechanisms are implicated in the progressive physiological decay of the organism. Surprisingly, transcriptional pathways involved in OS-response play a role also in the balance between proliferation and differentiation during embryonic development. Recently, it has been discovered a transcriptional network that triggers cell cycle arrest *in vitro* upon OS. This pathway involves two well-known aging-associated genes: *p53*, with its short isoform Δ40p53, and $p66^{Shc}$. Here we propose the use of two complementary model organisms, *Danio rerio* (zebrafish) and *Nothobranchius furzeri*, to unravel the p53/$p66^{Shc}$ pathway *in vivo*. We report here that Δ40p53 and $p66^{Shc}$ are conserved in these species, partially recapitulating mammalian functions. Moreover, our findings about the spatial and temporal regulation of $p66^{Shc}$ expression during zebrafish embryogenesis suggest that $p66^{Shc}$ has a role in neural development. Furthermore, we generated the first genetic model of Δ40p53 ablation in zebrafish, exploiting the CRISPR/Cas9 technology. The structure of the *p53 locus* in mouse does not allow to selectively knock-out Δ40p53 without depleting also p53 activity. Our $\Delta40p53^{-/-}$ zebrafish model shows the unique opportunity to characterize the biological functions of Δ40p53 isoform in physiological development in a context where p53 expression is maintained unaltered. Finally, we provide preliminary data showing that Δ40p53 modulates *in vivo* p53-dependent transcriptional response to stress.

Introduction

In the present thesis, we investigated the role of transcriptional response to oxidative stress (OS) in two, apparently opposite, physiological phenomena: aging and development. Several evidence shows that reactive oxygen species (ROS) take part in signalling pathways involved in the determination of aging and developmental processes. Among the various pathways, we focused on two genes participating in a recently discovered OS-induced transcriptional network that triggers cell cycle arrest *in vitro*: *p53* (with its short isoform Δ40p53) and $p66^{Shc}$. Our purpose is to dissect this pathway *in vivo*, taking advantage of two teleost fish as model organisms: *Danio rerio* and *Nothobranchius furzeri*.

1. The role of oxidative stress response in aging

Oxidative stress (OS), induced by reactive oxygen species (ROS), increases during lifespan and it is involved in the aging process. Aging is defined as the over-time increasing structural and functional decay of the organism, that leads to the increasing susceptibility to aging-related diseases (e.g. cancer, neurodegenerative diseases, metabolic syndrome), and ultimately death (López-Otín *et al*., 2013). According to the free radical theory of aging, postulated by Harman *et al*. in 1981, aging is strictly correlated to the accumulation of OS damage. ROS (e.g. hydrogen peroxide, superoxide anions and hydroxyl radicals) are physiologically produced by intracellular reactions, occurring in aerobic metabolism and involving oxygen as the final electron acceptor, although the environment is a major source of ROS. ROS generate reversible or irreversible modifications to

macromolecules, including DNA adducts, lipid peroxidation and protein carbonylation, impairing their functions. Defence mechanisms such as the activation of antioxidant enzymes (e.g. superoxide dismutase, catalase and glutathione peroxidase) have been positively selected to control intracellular ROS levels, but when ROS-induced damages accumulate, proliferation inhibition or apoptosis are triggered to avoid propagation of damaged cells (Giorgio *et al*., 2007). Nowadays, it is widely recognized that the permanent withdrawal of cells from cell cycle, known as cell senescence, is a biomarker of aging, since senescent cells accumulate in the aging organism. Tumour suppressor genes, such as *p53,* prevent uncontrolled proliferation and ultimately cancer, at the cost of impairing tissue replenishment and depleting the stem cell compartment (Campisi, 2005). Increased apoptosis is another hallmark of aging. It has been shown that ROS, produced within the mitochondrion, induce damage to mitochondrial DNA (mtDNA) that leads to mitochondrial dysfunction, thus increasing ROS production and susceptibility to permeability transition pore (PTP) and apoptosis (Pollack and Leeuwenburgh, 2001). Several tissues exhibit cell loss during aging: skeletal muscle undergoes atrophy, called sarcopenia, that has been correlated with apoptosis, while other organs as heart and kidney show an increase of apoptotic markers (e.g. cytocrome c and activated caspase-3) during aging (Tower, 2015).

According to these observations, it has been demonstrated that reduced OS is associated with delayed onset of aging phenotypes and lifespan prolongation in different long-lived mice models: (i) transgenic mice overexpressing mitochondrial catalase show reduced level of hydrogen peroxide and median and maximum lifespan extension (Shriner *et al.,*

2005); (ii) mice deficient for one allele of the Insuline Growth Factor 1-Receptor (IGF1-R) show both prolonged lifespan and resistance to OS (Holzenberger *et al*., 2003); (iii) the Ames dwarf mouse, that lacks growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin, lives ~40-60% longer and shows resistance to OS (Choski *et al*., 2011); (iv) mice overexpressing Klotho, a hormone that inhibits IGF1 signalling, are long-living and show increased expression of manganese superoxide dismutase (Yamamoto *et al*., 2005), (v) $p66^{Shc-/-}$ mice (Migliaccio *et al*., 1999) and mice overexpressing a truncated isoform of p53, named p44, (Maier *et al*., 2004) show modulation of lifespan associated with peculiar OS response. These two models will be discussed later.

The existence of several genetic models of aging demonstrates that aging might be determined by genes and that it cannot be merely considered the result of the progressive accumulation of environmental insults. Nevertheless, it has never been found a specific set of genes responsible for triggering organismal aging. This phenomenon is evidently associated to a complex interaction of genes that have been positively selected for their fundamental or positive role in the reproductive phase of life. According to the "antagonistic pleiotropy theory", which is still object of debate, aging can be considered as the result of the collateral and detrimental effect of genes selected for their positive role in promoting health during development and youth (Kirkwood and Austad, 2000).

2. The role of oxidative stress response in development

Development can be considered as the opposite process of aging. Nevertheless, it has been demonstrated that genetic pathways involved in

development are also implicated in aging (Somel *et al*., 2010). In particular, it has been shown that ROS and OS response are important for normal development in mammals through cellular signalling and control of cell fate. A "free radical theory of development" (Allen and Balin, 1989) has been postulated some years later than the more famous Harman's "free radical theory of aging". This theory proposes that ROS function as "morphogens". It is based on the observation that cells and tissues at various differentiation stages exhibit changes in intracellular ROS production and antioxidant defences, such as glutathione (GSH). This is produced at high level by poorly differentiated cells, favouring the maintenance of a reducing intracellular environment (Takahashi and Zeydel, 1982). More recently, it has been shown that intracellular ROS balance alters the functions of histone modifying enzyme during development, thus providing a mechanism through which ROS can drive gene expression and cell fate (Hitchler and Domann, 2007). Moreover, ROS contribute to trigger cellular differentiation after the initial high-rate proliferation driven by the reducing environment of the early post-implantation period in the mammalian embryo. Indeed, the balance of oxidative and reductive forces orchestrates signalling pathways in mammals (Dennery, 2007). *In vitro*, ROS are able to drive the cell fate of several cancerous and embryonic cell lines into various terminally differentiated cell types, from blood cells, to neurons and cardiomyocytes, by acting as signalling molecules and modulating the activity of several transcription factors. In contrast with these observations, it has been also proposed that ROS, produced at high levels by actively proliferating cells, are necessary to maintain uncontrolled proliferation of cancerous cells (Sauer *et al*., 2001). Finally, data produced by Dr. Migliaccio

at the European Institute of Oncology show that the aging protein $p66^{Shc}$, that has been shown to produce hydrogen peroxide and will be discussed in detail, is involved in the maintenance of the stem cell compartment in the mammary gland: mice lacking $p66^{Shc}$ show an increased capacity in self-renewal of proliferating mammary stem cells during aging, suggesting that $p66^{Shc}$ might be involved in asymmetrical cell division (Montariello *et al*., manuscript in preparation). Even though the molecular mechanism underlying the increased self-renewal capacity of the aging mammary gland in $p66^{Shc-/-}$ mice has to be elucidated, it is tempting to hypothesize that ROS produced by $p66^{Shc}$ participate in cell fate decision. This hypothesis can also link the role of $p66^{Shc}$ in aging with its role in development, explaining why a gene that shows detrimental functions in adult life has been maintained throughout evolution.

If the physiological role of ROS as signalling molecules in development and their function in controlling the switching from proliferation to differentiation is still to be investigated in depth, it is instead clear that abnormal exposure to ROS is associated with a large variety of birth defects. ROS are powerful teratogens: the teratogenic effect of substances such as ethanol (Peng *et al*., 2005) and thalidomide (Parman *et al*., 1999) resides in their ability to produce free radicals. In this context, it is clear the importance of genes involved in OS response during development: as an example, it has been demonstrated that the teratogenic effects of ROS are increased in a $p53^{-/-}$ genetic context (Wells *et al*., 2005). Recent data show that also zebrafish is responsive to OS during development. Moreover, the transcriptional response induced by OS is substantially conserved between fish and mammals (Hahn *et al.*, 2014).

Although the role of ROS in development has not been extensively studied, a growing bulk of evidence shows that the same aging-associated genes, involved in ROS response, can play a role also in development. We propose here the combined study of two well-known aging associated genes - *p53*, in particular its short isoform Δ40p53, and *p66^{Shc}* - in development and aging. We also propose the use of two animal models that, according to their characteristics, represent the best model for research on development or aging, respectively: *Danio rerio*, also known as zebrafish, and the short-living vertebrate *Nothobranchius furzeri*. In the following paragraphs, it will be discussed the state of the art on our chosen genes and models.

3. Oxidative stress-related genes in development and aging: the case of p53 and p66^{Shc}

3.1 p66^{Shc}

p66^{Shc} is the longest isoform encoded by the *shc1 locus*, whose discover dates back to 1992. The *shc1* gene was initially characterized as a novel proto-oncogene, encoding for two isoforms, namely p52^{Shc} and p46^{Shc}, involved in Ras signalling. These two isoforms, translated from the same mRNA through the usage of alternative ATGs, are phosphorylated by active tyrosine kinase receptors. Upon phosphorylation, p52^{Shc} and p46^{Shc} function as adaptor proteins, recruiting to the plasma membrane the GRB2-SOS complex, which mediates the activation of the Ras small GTPase, thus initiating the MAPK (Mitogen-Activated Protein Kinase) cascade (Pelicci *et al*., 1992).

The third isoform, p66^{Shc}, is translated from a different mRNA, expressed by its own promoter. p66^{Shc} contains the entire p52^{Shc}/p46^{Shc} sequence plus an additional N-terminal region (Ventura *et al*., 2002). Although p66^{Shc} is phosphorylated by active tyrosine kinase receptors, as the shorter isoforms encoded by *shc1*, it is not involved in Ras signalling. Therefore, p66^{Shc}, contrary to p52^{Shc}/p46^{Shc} does not contribute to stimulate proliferation (Migliaccio *et al*., 1997).

p66^{Shc} shares with p52^{Shc}/p46^{Shc}, from N-terminal to C-terminal, the PTB (Phospho-Tyrosine Binding) domain, the CH1 (Collagen Homologous 1) domain and the SH2 (Src Homologous) domain. Its unique amino-terminal domain, named CH2 (Collagen Homologous 2), is crucial to differentiate the role of p66^{Shc} from the shorter isoforms. In cultured mouse embryonic fibroblasts (MEFs), p66^{Shc} is rapidly phosphorylated on a serine residue present in its unique CH2 domain, upon treatment with hydrogen peroxide or UV, leading to its activation (Migliaccio *et al.*, 1999; Figure 1).

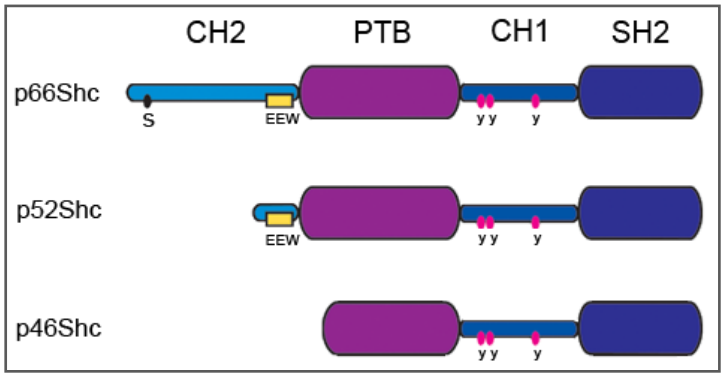


Figure 1. Modular domain organisation of Shc isoforms. p66^{Shc}, p52^{Shc} and p46^{Shc} share, from C-terminal to N-terminal, SH2, CH1 and PTB domains. p66^{Shc} shows a unique N-terminal domain, CH2, where the serine residue phosphorylated upon OS is located. The EEW is the amino-acidic core of the Cytochrome c binding domain, (CB), present in p66^{Shc} and p52^{Shc}, but absent in p46^{Shc} *(Atlas of Genetics and Cytogenetics in Oncology and Haematology)*.

At basal conditions, $p66^{Shc}$ is located within the intermembrane space of the mitochondrion, where it is bound to the TIM/TOM importing complex and to chaperonine Hsp70. Cellular stresses stimulate the release of $p66^{Shc}$ from this complex and promote its binding to the Cytochrome c (Cyt c). The Cyt c Binding Domain (CBD; Figure 1) has been mapped between the CH2 and the PTB domain. Bound to reduced Cyt c, $p66^{Shc}$ subtracs electrons from ETC to generate hydrogen peroxide from molecular oxygen. This event induces the opening of the Permeability Transition Pore (PTP) that triggers mitochondrion swelling and the release of Cyt c from the outer membrane, leading to the execution of apoptosis. The crucial role of $p66^{Shc}$ in the mitochondrial apoptosis pathway has been demonstrated both *in vitro*, where $p66^{Shc}$ oxidizes Cyt c, triggering mitochondrial swelling, and *in vivo*, taking advantage of the $p66^{Shc-/-}$ mouse model (Giorgio *et al*., 2005; Figure 2).

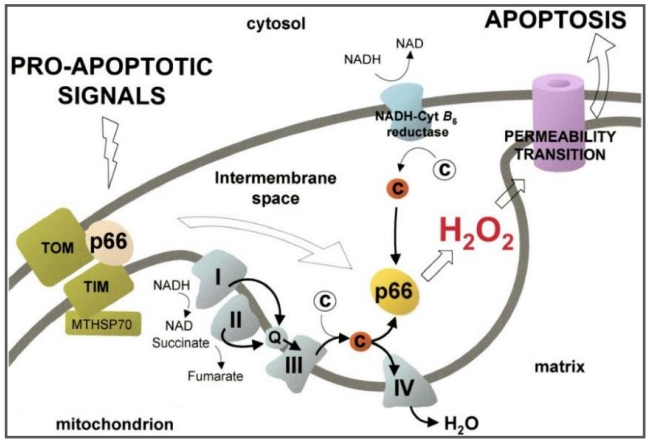


Figure 2. Model of $p66^{Shc}$ redox activity during mitochondrial apoptosis. Pro-apoptotic signals induce release of $p66^{Shc}$ from a putative inhibitory complex. Active $p66^{Shc}$ oxidises reduced Cyt c (red) and catalyses the reduction of O_2 to H_2O_2. PTP opening by H_2O_2 leads to mitochondrial swelling and apoptosis (Giorgio *et al*., 2005).

p66^{Shc} in aging

The p66$^{Shc-/-}$ mouse model, generated in 1999, is a genetic model of healthy aging. The deletion of p66^{Shc} improves general health in the aging mouse and it is associated with an elongation in lifespan, although this datum is still controversial (Migliaccio *et al*., 1999; Ramsey *et al*., 2014). *In vivo*, the deletion of p66^{Shc} is associated with: (i) increased resistance to OS-induced apoptosis upon treatment with paraquat, a potent pro-oxidant (Migliaccio *et al*., 1999); (ii) reduced apoptosis and tissue damage induced by acute ischemia (Zaccagnini *et al*., 2004); (iii) reduced nephropathies in a mouse model of diabetes (Menini *et al*., 2006); (iv) amelioration of the age-dependent decrease of endothelial functions (Francia *et al*., 2004); (v) amelioration of liver conditions in a mouse model of ethanol-induced hepatic steatosis (Koch *et al*., 2008); (vi) amelioration of cognitive functions in a mouse model of Alzheimer disease (Derungs *et al*., 2016); (vii) partial recovery of fertility, internal organ size and body weight in the progeric mouse model deficient for the Telomerase RNA component (TERC; Giorgio *et al*., 2016).

Although the molecular mechanisms underlying all the beneficial effects of p66^{Shc} deletion in mice are still to be elucidated, it has been demonstrated that p66^{Shc}, by increasing intracellular ROS levels, participates in various pathway known for being involved in aging. In particular, it has been shown that p66^{Shc} has a key role in the insulin pathway: insulin stimulation induces phosphorylation of p66^{Shc} and production of hydrogen peroxide. H_2O_2 in turn regulates the insulin signalling: in adipocytes derived from p66$^{Shc-/-}$ mice, insulin-dependent Protein Kinase B/AKT activation results attenuated and also FOXO

(Forkhead Box O) activity is impaired. These findings explain the reason why $p66^{Shc-/-}$ mice exhibit reduced fat accumulation and hepatic steatosis (Berniakovich *et al*., 2008).

A consistent body of evidence demonstrates also that $p66^{Shc}$ is regulated at epigenetic level: for example, the decreased epithelial dysfunctions associated with $p66^{Shc}$ deletion are mediated by (i) low-density lipoprotein cholesterol, that stimulates human $p66^{Shc}$ expression (Kim *et al*., 2012); (ii) SIRT1, that represses $p66^{Shc}$ in a model of hyperglycaemia-induced endothelial dysfunction, conferring an amelioration of the phenotype (Zhou *et al*., 2011), and (iii) homocysteine, which promotes $p66^{Shc}$ expression (Kim *et al*., 2011).

$p66^{Shc}$ in development

The fact that the ablation of $p66^{Shc}$ is associated with an amelioration of the aging phenotype in laboratory mice and in mouse models of human aging-associated diseases suggests that $p66^{Shc}$ has been evolutionary maintained for its positive functions exerted in development and/or reproductive phase of life. What these functions are is still unclear, even if it is possible to speculate that they could be related to $p66^{Shc}$ redox activity. Indeed, it is known that ROS play an important role in developmental processes such as cell differentiation (Dennery *et al*., 2007). On the other hand, it is evident as well that $p66^{Shc}$ is not necessary in embryogenesis, because $p66^{Shc-/-}$ mice are viable, $p66^{Shc-/-}$ embryos are not counterselected during gestation and no significant incidence of developmental abnormalities has been recorded (personal communication by Dr Giorgio).

For all these reasons, few efforts have been focused on understanding the role of $p66^{Shc}$ in development.

Nevertheless, there are evidence suggesting a function for $p66^{Shc}$ in neurogenesis. The expression regulation of the three isoforms of the *shc1* gene was investigated in 1997 through western blot and *in situ* hybridization in the brain of the developing mouse: all the three isoforms are exclusively expressed within the ventricular zone, the area were immature neuroblasts proliferate, being $p66^{Shc}$ the less represented isoform. On the contrary, in the post-natal adult brain the expression of Shc isoforms was detected only in the olfactory epithelium, where neuronal self-renewal occurs also in adulthood. Considering that $p66^{Shc}$, contrary to the other isoforms, is not involved in proliferation (Migliaccio *et al.*, 1999), these findings lead to the intriguing hypothesis that $p66^{Shc}$, in concert with $p52^{Shc}$/$p46^{Shc}$, has the role of orchestrating cell fate in neurogenesis (Conti *et al*., 1997). According with these observations, it has been found that in human and murine embryonic stem cells the overexpression of $p66^{Shc}$ anticipates the loss of pluripotency and induces the acquisition of a neuronal fate, through the regulation of the Wnt/β-catenin pathway (Papadimou *et al*., 2009). These data suggest that $p66^{Shc}$ might have a role in development and in particular in neuronal differentiation. Finally, it has been demonstrated that $p66^{Shc}$ has a role in metabolic adaptation: $p66^{Shc-/-}$ mice have been studied in a natural environment, subjected to food competition and low temperatures. In these conditions, $p66^{Shc}$ deletion resulted deleterious: as previously mentioned, $p66^{Shc}$ is involved in fat accumulation and energy metabolism; $p66^{Shc-/-}$ mice exposed to low temperatures resulted counterselected due to their inability to accumulate

fat, when food is not constantly available as it is in captivity. Moreover, it has been found a reduction in fertility under stressed conditions and in maternal cares (Giorgio *et al*., 2012). Taken together, these findings might explain the fact that $p66^{Shc}$ plays a positive role in adaptation, that becomes deleterious when mice are kept in controlled conditions such as absence of competition, constant warm temperature and food availability.

3.2 p53

Known for being the "guardian of the genome", p53 is the most frequently mutated protein in human cancer and one of the most intensively studied gene (Vogelstein *et al*., 2010). Nevertheless, the complete understanding of its numerous functions is still far from being achieved.

p53 is a transcription factor that acts as a tetramer in response to different stresses and it is a key regulator of DNA and protein damage response. In adult tissues, normally, p53 levels are very low: stress signals induce post-transcriptional modifications, such as acetylation, phosphorylation and sumoylation to specific amino-acidic residues, that stabilize p53 protein, avoiding its degradation and promoting its tetramerization (Bode and Dong, 2004).

p53 is active mainly in cell cycle control and different levels of stress and damage to macromolecules can trigger different p53-dependent transcriptional responses. They can lead to: (i) transient cell cycle arrest, through the activation of cyclin inhibitors such as *p21*, when damages are successfully repaired and the cell can recover its functions; (ii) apoptosis, through the activation of pro-apoptotic genes such as *bax* and *puma* and

negative regulation of anti-apoptotic genes such as *bcl-2*; (iii) permanent cell cycle arrest, known as cell senescence. Apoptosis and cell senescence are mainly chosen when damages to macromolecules cannot be repaired, to avoid propagation of damaged, potentially cancerous cells (Bargonetti and Manfredi, 2002).

However, it is still unclear how a single gene can orchestrate such a wide range of different pathways in response to a plethora of signals. This complexity can be explained by the finding that the *p53* gene encodes several p53 isoforms through the use of internal promoters, internal ribosomal entry site (IRES) and alternative splicing. These mechanisms are responsible for the production of the 12 p53 isoforms already identified in humans, that are distinguishable for the presence of different domains and specifically regulated. p53 isoforms are the result of the combinations among different N-terminal regions, that lack the entire Transcription Activation Domain (TAD) or even part of the DNA Binding Domain (DBD), and different C-terminal domains, where the Oligomerization Domain (OD) can be replaced by two different amino-acidic sequences. This surprising variability in p53 isoforms, that can or cannot associate each other or with canonical p53, that are specifically regulated among tissues and produced in response to different stimuli, explain why a single gene can be responsible for such a variety of functions (Figure 3; Khoury and Bourdon, 2011).

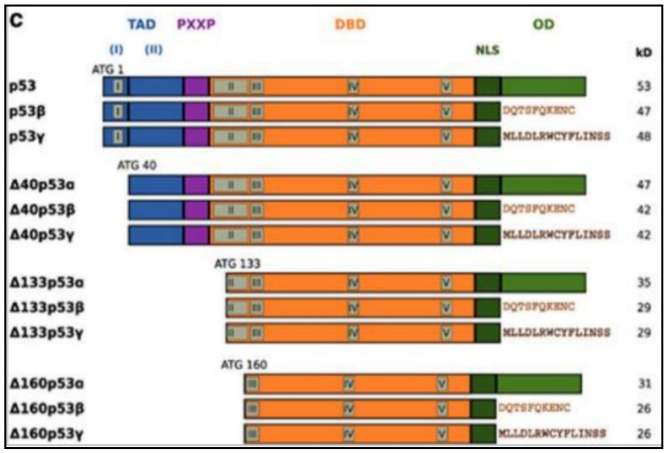


Figure 3. Human p53 protein isoforms. The canonical p53 protein contains a TAD (blue), a proline-rich domain (PXXP, purple), a DNA-binding domain (DBD, orange) and an OD (green) that encompasses a nuclear localization domain (NLS, green) and five regions conserved through evolution (I–V in grey boxes). Compared with p53, the Δ40p53 isoforms lack the first TAD, whereas the Δ133p53 and Δ160p53 isoforms lack the entire TAD and parts of the DBD. At the C-terminal, the *α*-peptide corresponds to the OD that can be replaced by new residues, the *β*- and *γ*-peptides (brown; modified from Khoury and Bourdon, 2011).

p53 in aging

Considering that apoptosis and cell senescence are known to be involved in aging, it is clear that p53 is active also in the regulation of aging processes: this makes p53 both the major defence against cancer and the road that leads to aging. The role of p53 in aging is demonstrated by several studies in mouse models of longevity. The first evidence that links genetically p53 and aging was obtained in a mouse model that expresses a truncated p53 protein, named $p53^{m}$. In presence of the wild-type allele, $p53^{m}$ confers an increase in the tumour suppressor activity exerted by p53, at the cost of a reduction in lifespan associated with early onset of aging phenotypes, such as general loss of body mass, atrophy of internal organs,

osteoporosis and kyphosis. The $p53^m$ allele has a deletion of the first six exons of the *p53* gene that leads to the loss of the two trans-activation domains. This determines the inability of $p53^m$ to exert a role of transcription factor *per se*, but also it leads to p53 stabilization due to the loss of the Mouse Double Minute 2 (MDM2) binding domain. MDM2 is the ubiquitin ligase that triggers p53 degradation. Moreover, $p53^m$ retains the ability to form tetramers with p53 due to the presence of an unaltered C-terminal oligomerization domain. As a consequence, the role of $p53^m$ in conferring a premature aging phenotype is determined by the presence of a wild-type p53 and it is the result of an increase in p53 activity and modulation of its transcriptional response. This demonstrates that p53 stabilization leads to cancer resistance at the cost of premature aging and reduced lifespan, probably due to the depletion of stem cell compartment and compromised tissue homeostasis (Tyner *et al*., 2002).

In contrast to this, another study in a different genetic model that harbours an extra copy of the entire *p53* gene, and named "super-p53", shows a completely different scenario. These mice are characterized by an increased resistance to cancer but are not subjected to premature aging or reduced lifespan. In this case, p53 is not stabilized and its activity is maintained at a basal level, but it results increased when p53 stabilization is stimulated by damage to DNA. Therefore, although the p53 pro-apoptotic response is enhanced upon stress exposure, the "super-p53" mice have a normal lifespan and do not show signs of premature aging due to premature loss of tissue homeostasis (Garcia-Cao *et al*., 2002).

In conclusion, p53 plays a complex role in lifespan and aging modulation: when p53 activity in enhanced, but the mechanisms ensuring a normal regulation of its turn-over are maintained, p53 promotes longevity. On the contrary, when it is constitutively active, p53 promotes aging without an increase in cancer susceptibility. Therefore, the p53-dependent transcriptional response is fine-tuned to avoid proliferation of damaged, potentially cancerous, cells but also to avoid the loss of stem cell compartments and preserve tissue homeostasis (Figure 4; Feng *et al*., 2011; Reinhardt and Schumacher, 2012).

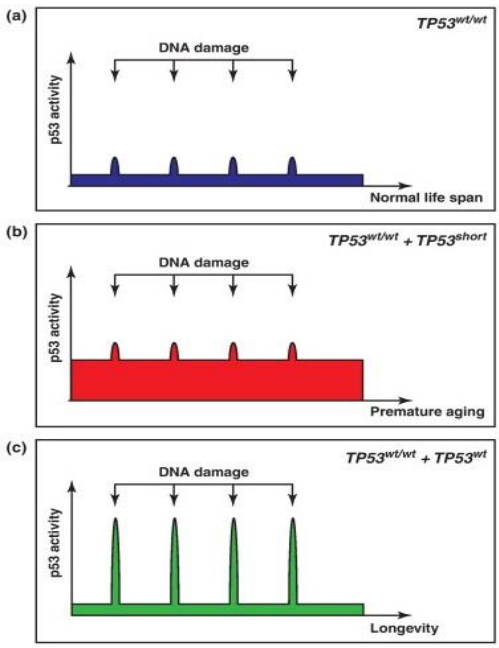


Figure 4. Activation modes of p53 determine the physiological outcome. (a) In wild-type cells, p53 exhibits low basal activity. Only upon stress signals, p53 is activated to prevent oncogenic transformation of cells. **(b)** Increased p53 activity, induced by the expression of short p53 isoforms, promotes cancer resistance, at the cost of impaired tissue regeneration and accelerated aging. **(c)** Low basal levels of p53, together with strong p53 activation only in response to stress, lead to cancer protection and normal lifespan (Reinhardt and Schumacher, 2012).

p53 in development

In 1992, it was generated a genetic model of p53 ablation: $p53^{-/-}$ mice develop normally, such as $p66^{Shc-/-}$ mice, suggesting that p53 has not a fundamental role in development (Donehower *et al*., 1992). Nevertheless, a

report of some years after demonstrated that a significant proportion of female $p53^{-/-}$ mice die during embryogenesis or in the period between birth and weaning. $p53^{-/-}$ female embryos fail to complete the normal development of neural tube and result affected of exencephaly, suggesting that p53 may play a role in neural development (Armstrong *et al*., 1995). Moreover, it has been shown that (i) constitutively active p53 is associated with embryonic lethality; (ii) a decrease in p53 expression levels leads to increased foetal lethality in embryos treated with teratogens (Choi and Donehower, 1999); (iii) p53 induces differentiation of mouse Embryonic Stem Cells (mESC) by suppressing *nanog* expression (Lin *et al*., 2005); (iv) p53 plays a regulatory role in directing primary neurons, oligodendrocytes, and PC12 cells toward either differentiation or apoptosis *in vitro* (Eizenberg *et al*., 1996). Altogether, these findings demonstrate that p53 could be involved in different developmental processes, even though p53 is not strictly necessary to complete development in mammals and, in absence of environmental stresses, other genes can compensate the lack of p53.

3.3 Δ40p53, or p44, a short isoform of p53

In our research, we focus our attention on a short isoform of p53, named Δ40p53, encoded by the *p53* gene. In mammals, the canonical p53 transcript encodes Δ40p53 thanks to an IRES located in exon 4 (Courtois *et al*., 2002), even if it has been reported an alternative splicing mechanism for Δ40p53 production in human (Ghosh *et al*., 2004). This isoform has a mass of about 44KDa in the mouse and 47KDa in human, and thus it will be referred to as “p44” hereafter. p44 is a N-terminally truncated isoform that lacks the first TAD, retaining the second TAD and all the p53 functional

domains and maintaining the ability to form oligomers with the canonical p53 protein. Together with the first TAD, p44 lacks the MDM2 binding domain: it has been demonstrated that p44 is unable to complex with MDM2 avoiding ubiquitination and degradation (Yin *et al*., 2002).

p44 expression depends on p53 post-transcriptional regulation: the canonical p53 and its short isoform share the same mRNA. Mouse embryonic fibroblast infected with p53 cDNA express both full-length p53 and its short isoform, while the same cDNA mutated at codon 40 is unable to produce the p44 protein. More in detail, the choice of the initiation site of translation depends on the interaction between p53 and its mRNA: the N-terminal domain of p53 binds a stem-loop structure located in the 5'UTR of its own mRNA. This interaction blocks p53 translation and causes the ribosome to choose the second IRES located in exon 4, leading to p44 translation. The production of p44 is regulated also by MDM2 that interacts with the N-terminal domain of p53, avoiding the binding of p53 protein with its own mRNA and leading to p53 translation (Yin *et al*., 2002; Ungewitter and Scrable, 2010).

The p44 isoform determines p53 functions: due to the lack of the first TAD, it is widely accepted that p44 is not able to drive by itself the transcription of p53 target genes (Courtois *et al*., 2002). Nevertheless, there is also evidence that p44 can be considered an autonomous transcription factor (Phang *et al*., 2015). However, it is known that p44, thanks to its intact oligomerization domain, has the ability to form oligomers with p53, determining its localization and fine-tuning its transcriptional activity: (i) the human p44 ortholog localizes into the cytoplasm and its overexpression drives p53 localization from the nucleus to the cytoplasm (Ghosh *et al*.,

2004); (ii) endoplasmic reticulum (ER) stress promotes the formation of p44/p53 oligomers leading to G2 arrest of the cell cycle (Bourougaa *et al*., 2010); (iii) the human p44 ortholog accumulates in response to genotoxic stress, modulating p53 post translational modification pattern and enhancing *p21* expression (Powell *et al*., 2008). The formation of oligomers between p44 and p53 depends on the delicate equilibrium between these two isoforms: high levels of p44 induce the formation of p44 homotetramers, compromising p53 functions. When p44 and p53 form heterotetramers (where the p53:p44 ratio is variable), this configuration is more stable than p53 homotetramers, because p44 lacks the MDM2 binding domain: this results in the modulation of p53 transcriptional activity (Ungewitter and Scrable, 2010).

p44 in aging

The role of p44 in aging has been demonstrated with the production of a mouse genetic model that overexpresses p44, named Tgp44: in this model, full-length p53 expression is maintained. The Tgp44 mice show premature signs of aging: as early as 4-month old, these mice show osteoporosis, kyphosis, infertility and reduced body size, and they die within the first year of age, without developing cancer. It has been demonstrated that the reduced susceptibility to cancer observed in these mice depends on the presence of p53, given the fact that $p53^{-/-}$ mice overexpressing p44 are susceptible to cancer as $p53^{-/-}$ mice. Cultured cells derived from adult Tgp44 mice exhibit enhanced p21 expression and increased levels of IFG-1 hormone and its receptor IGF-1R in mitotically active tissues. This finding indirectly demonstrates the fact that an altered p53:p44 ratio leads to a modification of the normal p53-dependent activity. This shows that, as in

invertebrate models like *Caenorhabditis elegans* and *Drosophila melanogaster*, the aging process is regulated by the insulin/IGF-1 signalling pathway also in mammals, where it is also regulated by p53 and particularly by its short isoform p44 (Maier *et al*., 2004).

p44 in development

Strikingly, it has been observed that p44 plays also an important role in development: in mESCs derived from wild-type mice, p53 and p44 levels are high and decrease with the loss of pluripotency. High levels of p44 are observed in embryos immediately after implantation, while full-length p53 is not expressed yet: therefore, p44 has been considered an embryonic isoform of p53, whose expression is high in pluripotent stem cells, decreasing as differentiation occurs. In mESCs, the downregulation of p44 expression leads to growth arrest because stem cells enter the G0/G1 phase, as it happens in terminally differentiated cells. This phenomenon is accompanied by a drastic decrease in the expression of two stem cell markers, *nanog* and *oct4*. On the contrary, mESCs derived from Tgp44 mice show a higher rate of proliferation than wild-type ones. These findings suggest that the dosage of p44 plays a crucial role in early mammalian development, in particular for the switch from pluripotency to differentiation. Also in development, p44 functions in concert with p53: these two proteins form heterodimers not only in somatic cells, but also in mESCs, where p44 evidently modulates p53 stability and transcriptional activity. More in detail, by the comparison of expression levels of several p53 targets in WT-mESCs and Tgp44-mESCs, it has been revealed that p44 interferes with the capacity of p53 to bind specific promoters, as those of *nanog* and *ifg-1R*. This leads to the overexpression of these genes. On these

bases, it is evident that the main function of p44 in the early stages of embryogenesis is to suppress p53 activity by preventing the trans-suppression of factors such as Nanog and IGF-1R, to preserve the pluripotency. When levels of p44 decrease, the negative regulation of p53 decreases as well, and ESCs begin to differentiate (Ungewitter and Scrable, 2010).

3.4 A link between p53/p44, p66^{Shc} and oxidative stress

A recent work demonstrated that the p44 isoform takes part in a pathway that involves also the p66^{Shc} redox protein. In response to OS, in mouse embryonic fibroblasts (MEFs) it is activated a p53/p44-p66^{Shc} dependent transcriptional program that is abrogated in p66$^{Shc-/-}$ mice. This transcriptional response leads to the repression of ~200 genes responsible for cell cycle progression between G2 and M phases and mitotic spindle checkpoint. These genes are normally downregulated during physiological aging in various tissues: their downregulation is responsible for the accumulation of senescent cells, a well-known hallmark of aging. These genes are downregulated also upon genotoxic stress (GS), but independently from p66^{Shc}: indeed, in MEFs derived from p66$^{Shc-/-}$ mice treated with a potent mutagen, doxorubicin, the GS-dependent transcriptional response is conserved. On the contrary, when the same cells are treated with hydrogen peroxide, this transcriptional response is lost. Importantly, OS is not only cause of DNA damage but causes damage to all macromolecules. This datum demonstrates that the transcriptional response to OS is p66^{Shc}-dependent.

Moreover, in MEFs derived from $p53^{-/-}$ mice, the transcriptional response is totally abrogated upon both OS and GS. In summary, the downregulation of the 200 genes involved in cell cycle progression is (i) p53-dependent; (ii) $p66^{Shc}$-independent in case of GS; (iii) $p66^{Shc}$-dependent in case of OS and protein/lipid damage. Notably, in $p66^{Shc-/-}$ mice, part of these genes is less downregulated in aging than in wild-type mice. $p66^{Shc-/-}$ mice are a model of healthy aging also because they accumulate less senescent cells, thanks to the abrogation of the $p66^{Shc}$-dependent transcriptional response to OS that leads to cell cycle arrest.

Furthermore, it is evident that p53 acts in response to stress in cooperation with different partners: not only $p66^{Shc}$ orchestrates p53-dependent transcriptional response to OS, but also p44. Indeed, in MEFs derived from Tgp44 mice, the p53/$p66^{Shc}$-dependent downregulation of G2/M transition genes is higher than in WT-MEFs. Additionally, in MEFs derived from Tgp44/$p66^{Shc-/-}$ mice, the transcriptional response to OS is abrogated, while it is maintained in case of GS. Therefore, the effect of $p66^{Shc}$ is epistatic to p44.

Finally, it has been observed that the deletion of $p66^{Shc}$ partially recovers the progeroid phenotype of Tgp44 mice. In conclusion, this work proposes that (i) p53 acts through different partners in response to different stress agents; (ii) the tumour suppression functions of p53 are exerted independently from $p66^{Shc}$ and p44; (iii) the role of p53 in aging is linked to the aging proteins p44 and $p66^{Shc}$ (Figure 5; Gambino *et al*., 2013).

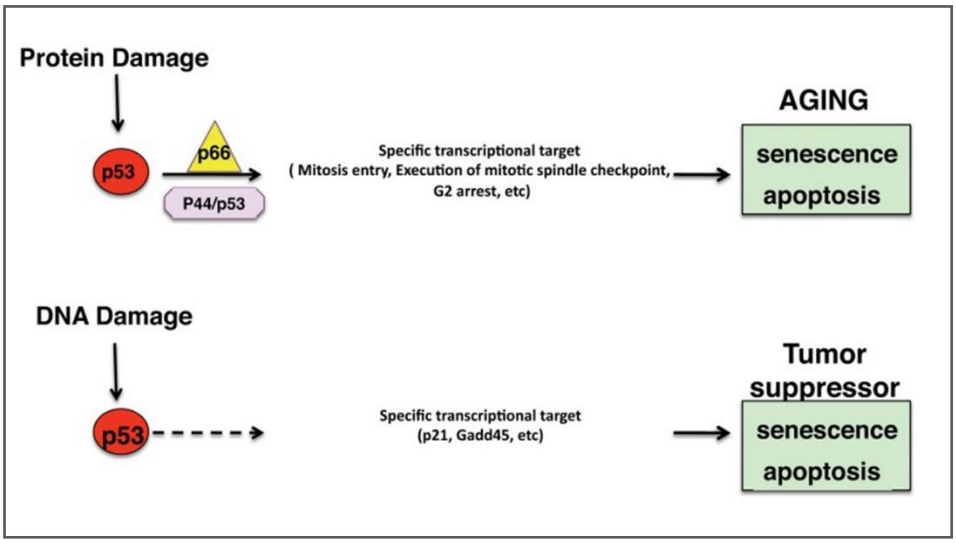


Figure 5. p53 exerts its double role in tumour suppression and aging through distinct pathways. Oxidative stress causes protein damage and activates a specific p53-dependent transcriptional response in concert with its short isoform p44 and $p66^{Shc}$. The downregulation of G2/M transition genes leads to cell senescence, apoptosis and ultimately aging. On the other hand, DNA damage stimulates p53 activity independently from $p66^{Shc}$ and p44. Different genes are involved in DNA damage response and they are responsible for tumour suppression (Migliaccio *et al.,* 2013).

4. The models: *Danio rerio* (zebrafish) and the short-living killifish *Nothobranchius furzeri*

As the final goal of this study is to investigate the role of the $p53/p66^{Shc}$ pathway in aging and development, we carefully chose the most suitable models for our research.

Invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* have a single homolog of the *p53-family* genes. *p53-family* is composed by *p53* and its isoforms, *p63* and *p73* with their

isoforms. In worms and insects, the single *p53-family* homolog is more closely related to *p63/p73* than *p53* and it can be considered an ancestor of the entire *p53-family*. It is with cartilaginous fish that can be found two *p53* paralogs: a *p53* gene and a hybrid *p63/p73* gene. Teleost fish, such as *Danio rerio* and *Nothobranchius furzeri*, have clear orthologs of *p53*, *p63* and *p73* genes (Belyi *et al*. 2010). Thus, considering also that p53 key amino-acidic residues are substantially conserved between fish and mammals, fish represent a good compromise among vertebrate and invertebrate models to study *in vivo* p53 functions.

$p66^{Shc}$ is instead a vertebrate protein. In *D. melanogaster* and *C. elegans*, two different *Shc-like* coding sequences can be found: they show a modular organization in which the PTB domain is followed by SH2 domain, but the CH1 and CH2 domains are not present (Luzi *et al*., 2000). It is only with teleost fish that the $p66^{Shc}$ gene is clearly recognizable. In fish, the modular organization of $p66^{Shc}$ is conserved: in particular, the key amino-acidic residues and the Cytochrome c binding domain, fundamental for $p66^{Shc}$ to exert its function as a redox protein, are present. Thus, fish represent the lowest model in evolution to study *in vivo* $p66^{Shc}$ functions.

Since our purpose is to investigate the role of these genes in development and aging, mice do not represent the best model, even if they are closer to humans: (i) mouse embryos develop *in utero*, thus whenever it is necessary to analyse them, the mother has to be sacrificed; (ii) embryo development lasts 3 weeks; (iii) a female mouse gives birth to only 6-12 pups per month; (iv) mice can live more than 2 years in captivity. Finally, it is important also to point out that both aging and development are systemic processes that involve the whole organism and cannot be properly

surrogated by *in vitro* culture. For all these reasons, we chose teleost fish as a model for this study: in the following paragraphs, it will be discussed the choice of two different species (*Danio rerio* and *Nothobranchius furzeri*) and the state of the art about p53/p44 and $p66^{Shc}$ in our models.

4.1 *Danio rerio* (zebrafish)

An overview on the available technologies

Danio rerio, hereafter referred to as "zebrafish", is nowadays widely considered the best model for the developmental biology and genetic studies. The zebrafish is a tropical fish native to South-East Asia; since 1960s, it has become increasingly important in scientific research because of its attractive features: (i) embryo development is extremely conserved in vertebrates and most of the genetic programmes involved are conserved among species; (ii) zebrafish females can lie a substantial number of eggs (200-300) every week; (iii) zebrafish embryos are completely transparent and develop externally – they are maintained in controlled conditions and can be checked whenever needed; (iv) embryos complete organogenesis within the third day post fertilization and begin to feed autonomously within the sixth day – this makes zebrafish one of the most rapidly developing vertebrates (Kimmel *et al*., 1995). Moreover, the zebrafish genome, which consists of 1.5 billion base-pairs (a half than human genome) and 26 thousand protein-coding genes (more than ever previously sequenced vertebrate) has been published in 2013: it appears that 71% of human proteins have an obvious ortholog in zebrafish (Howe *et al*, 2013).

Since zebrafish has been used in scientific research for more than 50 years, a wide range of techniques and protocols have been standardized

among laboratories and universally accepted by the scientific community. Toxicological studies have been successfully carried out in zebrafish embryos, as they can be exposed and subsequently analysed for their phenotype, physiology, metabolomics, transcriptomics, etc., by simply dissolving compounds in fish water: a wide range of compounds, dilutions and combinations can be tested altogether, taking advantage of the zebrafish high fecundity; for the same reason, it is considered a valuable tool for pharmacology and drug discovery (MacRae and Peterson, 2015). Genetic engineering techniques have been successfully applied in zebrafish: transient modulation of gene expression is extremely feasible. It is possible to microinject *in vitro*-synthetized mRNA molecules ("gain of function" experiments) or antisense oligonucleotides (named Morpholinos – "loss of function" experiments) in the one-cell stage embryo, so that the chosen gene results overexpressed or downregulated in all the developing embryo for the first 3-4 days of development, until the microinjected molecules are too much diluted or degraded (Rosen *et al*., 2009). Most importantly, the production of transgenic and knock-in lines, which allows the study of gene expression patterns and aberrant expression of wild-type and mutated genes, are routinely in zebrafish: a wide range of inducible and tissue-specific systems are available. On the other hand, gene knock-out has been challenging for years, since the use of zinc-finger nucleases and TALENs (Transcription Activator-Like Effector Nucleases) is particularly expensive: the arrival of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)-Cas9 technology, which is extremely efficient and cheap, has led to revolution in the field of genetic manipulation in zebrafish. Since its first

application in 2013, hundreds of knock-out zebrafish lines have been generated (Hwang *et al*., 2013; Li *et al*., 2016).

An overview on embryonic development

Zebrafish embryonic development has been extensively investigated by Kimmel and colleagues in the early 1990s: their works are still the most exhaustive in literature; it will be referred to Kimmel *et al*., 1995 for all the information provided below.

Zebrafish development can be divided into seven periods, from fertilization of the egg to the hatching of the larva. Each period will be briefly discussed below:

1. Zygote period (0 – 45'). Zebrafish egg is 0.7mm in diameter and telolecithal: 2/3 of its volume consists of yolk, the substance that provide nutrients to the developing embryo on top of it (animal pole). Microtubule arrays extend along the animal-vegetal axis to the vegetal pole, maintaining a contact between the blastomeres and the yolk cell. The first period starts with fertilization and ends with the formation of the first cleavage furrow. The cleavage occurs only in the blastodisc, the portion of yolk-free cytoplasm at the animal cap, that becomes visible at the end of this stage (Figure 6.a).
2. Cleavage period (45' – 2h and 15'). The divisions occur at 15-minute intervals and are defined meroblastic, because the blastodisc is divided only partially and the resulting blastomeres are interconnected by cytoplasmic bridges until the 8-cell stage. In this period, six synchronic divisions occur: at the end, the embryo counts 64 cells that form the blastoderm at the animal pole (Figure 6.b-g).

3. Blastula period (2h and 15' – 5h and 15'). This period starts from the 128-cell stage to the beginning of gastrulation. The first important event occurs at cycle 10 when the mid-blastula transition begins: the zygotic genome is now actively transcribed; cell divisions becomes asynchronous and cells acquire mobility. The second important event is the formation of the yolk syncytial layer: the inner cell layer of the blastoderm fuses with the yolk and divide forming extraembryonic tissues. When the epiboly begins, the blastodisc and the yolk syncytial layer move on the surface of the yolk to envelop it. This period ends when the cells of the blastoderm have covered the 30% of the yolk-cell (Figure 6.h-p).
4. Gastrula period (5h and 15' – 10h). Gastrulation begins when epiboly has reached the 50%. At this stage, a thickening is visible throughout the margin of the blastoderm: it is called "germ ring" and consists of a superficial layer, the epiblast, and an inner layer, the hypoblast. Convergence movements leads to the formation of a localized thickening called "embryonic shield". This is the zebrafish embryo organizer because it is able, when transplanted to a host embryo, to organize a secondary axis. In this period, the three germ layers distinguish each other; at the end of the movements of convergence and extension, the presumptive map of the embryo can be drawn. When gastrulation ends, the mesoderm (hypoblast) can be distinguished in axial mesoderm (notochord) and paraxial mesoderm (eyes, jaws, gills and somites), while the dorsal epiblast forms the neural keel (Figure 6.q-u).

5. Segmentation period (10h – 24h). During the first part of this period, the tail bud becomes visible at the caudal extremity of anterior-posterior axis. The somitogenesis begins; three somites develop per hour. The neural plate becomes thicker and the neural tube forms with secondary neurulation. At the end of this period, the yolk is divided into a spherical anterior portion (yolk ball) and a cylindric posterior portion (yolk extension). At the end of somitogenesis, the embryo counts 31 somites (Figure 6.v-z).
6. Pharyngula period (24h – 48h). In this period, the pharyngeal arches primordia become visible. The bilateral symmetry of the embryo becomes evident, as the long caudal fin. The brain is divided into five lobes and the melanophores, derived from the neural crests, start to differentiate: pigmentation of the eye starts at 26h. The head-trunk angle is 90° at the beginning of this period, but later this angle increases as a consequence of straightening of the embryo. The heart beating and circulation can be clearly distinguished; at the end of this period the heart is completely formed and the primordia of all the organs are present (Figure 7.a-b).
7. Hatching period (48h – 72h). Zebrafish embryos hatch asynchronically, between 48h and 72h. The pectoral fins and the jaws become visible. The cranial cartilages form, as the branchial arches; at end of this period, the swim bladder starts to be visible. After 72h embryos are defined as larvae and can autonomously swim. After 120h, the yolk is consumed and zebrafish larvae can eat autonomously (Figure 7.b-c).

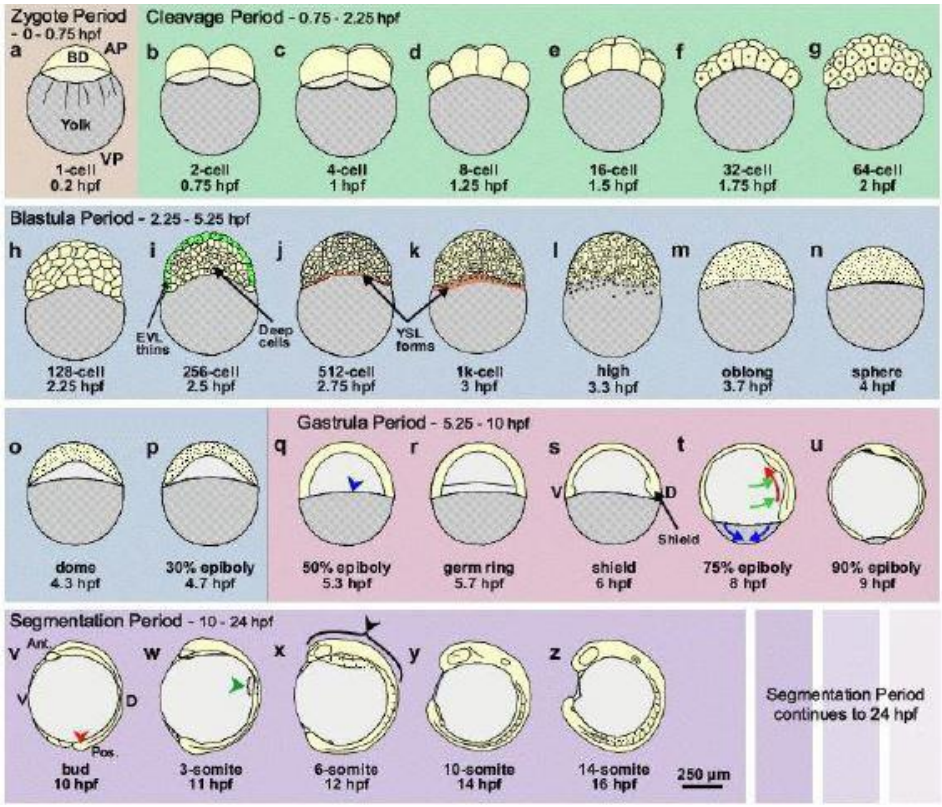


Figure 6. Stages of early embryonic development in zebrafish. Zebrafish development can be divided into seven periods. This is a schematic representation of the changes occurring throughout the first five periods, from zygote to segmentation stages (Web and Miller, 2007).

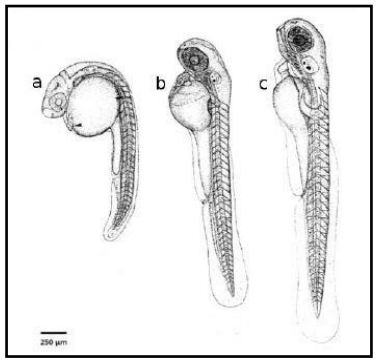


Figure 7. Stages of late embryonic development in zebrafish. This is a schematic representation of the changes occurring throughout the last three periods, from pharyngula to hatching stages (Kimmel *et al.,* 1995).

p66^{Shc} and p53/p44 in zebrafish

The only data present in literature suggest a role of Shc proteins in postnatal angiogenesis: it has been shown that specific knockdown of p52^{Shc}/p46^{Shc} in zebrafish embryos leads to defects of intersegmental vessel sprouting angiogenesis (Sweet *et al*., 2012). The aging-associated isoform of Shc, p66^{Shc}, has never been investigated in fish. Nevertheless, through bioinformatic analysis it has emerged that the *shc locus* in zebrafish shows conservation with mammals: in Ensemble genome browser is present a protein coding putative transcript for p66^{Shc} (ENSDART00000098400). Ensemble prediction was used as a reference in this work for molecular cloning of the p66^{Shc} full-length cDNA.

On the contrary, p53 has been extensively investigated in zebrafish. In this fish, as in mammals, p53 controls cell cycle in response to different stresses: (i) the zebrafish p53 protein shows 48% similarity with human p53 and, as in mammals, it is expressed during embryogenesis (Cheng *et al*., 1997); (ii) p53 knockdown does not affect embryonic development, but it impairs DNA damage response upon UV radiation (Langheinrich *et al*., 2002; (iii) p53 accumulates in zebrafish embryos upon treatments with ionizing radiations and a known p53-activating agent, the chemotherapeutic drug R-roscovitine (Lee *et al*., 2008); (iv) a zebrafish p53 mutant line (named p53^{M214K}), that harbours a missense mutation found in human cancer, does not show p53-dependent response to ionizing radiation and mutant embryos fails to undergo apoptosis, while mutant adults are prone to develop malignant peripheral neural sheath tumours (Berghmans *et al*., 2005); (v) double mutants that harbour p53^{M214K} and a constitutive active form of B-RAF are prone to develop melanomas (Ceol *et al*., 2008); (vi)

double mutants that harbour $p53^{M214K}$ and a constitutive active form of RAS are prone to develop embryonic rhabdomyosarcomas (Langenau *et al.*, 2007).

The existence of a p44 ortholog in zebrafish has been shown by Davidson and colleagues in 2010: in this work, the authors isolated, through 3'-RACE PCR, a 1.7kb transcript produced by alternative splicing of the p53 mRNA. This 1.7kb transcript shows an in-frame STOP codon that does not allow full-length p53 translation. It also shows a downstream ATG that encodes an N-terminal p53 isoform that lacks the first TAD, as mammalian p44, showing an alternative N-terminal amino-acid sequence with no similarity with other zebrafish proteins (Figure 8).

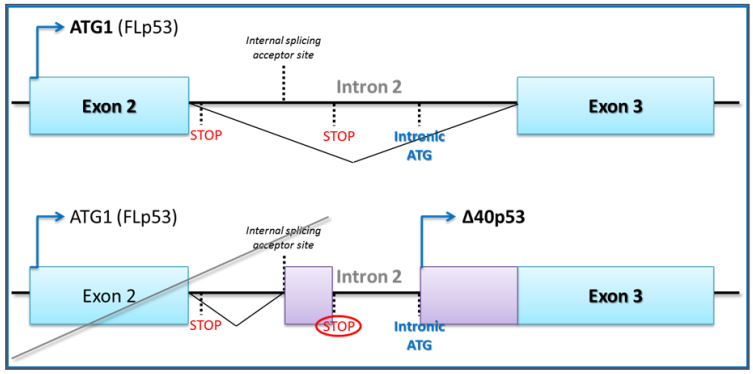


Figure 8. Illustration of the mechanism proposed by Davidson *et al.* for p44 production in zebrafish. Full-length p53 (FLp53) ATG is located in exon 2. When intron 2 is spliced, FLp53 is produced. On the contrary, when intron 2 is retained, the STOP codon located in intron 2 blocks FLp53 production (as suggested by the diagonal line, the prematurely truncated polypeptide undergoes degradation). In the same intron is located, downstream to the STOP codon, an ATG that is used to translate p44 (Δ40p53): the resulting protein shares the same sequence of FLp53, with the exception of the first transactivation domain.

The microinjection of p44 mRNA in wild-type embryos is associated with severe developmental abnormalities, such as hypoplasia of the head, eyes and somites, and marked embryonic lethality within the seventh day post fertilization. Interestingly, the same phenotype is obtained with the microinjection of human p44 ortholog. On the contrary, microinjection of p44 mRNA in $p53^{M214K}$ embryos does not lead to any morphological abnormalities, suggesting that, as previously shown in mammals, the p44 isoform acts only in cooperation with FLp53. Moreover, p44 overexpression is evident after treatment with ionizing radiation: as in mammals, in zebrafish this isoform is overexpressed in response to stress. As its mammalian ortholog, p44 drives the overexpression of p21 in concert with p53; in absence of p53, p44 alone is not able to drive p21 expression. Finally, knockdown of p21 in p44-overexpressing embryos leads to a partial rescue of embryonic abnormalities (Davidson *et al*., 2010). In summary, this work shows that zebrafish p44 recapitulates mammalian p44 functions: nevertheless, further efforts are necessary to investigate the role of p44 in development, under normal conditions and upon stress. Finally, it is important to notice that zebrafish has the unique opportunity to generate a knock-out model of p44. The alternative splicing mechanism through which p44 is produced gives the chance to selectively knock-out p44 in this organism, without interfering with p53 sequence: indeed, in mice it has been observed that a point mutation introduced to mutagenize the p44 ATG leads to a dramatic reduction of the p53 mRNA level, due to its instability (Melis *et al*., 2011). Considering this, a genetic model of selective p44 ablation is not feasible in mice.

4.1 The short-living killifish *Nothobranchius furzeri*

The animal models for research on aging range from invertebrates to mammals, which lifespans range from few weeks to years.

Historically, the worm *Caenorhabditis elegans*, with a mean lifespan of 18-20 days, was the first model to be genetically engineered to discover genes that regulate the aging process: as an example, the involvement of insulin/IGF-1 signalling pathway in aging was revealed in this organism (Tissenbaum *et al*., 1998). Although *C. elegans* shows enormous advantages in aging research (such as the extremely short lifespan, easy genetic manipulation, the feasibility of high-throughput genetic screenings for RNA interference, the possibility to test simultaneously a variety of molecules) it is an invertebrate organism that cannot completely recapitulate vertebrate physiology (Olsen *et al*. 2006).

Recently, mice have been extensively used in aging research, considering also the increasing interest in neurodegenerative diseases and aging-dependent cognitive decline; but the expensive costs for their maintenance and the median lifespan of 2 years are obvious disadvantages, together with the rising ethical pressure on replacing mammals, whenever possible, with lower vertebrate or invertebrate model organisms.

Zebrafish has a median lifespan of 3 years and a maximum lifespan of 5.5 years (Gerhard *et al*., 2002), even though these numbers are influenced by housing conditions and differences in strains. It has been reported that zebrafish recapitulates some of the mammalian aging features: spinal curvature, accumulation of oxidative damages to proteins in the muscle, accumulation of senescence-associated β-galactosidase. Nevertheless, the

study of 2-year-old wild-type fish (that cannot be defined old yet) in comparison with very young fish of 4-6 months failed to detect differences in some aging-associated phenotypes, such as the decrease in telomerase activity, the depletion of the stem cell compartment and the accumulation of the aging marker lipofuscin. For this reason, it has been proposed that zebrafish represents a model of "very gradual senescence" (Kishi *et al*., 2003). This issue, together with the prolonged median lifespan, are the main problems that counteract the use of zebrafish in aging research. However, it is important to point out that recently a genetic model of shortened lifespan and premature aging has been produced: the telomerase-deficient (*tert*$^{-/-}$) zebrafish (Henriques *et al*., 2013; Anchelin *et al*., 2013) represents a new tool that will improve the use of this species in aging research.

An overview on **Nothobranchius furzeri** *life cycle and age-related phenotypes*

In this work, we chose an emerging model organism: the short-living killifish *Nothobranchius furzeri*, first proposed as a model for aging research in 2003 (Valdesalici and Cellerino, 2003). This fish is characterized by an extremely short lifespan that ranges, depending on the laboratory strain, from a minimum of 3 months to a maximum of 12 months: this is the result of the evolutional adaptation of *N. furzeri* to its habitat. The habitat of this killifish consists of seasonal water bodies in Zimbabwe and Mozambique, where a long dry season is followed by a brief rainy season: during the latter, ephemeral ponds of water appear on the riverside. In this season, *N. furzeri* embryos hatch, they quickly grow and reach sexual maturity at one month of age. Killifish reproduce and die before the ponds dry. Killifish

embryos are adapted to survive the dry season: they do not develop in water, but in humid or dry mud. Depending on the environmental conditions (e.g. hypoxia and low temperature), embryos can also enter diapause, a state of arrested development (dormancy) during which oxygen consumption is reduced at the minimal level that guarantees survival. With the new rainy season, killifish embryos, finally in contact with water, hatch. (Levels *et al*., 1986; Valdesalici and Cellerino, 2003; Kim *et al*., 2016).

Thanks to the efforts of *Nothobranchius* researchers, we have now the possibility to use sufficiently standardized protocols for laboratory husbandry of this fish. In captivity, the reduced competition and the controlled conditions allows *N. furzeri* to live longer, even though it remains an annual fish. It is interesting to point out that, nevertheless, the reproductive phase of this killifish is not extended by captivity: it becomes fertile at one month of age and sterile when it is 6-month-old. After the reproductive phase, this killifish rapidly ages showing several aging-associated phenotypes in common with mammals. It is important to point out that, in captivity, killifish reproduction is continuous and not seasonal, even if they have a lower fecundity than zebrafish: *N. furzeri* females lie a maximum of ~30 eggs 2/3 times a week. In laboratory conditions, killifish embryos usually skip diapause, but their development still takes a long time if compared with zebrafish: they hatch after ~3 weeks post-fertilization. For these reasons, including the fact that some fundamental techniques of developmental biology such as *in situ* hybridization and transient modulation of gene expression are very far from being set up, *N. furzeri* is not considered, nowadays, an advantageous model for developmental biology. Nevertheless, recent efforts were made to describe killifish

development, especially in relation with the interesting phenomenon of diapause (Dolfi *et al*., 2014; Cellerino *et al*., 2015).

As previously mentioned, different *N. furzeri* laboratory strains are available. The first strain is named MZM 04/10: it is original from Southern Mozambique, collected in 2010 and thus inbred for less than ten years. It has a median lifespan of 6-9 months and a maximum lifespan of 12 months. The second strain is named GRZ: it is original from the Gona Re Zhou National Park in Zimbabwe and derived from a naturally inbred population, collected in 1969. This strain is characterized by an extremely short lifespan of 3-6 months. Interestingly, both MZM and GRZ strains reach sexual maturity at ~1 month of age, suggesting that the shorten lifespan observed in GRZ depends on accelerated physiological decay and not on accelerated development (Terzibasi *et al*., 2008). This has also been confirmed by several works: the GRZ strain ages faster than MZM and aging-associated features appear earlier in GRZ than in MZM (Di Cicco *et al*., 2011; Baumgart *et al*., 2012). The extremely short lifespan observed in the GRZ strain is not the result of a single mutation: quantitative trait loci (QTL) analyses revealed that this trait has a complex multigenic origin (Kirschner *et al*., 2012, Cellerino *et al*., 2015).

Several efforts have been done by *Nothobranchius* researchers to demonstrate that this fish recapitulates mammalian aging. From a macroscopic point of view, it is extremely easy to recognize aging-related phenotypes in old fish. After the reproductive phase, fish begin to appear more and more emaciated: males lose their colouration, the spinal curvature increases, fins become deteriorated. They also spend less and less time exploring the environment: locomotor activity and learning

performance tests show that old fish encounter rapidly cognitive decline (Genade *et al*., 2005; Valenzano *et al*., 2006b). Histopathological analyses show that, at the basis of this progressive systemic decline, there are aging-related lesions in multiple organs: kidneys are subjected to nephrocalcinosis and, thus, reduced functionality, heart undergoes hypertrophy and liver undergoes steatosis: these lesions are common in old vertebrates, including humans (Di Cicco *et al*., 2011; Cellerino *et al*., 2015). An important aspect in *Nothobranchius* aging is the age-related onset of spontaneous tumours, that are rare in other teleost fish: in ~50% of old killifish were found neoplastic lesions primarily in the liver (hepatomas and cellular hepatocarcinomas) and kidney (Di Cicco *et al*., 2011).

Nothobranchius has been shown to accumulate with age several well-known cellular and molecular aging biomarkers. Lipofuscin is an autofluorescent pigment detected in lysosomes of old invertebrates and vertebrates, including humans, as a consequence of lipid peroxidation: this pigment accumulates in the liver and brain of N. *furzeri* (Terzibasi *et al*., 2008 and 2009). β-galactosidase activity increases with age in mammals, as a result of the accumulation of cells that have lost their replicative potential: senesce-associated β-galactosidase staining strikingly increases with age also in the skin of *N. furzeri* (Genade *et al*., 2005), whereas killifish liver shows an increasing number of apoptotic cells (Di Cicco *et al*., 2011). *Nothobranchius* brain shows age-dependent neurodegeneration: a well-known marker of degenerating neurons, Fluoro-Jade B, accumulates in the brain of aging fish (Valenzano *et al*., 2006b); as in mammals, adult neurogenesis is impaired in the old killifish, due to an evident decrease in stem cell activity (Terzibasi Tozzini *et al*., 2012); finally, gliosis (hypertrophy

of several types of glial cells) dramatically increases in old fish as in humans (Terzibasi Tozzini *et al*., 2012). Moreover, *N. furzeri* telomere length is similar to humans and telomeres shorten with age in this fish (Hartmann *et al*., 2009). Another molecular biomarker of aging is the reduction of mitochondrial DNA (mtDNA) copy number, due to the age-dependent increase of mtDNA instability: mtDNA copy number decreases with age in *Nothobranchius* tissues (Hartmann *et al*., 2011). Finally, genome-wide analysis of microRNA expression reveals that they are regulated during aging in the *N. furzeri* brain: in particular, miRNAs that enhance the proto-oncogene *myc* activity are downregulated with age, whereas miRNAs that enhance the tumour suppressor *p53* activity are upregulated. This age-dependent regulation of miRNA expression is conserved between *Nothobranchius* and primates (Baumgart *et al*., 2012). Whole-genome transcript regulation has also been investigated in the aging brain of *N. furzeri*, revealing strong conservation with humans in terms of downregulated and upregulated genes; this analysis also reveals uncharacterized genes that are potentially new genetic determinants of aging (Baumgart *et al*., 2014).

An overview on the available technologies

Thanks to its short lifespan, *N. furzeri* represents a valid model for longitudinal studies, in which the same animals are monitored from birth to death. For this reason, several non-genetic interventions have been applied to modulate *Nothobranchius* lifespan: (i) lowering water temperature results in prolonging lifespan and decelerating the onset of aging biomarkers (Valenzano *et al*., 2006a); (ii) resveratrol administration with standard diet extends *N. furzeri* lifespan by the 30% and delays

neurodegeneration (Valenzano *et al*., 2006b); (iii) dietary restriction extends median lifespan and slows down aging of the GRZ strain, whereas it increases only maximum lifespan of the MZM strain (Terzibasi *et al*., 2009); (iv) treating *N. furzeri* with very low doses of rotenone, which interferes with the mitochondrial ETC, results in 15% lifespan prolongation (Baumgart *et al*., 2016). These studies prove that *N. furzeri* is a useful model for the investigation of the effects of diets and drugs on lifespan and aging. Nevertheless, a valid experimental model has to provide the opportunity to apply genetic interventions.

Recently, two fundamental bioinformatic tools were provided to *Nothobranchius* researchers: sequenced, assembled and annotated genome (Reichwald *et al*., 2015; Valenzano *et al*., 2015) and transcriptome for several tissues (Petzold *et al*., 2013; Valenzano *et al*., 2015). Moreover, a protocol for microinjection in *Nothobranchius* eggs has been set up (Hartmann and Englert, 2012) and transgenesis has been successfully applied in this fish using the Tol2 transposon system. Transgenic lines, that express the green fluorescent protein (GFP) under different promoters, have been generated, proving that *N. furzeri* can grow up as a model for genetic screenings (Valenzano *et al*., 2011; Allard *et al*., 2013). Most importantly, the CRISPR/Cas9 technique has been used in this model to generate several knock-out lines of aging genes involved in nutrient sensing, genomic stability, mitochondrial functions, cell senescence and proteostasis. The phenotype of a knock-out line deficient for the telomere reverse transcriptase (TERT) enzyme has been investigated: as previously mentioned, *N. furzeri*'s telomeres are similar in length to humans'; *tert*$^{-/-}$ killifish undergo age-dependent telomere shortening and develop atrophied

gonads and impaired self-renewal of stem cells, showing severe dysfunctions in actively proliferating tissues such as testes, blood and intestine. The use of CRISPR/Cas9 combined with homology directed repair allowed the generation of a knock-out/knock-in killifish line harbouring a mutation in the *tert* gene that corresponds to the mutation found in human dyskeratosis congenita (Harel *et al*., 2015; Kim *et al*., 2016).

In conclusion, initial research on *N. furzeri* provided evidence that this fish represents a model of accelerated senescence that recapitulates in less than one year all the hallmarks of mammalian aging that have been searched so far. Recently, several fundamental tools and techniques have been set up in this fish, so that *N. furzeri* is ready to become the most appealing model to investigate genetics of aging.

p66^{Shc} and p53/p44 in Nothobranchius furzeri

All the knowledge about the conservation of p66^{Shc}, p53 and its short isoform p44, including molecular cloning and sequence analysis, has been generated with this work. cDNA sequences of p66^{Shc} and p53 have been retrieved using the *N. furzeri* transcriptome browser (Petzold *et al*., 2013), searching for contigs showing similarity with zebrafish and mammalian orthologs. Genomic sequences of *p66^{Shc}* and *p53* were a courtesy of Dr A. Cellerino, since when this work began the *Nothobranchius* genome was not published yet.

Aim of the project

Oxidative stress (OS) induced by reactive oxygen species (ROS) increases during lifespan and it is involved in the aging process (Campisi, 2005). ROS are also important for normal development, since the balance of oxidative and reductive forces contributes to fine tune the balance of proliferation and differentiation (Dennery, 2007). On these bases, key regulators of intracellular ROS levels have a role in both aging and development: two well-known players in OS response are $p66^{Shc}$ and p53. $p66^{Shc}$ is activated upon OS and it is responsible for p53-dependent apoptosis. $p66^{Shc-/-}$ mice are resistant to OS-induced apoptosis (Migliaccio *et al*., 1999) and they are considered a model of healthy aging (Zaccagnini *et al.*, 2004; Menini *et al.*, Koch *et al*., 2008). The role of $p66^{Shc}$ in development has not been investigated, but some evidence suggests that it can have a function in neuronal differentiation (Conti *et al.*, 1997; Papadimou *et al.*, 2009). p53 is universally known for its role in tumor suppression, but little is known on its role in aging: it has emerged that the p44 isoform of p53, which is also present in zebrafish (Davidson *et al*., 2010), could be the key regulator of p53 role in stress response (Bourougaa *et al.*, 2010) and aging (Maier *et al*., 2004). Intriguingly, the p44 isoform appears to have an opposite role on p53 during development, when it is involved in cell proliferation, blocking p53-dependent differentiation (Ungewitter and Scrable, 2010). Recently, it has been demonstrated that p44/p53 and $p66^{Shc}$ participate altogether in a transcriptional regulation network that triggers, specifically upon OS, *in vitro* cell cycle arrest, through downregulation of G2/M genes (Gambino *et al*., 2013). Taking advantage of two animal models, the teleost fish *Danio*

rerio and *Nothobranchius furzeri*, this work has been tailored to address two main objectives:

1. Evaluating whether, in our models, p53/p44 and $p66^{Shc}$ are present and conserved, through molecular cloning of the ortholog genes, sequence analyses, characterization of their expression patterns and functional analyses.
2. Using the CrispR-Cas9 technique to produce a genetic model of p44 ablation in zebrafish, in order to elucidate the role of this isoform in the orchestration of p53-dependent stress response in development.

Main results

The first part of my project has been focused on the identification through molecular cloning and sequence analyses of the *Danio rerio* ortholog of $p66^{Shc}$ and the *Nothobranchius furzeri* orthologs of p53, p44 and $p66^{Shc}$. These results are collected in the paper published on *Aging and Disease,* included in Part II of the present thesis. Here below the main findings obtained:

1. A $p66^{Shc}$ isoform of the *shc1* gene is expressed in *D. rerio* and *N. furzeri.*
2. Sequence analyses of $p66^{Shc}$ and p53 orthologs of *D. rerio* and *N. furzeri* show that the key aminoacidic residues and domains are conserved between these fish and mammals. This fact suggests that fish proteins can recapitulate mammalian ortholog functions.

The second part of my project was focused on the initial characterization of the role of (i) $p66^{Shc}$ in zebrafish development and (ii) *N. furzeri* p53/p44 in stress response. The results obtained are not organized in a manuscript and they are included in Part III of this thesis. Here below the main findings obtained:

1. <u>$p66^{Shc}$ expression is restricted to the cephalic area in zebrafish development</u>:
    a. At 24hpf, $p66^{Shc}$ expression is ubiquitous, but concentrated in the head, in the trigeminal placodes and in Rohon-Beard neurons, transient mechanoreceptors located dorsally in the

spinal cord, that undergo programmed cell death after 24hpf (Reyes *et al.,* 2004).

b. At 48hpf, $p66^{Shc}$ expression is localized (i) in the brain and in particular in the optic tectum, (ii) in the ciliary marginal zone of the retina, where retinal stem cells and retinal progenitor cells are located (Moshiri *et al.,* 2004), and (iii) in the trigeminal ganglion, where the nuclei of the touch-sensory neurons of the fifth cranial nerve are located (Kimmel *et al.,* 1995).

c. At 72hpf and 96hpf, $p66^{Shc}$ expression is still restricted to the cephalic area and it is concentrated in the optic tectum, in the ciliary marginal zone of the retina and in the trigeminal ganglion.

2. $p66^{Shc}$ was successfully discriminated from $p52^{Shc}$: at 24hpf and 48hpf, $p52^{Shc}$ expression is not spatially restricted, being ubiquitously distributed in the embryo.
3. $p66^{Shc}$ mRNA levels increases at later developmental stages: mRNA levels of $p66^{Shc}$ are very low at early developmental stages and dramatically increases reaching a peak at 48hpf-72hpf. On the contrary, mRNA levels of $p52^{Shc}$ are stable over time.
4. p44 and p53 share the same expression pattern: at 48hpf, p53 and p44 are both expressed (i) in the midbrain-hindbrain boundary; (ii) in the retina; (iii) in the fin buds; (iv) in the pharyngeal arches; (v) in the otic vesicles and (vi) in the hatching glands.
5. *Nothobranchius* $p66^{Shc}$ is expressed in adult tissues: we cloned the *N. furzeri* ortholog of $p66^{Shc}$ and proved that it is expressed in several adult tissues (brain, heart, liver, intestine and muscle).

6. *Nothobranchius* p53 (Np53) and p44 (Np44) partially recapitulate mammalian p53/p44 functions in oxidative stress response:
    a. We identified the putative Np44 by sequence analysis of *p53* genomic sequence; the full-length cDNA sequence of Np44 has been cloned and found expressed in several adult tissues (brain, heart, intestine and testes).
    b. In $p53^{-/-}$ MEFs reconstituted with either Np53 or Np44 and treated with H_2O_2 or doxorubicin, cell senescence is induced by Np53 also at basal conditions, whereas it is induced by Np44 only upon treatments.
    c. In $p53^{-/-}$ MEFs reconstituted with either Np53 or Np44 and treated with H_2O_2 or doxorubicin: (i) G2/M genes are not downregulated by Np53 upon treatments; (ii) G2/M genes are downregulated by Np44 upon treatment with H_2O_2 but not doxorubicin; (iii) p21 upregulation is induced by Np53 upon both the treatments; (iv) p21 upregulation is mildly induced by Np44 only upon doxorubicin treatment.

The third part of this work has been focused on the generation and initial characterization of the $p44^{-/-}$ model in *D. rerio* (zebrafish). These results are collected in a manuscript in preparation; this will be concluded with the results of functional experiments planned to unravel the role of p44 in stress response during development. Here below the main findings already obtained:

1. Three different $p44^{-/-}$ lines have been generated in zebrafish taking advantage of the CRISPR/Cas9 technique. In these three lines, the correct splicing of full-length p53 is unaltered.

2. In the $p44^{-/-}$ line used for further investigations, mRNA levels of full-length p53 are comparable to those of wild-type (WT; data obtained with Real-Time RT-PCR).
3. Preliminary data obtained by semiquantitative RT-PCR data show that:
    a. p44 and Δ113p53 mRNA levels are comparable between $p44^{-/-}$ and WT.
    b. Δ113p53 expression is low at mid-blastula transition, then it reaches a peak at gastrulation and it slightly decreases until 120hpf.
4. A preliminary experiment in which $p44^{-/-}$ and WT embryos were treated with the p53-activating agent R-roscovitine (Lu *et al.*, 2001; Guo *et al.*, 2010) shows that: (i) phenotypic differences are not detectable between $p44^{-/-}$ and WT embryos upon treatment; (ii) p44 expression is induced in a dose-dependent manner, in particular in WT embryos; (iii) p21 expression is poorly induced upon treatment with both doses; (iv) p53 expression is stable; (v) Δ113p53 dramatically increases in $p44^{-/-}$ and WT embryos in a dose-dependent manner; (vi) $p66^{Shc}$ expression is stable in WT embryos upon treatment, while it results strongly induced in the $p44^{-/-}$.

Conclusions and future perspectives

The role of $p66^{Shc}$ in aging has been extensively investigated, but the mechanisms involved are still to be elucidated. Our aim is to investigate $p66^{Shc}$ genetics taking advantage of the short-living vertebrate *Nothobranchius furzeri*. This teleost fish is an emerging model in which the generation of transgenic (Valenzano *et al.*, 2011; Allard *et al.*, 2013) and CRISPR/Cas9-mediated knock-out lines (Harel *et al.*, 2015) are feasible, as well as screenings of compounds affecting lifespan and healthspan (Valenzano *et al.*, 2006b). We used the *N. furzeri* transcriptome browser (Petzold *et al.,* 2013) to identify the ortholog of $p66^{Shc}$ ($Np66^{Shc}$) and proved that it is expressed in several adult tissues. Our sequence analyses show that the aminoacidic domains important for $p66^{Shc}$ to exert its functions are conserved in *N. furzeri*. This is the basis for further functional analyses: heterologous complementation experiments in $p66^{Shc-/-}$ mammalian cells will be useful to elucidate whether $Np66^{Shc}$ is able to initiate OS response, as in mammals. Further investigations include *in vivo* analyses of $Np66^{Shc}$ expression changes under different environmental conditions or stresses and the generation of a genetic model of $Np66^{Shc}$ overexpression or ablation.

Since $p66^{Shc-/-}$ mice are viable and $p66^{Shc-/-}$ embryos are not counterselected during gestation, few attentions have been focused on understanding whether $p66^{Shc}$ may have a function in development. The recent debate on differences in phenotypic effects obtained with gene knockdown and mutagenesis highlighted that mechanisms of genetic compensation may explain why deleterious mutations have sometimes no

morphological effects, while gene knockdown leads to abnormal development: mutations in the genome can trigger up- or downregulation of genes related to the ablated one (Rossi *et al.*, 2015). This means that the ablated gene has not to be considered "necessary", but, nevertheless, it is "functional". In fact, although $p66^{Shc}$ is not necessary to complete embryogenesis, there are some evidence suggesting a function in neuronal differentiation (Conti *et al.*, 1997; Papadimou *et al.*, 2009). Through bioinformatic analysis it has emerged that the *shc locus* in zebrafish shows conservation with mammals. Ensemble prediction was used as a reference for molecular cloning of the $p66^{Shc}$ full-length cDNA, demonstrating for the first time that this isoform is expressed in zebrafish and that the functional domains present in the N-terminal sequence unique to $p66^{Shc}$ are conserved. Moreover, Real-Time RT-PCR and whole-mount *in situ* hybridization analyses on zebrafish embryos show that the shorter and the longest isoforms of *shc1* are differently regulated in zebrafish development. $p52^{Shc}$ behaves as a housekeeping gene, whereas $p66^{Shc}$ is temporally regulated: its mRNA levels reach a peak between 48hpf and 72hpf, during the organogenesis process, when areas of active cell proliferation become more and more restricted and the vast majority of cell types are differentiated (Kimmel *et al.,* 1995). Whole-mount *in situ* hybridization of $p66^{Shc}$ shows that its expression is concentrated, until 24hpf, in neural crest-derived structures. After 48hpf, interestingly, $p66^{Shc}$ is expressed only in the cephalic area: (i) in the optic tectum, where neural progenitors are located (Ito *et al.,* 2010); (ii) in the ciliary marginal zone of the retina, where retinal stem cells and retinal progenitor cells are located (Moshiri *et al.,* 2004) and (iii) in the trigeminal ganglion. Our findings are in agree with

literature data and they suggest that $p66^{Shc}$ might be involved in brain development and in the specification of neural crest-derived structures, where it appears to be strongly expressed. Experiments of double *in situ* hybridization of $p66^{Shc}$ together with neural stem cell markers (e.g. *musashi-1*; Sakakibara *et al.,* 1996) and neural crest markers (e.g. *islet-1*; Inoue *et al.,* 2004) will confirm that $p66^{Shc}$ is localized in these cell types.

The tumour suppressor p53 is involved, as $p66^{Shc}$, in stress response, lifespan and aging modulation. p44 is a short isoform of p53 that has been demonstrated to orchestrate p53 functions in stress response and aging. As for $p66^{Shc}$, our aim is to take advantage of *N. furzeri* to investigate the mechanisms through which p44 acts on p53. We identified the putative p44 ortholog by sequence analysis of the *N. furzeri p53* genomic *locus*. We demonstrated that the full-length cDNA sequence of Np44 is expressed at low levels in several *N. furzeri* adult tissues. Moreover, our preliminary results of heterologous complementation experiments in $p53^{-/-}$ MEFs reconstituted with either Np53 or Np44 show that: (i) Np53 induces cell senescence upon OS and genotoxic stress (GS), but also at basal conditions; (ii) Np53 is not able to downregulate the 31 G2/M genes but it induces the upregulation of p21; (iii) Np44 is able to induce the downregulation of G2/M genes only upon OS. Future experiments have to be planned in order to modulate Np53 expression in $p53^{-/-}$ MEFs: in fact, Np53 is able to induce cell cycle arrest also at basal conditions. This makes the other results difficult to interpret. In mammals, p44 is implicated in OS response (Gambino *et al.*, 2013). This fact suggests that Np44 might recapitulate mammalian p44 functions in orchestrating the p53-dependent transcriptional response to different kinds of stress agents. *In vivo*

experiments in *N. furzeri* will clarify whether Np44 is indeed activated by OS and in which tissues is preferentially expressed upon OS and at basal conditions. Moreover, being *N. furzeri* the shortest-living vertebrate model organism, a $p44^{-/-}$ model in this fish will be also fundamental to deeply investigate the role of p44 in aging and lifespan modulation.

In this study, we analysed p53 and p44 expression patterns during zebrafish development through *in situ* hybridization. In mammals, the main function of p44 during early embryogenesis is to suppress p53 activity to preserve pluripotency (Ungewitter and Scrable, 2010). p44 and p53 share the same expression patterns in 48hpf embryos. Both the isoforms are expressed in areas of persistent cell proliferation, such as the midbrain-hindbrain boundary (Wulliman and Knipp, 2000) and the fin buds (Nomura *et al.,* 2006). It is not surprising that p53 and p44 are expressed in the same areas, considering that (i) p44 has been described as a splicing isoform of p53 (Davidson *et al.,* 2010) and (ii) it is demonstrated that p44 has to interact with p53 to exert its functions (Maier *et al.,* 2004; Ungewitter and Scrable, 2010). Interestingly, both p53 and p44 are expressed in areas of extensive cell proliferation: this observation is consistent with previous data showing that p44 and p53 cooperate during embryogenesis in maintaining the balance between proliferation and differentiation (Ungewitter and Scrable, 2010).

Importantly, the alternative splicing mechanism through which p44 is produced in zebrafish gives the chance to selectively knock-out p44, without interfering with p53 sequence. In mice, it has been observed that a point mutation introduced within the p44 ATG leads to a dramatic reduction of the p53 mRNA level due to its instability (Melis *et al.*, 2011).

Considering this, a genetic model of selective p44 ablation is not feasible in mice. Taking advantage of the CRISPR/Cas9 technology, we generated three different stable lines of $p44^{-/-}$ zebrafish in which the full-length p53 mRNA is unaltered. Real-Time RT-PCR of p53 at different developmental stages shows that p53 mRNA levels are comparable between $p44^{-/-}$ and wild-type (WT) embryos at each developmental stage. This data demonstrates that in our model only p44 is depleted.

Preliminary data obtained by semiquantitative RT-PCR show that mRNA levels of p44 and the shortest isoform of p53, Δ113p53, are comparable between $p44^{-/-}$ and WT embryos and that the two isoforms are differently regulated. Preliminary data obtained by treating $p44^{-/-}$ and WT embryos at gastrulation stage with the p53-activating agent R-roscovitine (Lu *et al.*, 2001; Guo *et al.*, 2010) suggests that p44 expression is dramatically induced by this drug. Moreover, the absence of phenotypic differences between $p44^{-/-}$ and WT upon treatment might be the result of a genetic compensation mechanism. In particular, $p66^{Shc}$ expression is induced in a dose-dependent manner in treated $p44^{-/-}$ embryos. Further experiments are needed to confirm our hypothesis. Other p53-activating agents will be tested in order to evaluate how p44 orchestrates stress response in development. In particular, we will induce OS and GS response in zebrafish embryos to validate *in vivo* the hypothesis that p44 is in particular involved in transcriptional regulation in response to protein damage (Bourougaa *et al.*, 2010; Gambino *et al.*; 2013).

References

Allard, J.B.; Kamei, H. and Duan, C. 2013. Inducible transgenic expression in the short-lived fish Nothobranchius furzeri. ***J Fish Biol,*** 82(5):1733-8.

Allen, R.G. and Balin, A.K. 1989. Oxidative influence on development and differentiation: an overview of a free radical theory of development. ***Free Radic Biol Med***, 6(6):631-61.

Anchelin, M.; Alcaraz-Pérez, F.; Martínez, C.M.; Bernabé-García, M.; Mulero, V. and Cayuela, M.L. 2013. Premature aging in telomerase-deficient zebrafish. ***Dis Model Mech***, 6(5):1101-12.

Armstrong, J.F.; Kaufman, M.H.; Harrison, D.J. and Clarke, A.R. 1995. High-frequency developmental abnormalities in p53-deficient mice. ***Curr Biol***, 5(8):931-6.

Bargonetti, J. and Manfredi, J.J. 2002. Multiple roles of the tumor suppressor p53. ***Curr Opin Oncol***, 14(1):86-91.

Baumgart M.; Groth, M.; Priebe, S.; Appelt, J.; Guthke, R.; Platzer, M. and Cellerino, A. 2012. Age-dependent regulation of tumor-related microRNAs in the brain of the annual fish Nothobranchius furzeri. ***Mech Ageing Dev***, 133(5):226-33.

Baumgart, M.; Groth, M.; Priebe, S.; Savino, A.; Testa, G.; Dix, A.; Ripa, R.; Spallotta, F.; Gaetano, C.; Ori, M.; Terzibasi Tozzini, E.; Guthke, R.; Platzer, M. and Cellerino, A. 2014. RNA-seq of the aging brain in the short-lived fish N. furzeri - conserved pathways and novel genes associated with neurogenesis. ***Aging Cell***, 13(6):965-74.

Baumgart, M.; Priebe, S.; Groth, M.; Hartmann, N.; Menzel, U.; Pandolfini, L.; Koch, P.; Felder, M.; Ristow, M.; Englert, C.; Guthke, R.; Platzer, M. and Cellerino A. 2016. Longitudinal RNA-Seq Analysis of Vertebrate Aging Identifies Mitochondrial Complex I as a Small-Molecule-Sensitive Modifier of Lifespan. ***Cell Syst***, 2(2):122-32.

Belyi, V.A.; Ak, P.; Markert, E.; Wang, H.; Hu, W.; Puzio-Kuter, A. and Levine, A.J. 2010. The origins and evolution of the p53 family of genes. ***Cold Spring Harb Perspect Biol***, 2(6):a001198.

Berghmans, S.; Murphey, R.D.; Wienholds, E.; Neuberg, D.; Kutok, J.L.; Fletcher, C.D.; Morris, J.P.; Liu, T.X.; Schulte-Merker, S.; Kanki, J.P.; Plasterk, R.; Zon, L.I. and Look, A.T. 2005. tp53 mutant zebrafish

develop malignant peripheral nerve sheath tumors. ***Proc Natl Acad Sci USA***, 102(2):407-12.

Berniakovich, I.; Trinei, M.; Stendardo, M.; Migliaccio, E.; Minucci, S.; Bernardi, P.; Pelicci, P.G. and Giorgio, M. 2008. p66Shc-generated oxidative signal promotes fat accumulation. ***J Biol Chem***, 283(49):34283-93.

Bode, A.M. and Dong, Z. 2004. Post-translational modification of p53 in tumorigenesis. ***Nat Rev Cancer***, 4(10):793-805.

Bourougaa, K.; Naski, N.; Boularan, C.; Mlynarczyk, C.; Candeias, M.M.; Marullo, S. and Fåhraeus, R. 2010. Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47. ***Mol Cell,*** 38(1):78-88.

Campisi, J. 2005. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. ***Cell,*** 120(4):513-22.

Cellerino, A.; Valenzano, D.R. and Reichard, M. 2015. From the bush to the bench: the annual Nothobranchius fishes as a new model system in biology. ***Biol Rev Camb Philos Soc***, 91(2):511-33.

Ceol, C.J.; Houvras, Y.; White, R.M. and Zon, L.I. 2008. Melanoma biology and the promise of zebrafish. ***Zebrafish***, 5(4):247-55.

Cheng, R.; Ford, B.L.; O'Neal, P.E.; Mathews, C.Z.; Bradford, C.S.; Thongtan, T.; Barnes, D.W.; Hendricks, J.D. and Bailey, G.S. 1997. Zebrafish (Danio rerio) p53 tumor suppressor gene: cDNA sequence and expression during embryogenesis. ***Mol Mar Biol Biotechnol***, 6(2):88-97.

Choi, J. and Donehower, L.A. 1999. p53 in embryonic development: maintaining a fine balance. ***Curr Mol Life Sci***, 55(1):38-47.

Choksi, K.B.; Nuss. J.E.; DeFord, J.H. and Papaconstantinou, J. 2011. Mitochondrial electron transport chain functions in long-lived Ames dwarf mice. ***Aging (Albany NY)***, 3(8):754-67.

Conti, L.; De Fraja, C.; Gulisano, M.; Migliaccio, E.; Govoni, S. and Cattaneo, E. 1997. Expression and activation of SH2/PTB-containing ShcA adaptor protein reflects the pattern of neurogenesis in the mammalian brain. ***Proc Nat Acad Sci USA***, 94(15):8185-90.

Courtois, S.; Verhaegh, G.; North, S.; Luciani, M.G.; Lassus, P.; Hibner, U.; Oren, M. and Hainaut P. 2002. DeltaN-p53, a natural isoform of p53

lacking the first transactivation domain, counteracts growth suppression by wild-type p53. ***Oncogene***, 21(44):6722-8.

Davidson, W.R., Kari, C., Ren, Q., Daroczi, B., Dicker, A.P. and Rodeck, U. 2010. Differential regulation of p53 function by the N-terminal ΔNp53 and Δ113p53 isoforms in zebrafish embryos. ***BMD Dev Biol,*** 10:102.

Dennery, P.A. 2007. Effects of oxidative stress on embryonic development. Birth Defects Res C ***Embryo Today***, 81(3): 155-162.

Derungs, R; Camici, G.G.; Spescha, R.D.; Welt, T.; Tackenberg, C.; Späni, C.; Wirth, F.; Grimm, A.; Eckert, A.; Nitsch, R.M. and Kulic, L. 2016. Genetic ablation of the p66Shc adaptor protein reverses cognitive deficits and improves mitochondrial function in an APP transgenic mouse model of Alzheimer's disease. ***Mol Psychiatry***, 22(4):605-614.

Di Cicco, E.; Tozzini, E.T.; Rossi, G. and 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(4):249-56.

Dolfi, L.; Ripa, R. and Cellerino, A. 2014. Transition to annual life history coincides with reduction in cell cycle speed during early cleavage in three independent clades of annual killifish. ***Evodevo***, 5:32.

Donehower, L.A.; Harvey, M.; Slagle, B.L; McArthur, M.J.; Montgomery, C.A. Jr; Butel, J.S. and Bradley, A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. ***Nature***, 356(6366):215-21.

Eizenberg, O.; Faber-Elman, A.; Gottlieb, E.; Oren, M.; Rotter, V. and Schwartz, M. 1996. p53 plays a regulatory role in differentiation and apoptosis of central nervous system-associated cells. ***Mol Cell Biol***, 16(9):5178-85.

Feng, Z.; Lin, M. and Wu, R. 2011. The Regulation of Aging and Longevity: A New and Complex Role of p53. ***Genes Cancer***, 2(4): 443-452.

Francia, P.; delli Gatti, C.; Bachschmid, M.; Martin-Padura, I.; Savoia, C.; Migliaccio, E.; Pelicci, P.G.; Schiavoni, M.; Lüscher, T.F.; Volpe, M. and Cosentino F. 2004. Deletion of p66shc gene protects against age-related endothelial dysfunction. ***Circulation***, 110(18):2889-95.

Gambino, V.; De Michele, G.; Venezia, O.; Migliaccio, P.; Dall'Olio, V.; Bernard, L.; Minardi, S.P.; Della Fazia, M.A.; Bartoli, D.; Servillo, G.;

Alcalay, M.; Luzi, L.; Giorgio, M.; Scrable, H.; Pelicci, P.G. and Migliaccio, E. 2013. Oxidative stress activates a specific p53 transcriptional response that regulates cellular senescence and aging. ***Aging Cell,*** 12(3):435-45.

García-Cao, I.; García-Cao, M.; Martín-Caballero, J.; Criado, L.M.; Klatt, P.; Flores, J.M.; Weill, J.C.; Blasco, M.A. and Serrano, M. 2002. "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. ***EMBO J***, 21(22):6225-35.

Genade, T.; Benedetti, M.; Terzibasi, E.; Roncaglia, P.; Valenzano, D.R.; Cattaneo, A. and Cellerino, A. 2005. Annual fishes of the genus Nothobranchius as a model system for aging research. ***Aging Cell***, 4(5):223-33.

Gerhard, G.S.; Kauffman, E.J.; Wang, X.; Stewart, R.; Moore, J.L.; Kasales, C.J.; Demidenko, E.; Cheng, K.C. 2002. Life spans and senescent phenotypes in two strains of Zebrafish (Danio rerio). ***Exp Gerontol***, 37(8-9):1055-68.

Ghosh, A.; Stewart, D. and Matlashewski, G. 2004. Regulation of human p53 activity and cell localization by alternative splicing. ***Mol Cell Biol,*** 24(18):7987-97.

Giorgio, M.; Berry, A.; Berniakovich, I.; Poletaeva, I.; Trinei, M.; Stendardo, M.; Hagopian, K.; Ramsey, J.J.; Cortopassi, G.; Migliaccio, E.; Nötzli, S.; Amrein, I.; Lipp, H.P.; Cirulli, F. and Pelicci, P.G. 2012. The p66Shc knocked out mice are short lived under natural condition. ***Aging Cell***, 11(1):162-8.

Giorgio, M.; Migliaccio, E.; Orsini, F.; Paolucci, D.; Moroni, M.; Contursi, C.; Pelliccia, G.; Luzi, L.; Minucci, S.; Marcaccio, M.; Pinton, P.; Rizzuto, R.; Bernardi, P.; Paolucci, F. and Pelicci, P.G. 2005. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. ***Cell***, 122(2):221-33.

Giorgio, M.; Stendardo, M.; Migliaccio, E. and Pelicci, P.G. 2016. P66SHC deletion improves fertility and progeric phenotype of late-generation TERC-deficient mice but not their short lifespan. ***Aging Cell***, 15(3):446-54.

Giorgio, M.; Trinei, M.; Migliaccio, E. and Pelicci, P.G. 2007. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? ***Nat Rev Mol Cell Biol,*** 8(9):722-8.

Guo, L.; Chua, J.; Vijayakumar, D.; Lee, K.C.; Lim, K.; Eng, H.; Ghadessy, F.; Coomber, D. and Lane, D.P. 2010. Detection of the 113p53 protein isoform: a p53-induced protein that feeds back on the p53 pathway to modulate the p53 response in zebrafish. ***Cell Cycle***, 9(10):1998-2007.

Hahn, M.E.; McArthur, A.G.; Karchner, S.I.; Franks, D.G.; Jenny, M.J.; Timme-Laragy, A.R.; Stegeman, J.J.; Woodin, B.R.; Cipriano, M.J. and Linney, E. 2014. The Transcriptional Response to Oxidative Stress during Vertebrate Development: Effects of tert-Butylhydroquinone and 2,3,7,8-Tetrachlorodibenzo-p-Dioxin. ***PLoS One***, 9(11): e113158.

Harel, I.; Benayoun, B.A.; Machado, B.; Singh, P.P.; Hu, C.K.; Pech, M.F.; Valenzano, D.R.; Zhang, E.; Sharp, S.C.; Artandi, S.E. and Brunet, A. 2015. A platform for rapid exploration of aging and diseases in a naturally short-lived vertebrate. ***Cell,*** 160(5):1013-26.

Harman, D. 1981. The aging process. ***Proc Natl Acad Sci USA***, 78(11): 7124-7128.

Hartmann, N. and Englert, C. 2012. A microinjection protocol for the generation of transgenic killifish (Species: Nothobranchius furzeri). ***Dev Dyn***, 241(6):1133-41.

Hartmann, N.; Reichwald, K.; Wittig, I.; Dröse, S.; Schmeisser, S.; Lück, C.; Hahn, C.; Graf, M.; Gausmann, U.; Terzibasi, E.; Cellerino, A.; Ristow, M.; Brandt, U.; Platzer, M. and Englert, C. 2011. Mitochondrial DNA copy number and function decrease with age in the short-lived fish Nothobranchius furzeri. ***Aging Cell***, 10(5):824-31.

Henriques, C.M.; Carneiro, M.C.; Tenente, I.M.; Jacinto, A. and Ferreira, M.G. 2013. Telomerase is required for zebrafish lifespan. ***PLoS Genet***, 9(1):e1003214.

Hitchler, M.J. and Domann, F.E. An epigenetic perspective on the free radical theory of development. ***Free Radic Biol Med***, 43(7):1023-36.

Holzenberger, M.; Dupont, J.; Ducos, B.; Leneuve, P.; Geloen, A.; Even, P. C.; Cervera, P. and Le Bouc, Y. 2003. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. ***Nature***, 421(6919): 182-187.

Howe, K.; Clark, M.D., Torroja, C.F.; […] and Stemple, D.L. 2013. The zebrafish reference genome sequence and its relationship to the human genome. ***Nature***, 496(7446):498-503.

Hwang, W.Y.; Fu, Y.; Reyon, D.; Maeder, M.L.; Tsai, S.Q.; Sander, J.D.; Peterson, R.T.; Yeh, J.R. and Joung, J.K. 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. ***Nat Biotechnology***, 31(3):227-9.

Inoue, A.; Takahashi, M.; Hatta, K.; Hotta, Y. and Okamoto H. 1994. Developmental regulation of islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. ***Dev Dyn***, 199(1):1-11.

Khoury, M.P. and Bourdon, J.C. 2011. p53 Isoforms: An Intracellular Microprocessor? ***Genes Cancer***, 2(4):453-65.

Kim, C.S.; Kim, Y.R.; Naqvi, A.; Kumar, S.; Hoffman, T.A.; Jung, S.B.; Kumar, A.; Jeon, B.H.; McNamara, D.M. and Irani, K. 2011. Homocysteine promotes human endothelial cell dysfunction via site-specific epigenetic regulation of p66shc. ***Cardiovasc Res***, 92(3):466-75.

Kim, Y.; Nam, H.G. and Valenzano, D.R. 2016. The short-lived African turquoise killifish: an emerging experimental model for ageing. ***Dis Model Mech***, 9(2):115-29.

Kim, Y.R.; Kim, C.S.; Naqvi, A.; Kumar, A.; Kumar, S.; Hoffman, T.A. and Irani, K. 2012. Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction. ***Am J Physiol Heart Circ Physiol***, 303(2):H189-96.

Kimmel, C.B.; Ballard, W.W.; Kimmel, S.R.; Ullmann, B. and Schilling, T.F. 1995. Stages of embryonic development of the zebrafish. ***Dev Dyn,*** 203(3):253-310.

Kirkwood, T.B. and Austad, S.N. 2000. Why do we age? ***Nature***, 408(6809):233-8.

Kirschner, J.; Weber, D.; Neuschl, C.; Franke, A.; Böttger, M.; Zielke, L.; Powalsky, E.; Groth, M.; Shagin, D.; Petzold, A.; Hartmann, N.; Englert, C.; Brockmann, G.A.; Platzer, M.; Cellerino, A. and Reichwald K. 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(2):252-61.

Kishi, S.; Uchiyama, J.; Baughman, A.M.; Goto, T.; Lin, M.C. and Tsai, S.B. 2003. The zebrafish as a vertebrate model of functional aging and very gradual senescence. ***Exp Gerontol***, 38(7):777-86.

Koch, O.R.; Fusco, S.; Ranieri, S.C.; Maulucci, G.; Palozza, P.; Larocca, L.M.; Cravero, A.A.; Farre', S.M.; De Spirito, M.; Galeotti, T. and Pani, G. 2008. Role of the life span determinant P66(shcA) in ethanol-induced liver damage. ***Lab Invest***, 88(7):750-60.

Langenau, D.M.; Keefe, M.D.; Storer, N.Y.; Guyon, J.R.; Kutok, J.L.; Le, X.; Goessling, W.; Neuberg, D.S.; Kunkel, L.M. and Zon, L.I. 2007. Effects of RAS on the genesis of embryonal rhabdomyosarcoma. ***Genes Dev***, 21(11):1382-95.

Langheinrich, U.; Hennen, E.; Stott, G. and Vacun, G. 2002. Zebrafish as a model organism for the identification and characterization of drugs and genes affecting p53 signaling. ***Curr Biol***, 12(23):2023-8.

Lee, K.C.; Goh, W.L.; Xu, M.; Kua, N.; Lunny, D.; Wong, J.S.; Coomber, D.; Vojtesek, B.; Lane, E.B. and Lane, D.P. 2008. Detection of the p53 response in zebrafish embryos using new monoclonal antibodies. ***Oncogene***, 27(5):629-40.

Levels, P.J.; Gubbels, R.E. and Denucé, J.M. 1986. Oxygen consumption during embryonic development of the annual fish Nothobranchius korthausae with special reference to diapause. ***Comp Biochem Physiol A Comp Physiol***, 84(4):767-70.

Li, M.; Zhao, L.; Page-McCaw, P.S. and Chen, W. 2016. Zebrafish Genome Engineering Using the CRISPR-Cas9 System. ***Trends Genet***, 32(12):815-827.

Lin, T.; Chao, C.; Saito, S.; Mazur, S.J.; Murphy, M.E.; Appella, E. and Xu, Y. 2005. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. ***Nat Cell Biol***, 7(2):165-71

López-Otín, C.; Blasco, M.A.; Partridge, L.; Serrano, M. and Kroemer, G. 2013. The hallmarks of aging. ***Cell***, 153(6):1194-217.

Lu, W.; Chen, L.; Peng, Y. and Chen, J. 2001. Activation of p53 by roscovitine-mediated suppression of MDM2 expression. ***Oncogene***, 20(25):3206-16.

Luzi, L.; Confalonieri, S.; Di Fiore, P.P. and Pelicci, P.G. 2000. Evolution of Shc functions from nematode to human. ***Curr Opin Genet Dev***, 10(6):668-74.

MacRae, C.A. and Peterson, R.T. 2015. Zebrafish as tools for drug discovery. ***Nat Rev Drug Discov***, 14(10):721-31.

Maier, B.; Gluba, W.; Bernier, B.; Turner, T.; Mohammad, K.; Guise, T.; Sutherland, A.; Thorner, M. and Scrable, H. 2004. Modulation of mammalian life span by the short isoform of p53. ***Genes Dev,*** 18(3):306-19.

Melis, J.P.; Hoogervorst, E.M.; van Oostrom, C.T.; Zwart, E.; Breit, T.M.; Pennings, J.L.; de Vries, A. and van Steeg, H. 2011. Genotoxic exposure: novel cause of selection for a functional ΔN-p53 isoform. ***Oncogene,*** 30(15):1764-72.

Menini, S.; Amadio, L.; Oddi, G.; Ricci, C.; Pesce, C.; Pugliese, F.; Giorgio, M.; Migliaccio, E.; Pelicci, P.; Iacobini, C. and Pugliese, G. 2006. Deletion of p66Shc longevity gene protects against experimental diabetic glomerulopathy by preventing diabetes-induced oxidative stress. ***Diabetes***, 55(6):1642-50.

Migliaccio, E.; Giorgio, M. and Pelicci, P.G. 2013. p53 and aging: role of p66Shc. ***Aging (Albany NY)***, 5(7):488-9.

Migliaccio, E.; Giorgio, M.; Mele, S.; Pelicci, G.; Reboldi, P.; Pandolfi, P.P.; Lanfrancone, L. and Pelicci, P.G. 1999. The p66shc adaptor protein controls oxidative stress response and life span in mammals. ***Nature***, 402(6759): 309-313.

Migliaccio, E.; Mele, S.; Salcini, A.E.; Pelicci, G.; Lai, K.M.; Superti-Furga, G.; Pawson, T.; Di Fiore, P.P.; Lanfrancone, L. and Pelicci, P.G. 1997. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. ***EMBO J***, 16(4): 706-716.

Moshiri, A.; Close, J. and Reh, T.A. 2004. Retinal stem cells and regeneration. Int ***J Dev Biol***, 48(8-9):1003-14.

Nomura, R.; Kamei, E.; Hotta, Y.; Konishi, M.; Miyake, A. and Itoh N. 2006. Fgf16 is essential for pectoral fin bud formation in zebrafish. ***Biochem Biophys Res Commun***, 347(1):340-6.

Olsen, A.; Vantipalli, M.C. and Lithgow, G.J. 2006. Using Caenorhabditis elegans as a model for aging and age-related diseases. ***Ann NY Acad Sci***, 1067:120-8.

Papadimou, E.; Moiana, A.; Goffredo, D.; Koch, P.; Bertuzzi, S.; Brüstle, O.; Cattaneo, E. and Conti, L. 2009. p66(ShcA) adaptor molecule accelerates ES cell neural induction. ***Mol Cell Neurosci***, 41(1):74-84.

Parman, T.; Wiley, M.J. and Wells, P.G. 1999. Free radical-mediated oxidative DNA damage in the mechanism of thalidomide teratogenicity. ***Nat Med***, 5(5):582-5.

Pelicci, G.; Lanfrancone L.; Grignani, F.; McGlade, J.; Cavallo, F.; Forni, G.; Nicoletti, I.; Grignani, F.; Pawson, T. and Pelicci, P.G. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. ***Cell***, 70(1):93-104.

Peng, Y.; Kwok, K.H.; Yang, P.H.; Ng, S.S.; Liu, J.; Wong, O.G.; He, M.L.; Kung, H.F. and Lin, M.C. 2005. Ascorbic acid inhibits ROS production, NF-kappa B activation and prevents ethanol-induced growth retardation and microencephaly. ***Neuropharmacology***, 48(3):426-34.

Petzold, A.; Reichwald, K.; Groth, M.; Taudien, S.; Hartmann, N.; Priebe, S.; Shagin, D.; Englert, C. and Platzer, M. 2013. The transcript catalogue of the short-lived fish Nothobranchius furzeri provides insights into age-dependent changes of mRNA levels. ***BMC Genomics,*** 14:185.

Phang, B.H.; Othman, R.; Bougeard, G.; Chia, R.H.; Frebourg, T.; Tang, C.L.; Cheah, P.Y. and Sabapathy, K. 2015. Amino-terminal p53 mutations lead to expression of apoptosis proficient p47 and prognosticate better survival, but predispose to tumorigenesis. ***Proc Natl Acad Sci USA***, 112(46):E6349-58.

Pollack, M. and Leeuwenburgh, C. 2001. Apoptosis and aging: role of the mitochondria. ***J Gerontol A Biol Sci Med Sci***, 56(11):B475-82.

Powell, D.J.; Hrstka, R.; Candeias, M.; Bourougaa, K.; Vojtesek, B. and Fåhraeus R. 2008. Stress-dependent changes in the properties of p53 complexes by the alternative translation product p53/47. ***Cell cycle,*** 7(7):950-9.

Ramsey, J.J.; Tran, D.; Giorgio, M.; Griffey, S.M.; Koehne, A.; Laing, S.T.; Taylor, S.L.; Kim, K.; Cortopassi, G.A.; Lloyd, K.C.; Hagopian, K.; Tomilov, A.A.; Migliaccio, E.; Pelicci, P.G. and McDonald, R.B. 2014.

The influence of Shc proteins on life span in mice. ***J Gerontol A Biol Sci Med Sci***, 69(10):1177-85.

Reichwald, K.; Petzold, A.; Koch, P.; Downie, B.R.; Hartmann, N.; Pietsch, S.; Baumgart, M.; Chalopin, D.; Felder, M.; Bens, M.; Sahm, A.; Szafranski, K.; Taudien, S.; Groth, M.; Arisi, I.; Weise, A.; Bhatt, S.S.; Sharma, V.; Kraus, J.M.; Schmid, F.; Priebe, S.; Liehr, T.; Görlach, M.; Than, M.E.; Hiller, M.; Kestler, H.A.; Volff, J.N.; Schartl, M.; Cellerino, A.; Englert, C. and Platzer, M. 2015. Insights into Sex Chromosome Evolution and Aging from the Genome of a Short-Lived Fish. ***Cell***, 163(6):1527-38.

Reinhardt, H.C. and Schumacher, B. 2012. The p53 network: cellular and systemic DNA damage responses in aging and cancer. ***Trends Genet***, 28(3):128-36.

Reyes, R.; Haendel, M.; Grant, D.; Melancon, E. and Eisen, J.S. 2004. Slow degeneration of zebrafish Rohon-Beard neurons during programmed cell death. ***Dev Dyn***, 229(1):30-41.

Rosen, J.N.; Sweeney, M.F. and Mably, J.D. 2009. Microinjection of zebrafish embryos to analyze gene function. ***J Vis Exp***, (25).

Rossi, A.; Kontarakis, Z.; Gerri, C.; Nolte, H.; Hölper, S.; Krüger, M. and Stainier, D.Y. 2015. Genetic compensation induced by deleterious mutations but not gene knockdowns. ***Nature***, 524(7564):230-3.

Sakakibara, S.; Imai, T.; Hamaguchi, K.; Okabe, M.; Aruga, J.; Nakajima, K.; Yasutomi, D.; Nagata, T.; Kurihara, Y.; Uesugi, S.; Miyata, T.; Ogawa, M.; Mikoshiba, K. and Okano, H. 1996. Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. ***Dev Biol***, 176(2):230-42.

Sauer, H.; Wartenberg, M. and Hescheler J. 2001. Reactive oxygen species as intracellular messengers during cell growth and differentiation. ***Cell Physiol Biochem***, 11(4):173-86.

Schriner, S.E.; Linford, N.; Martin, G.M.; Treuting, P.; Ogburn, C.E.; Emond, M.; Coskun, P.E.; Ladiges, W.; Wolf, N.; Van Remmen, H.; Wallace, D.C. and Rabinovitch, P.S. 2005. Extension of murine life span by overexpression of catalase targeted to mitochondria. ***Science***, 308(5730):1909-11.

Somel, M.; Guo, S.; Fu, N.; Yan, Z.; Hu, H.Y.; Xu, Y.; Yuan, Y.; Ning, Z.; Hu, Y.; Menzel, C.; Hu, H.; Lachmann, M.; Zeng, R; Chen, W. and Khaitovich, P. 2010. MicroRNA, mRNA, and protein expression link development and aging in human and macaque brain. ***Genome Res***, 20(9):1207-18.

Sweet, D.T.; Chen, Z.; Wiley, D.M.; Bautch, V.L. and Tzima, E. 2012. The adaptor protein Shc integrates growth factor and ECM signaling during postnatal angiogenesis. ***Blood,*** 119(8):1946-55.

Takahashi, S. and Zeydel, M. 1982. gamma-Glutamyl transpeptidase and glutathione in aging IMR-90 fibroblasts and in differentiating 3T3 L1 preadipocytes. ***Arch Biochem Biophys***, 214(1):260-7.

Terzibasi, E.; Lefrançois, C.; Domenici, P.; Hartmann, N.; Graf, M. and Cellerino, A. 2009. Effects of dietary restriction on mortality and age-related phenotypes in the short-lived fish Nothobranchius furzeri. ***Aging Cell***, 8(2):88-99.

Terzibasi, E.; Valenzano, D.R.; Benedetti, M.; Roncaglia, P.; Cattaneo, A.; Domenici, L. and Cellerino, A. 2008. Large differences in aging phenotype between strains of the short-lived annual fish Nothobranchius furzeri. ***PLoS One***, 3(12):e3866.

Tissenbaum, H.A. and Ruvkun, G. 1998. An insulin-like signaling pathway affects both longevity and reproduction in Caenorhabditis elegans. ***Genetics***, 148(2):703-17.

Tower, J. 2015. Programmed cell death in aging. ***Ageing Res Rev***, 23(Pt A):90-100.

Tozzini, E.T.; Baumgart, M.; Battistoni, G. and Cellerino, A. 2012. Adult neurogenesis in the short-lived teleost Nothobranchius furzeri: localization of neurogenic niches, molecular characterization and effects of aging. ***Aging Cell***, 11(2):241-51.

Tyner, S.D.; Venkatachalam, S.; Choi, J.; Jones, S.; Ghebranious, N.; Igelmann, H.; Lu, X.; Soron, G.; Cooper, B.; Brayton, C.; Park, S.H.; Thompson, T.; Karsenty, G.; Bradley, A. and Donehower, L.A. 2002. p53 mutant mice that display early ageing-associated phenotypes. ***Nature***, 415(6867):45-53.

Ungewitter, E. and Scrable, H. 2010. Delta40p53 controls the switch from pluripotency to differentiation by regulating IGF signaling in ESCs. ***Genes Dev,*** 24(21):2408-19.

Valdesalici, S. and Cellerino, A. 2003. Extremely short lifespan in the annual fish Nothobranchius furzeri. ***Proc Biol Sci***, 270 Suppl 2:S189-91.

Valenzano, D.R.; Benayoun, B.A.; Singh, P.P.; Zhang, E.; Etter, P.D.; Hu, C.K.; Clément-Ziza, M.; Willemsen, D.; Cui, R.; Harel, I.; Machado, B.E.; Yee, M.C.; Sharp, S.C.; Bustamante, C.D.; Beyer, A.; Johnson, E.A. and Brunet, A. 2015. The African Turquoise Killifish Genome Provides Insights into Evolution and Genetic Architecture of Lifespan. ***Cell***, 163(6):1539-54.

Valenzano, D.R.; Sharp, S. and Brunet, A. 2011. Transposon-Mediated Transgenesis in the Short-Lived African Killifish Nothobranchius furzeri, a Vertebrate Model for Aging. ***G3 (Bethesda),*** 1(7):531-8.

Valenzano, D.R.; Terzibasi, E.; Cattaneo, A.; Domenici, L. and Cellerino, A. 2006a. Temperature affects longevity and age-related locomotor and cognitive decay in the short-lived fish Nothobranchius furzeri. ***Aging Cell***, 5(3):275-8.

Valenzano, D.R.; Terzibasi, E.; Genade, T.; Cattaneo, A.; Domenici, L. and Cellerino, A. 2006b. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. ***Curr Biol,*** 16(3):296-300.

Ventura, A.; Luzi, L.; Pacini, S.; Baldari, C.T. and Pelicci, P.G. 2002. The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. ***J Biol Chem***, 277(25):22370-6.

Vogelstein, B.; Sur, S. and Prives, C. 2010. p53: The Most Frequently Altered Gene in Human Cancers. ***Nat Education***, 3(9):6

Webb, S.E. and Miller, A.L. 2007. Ca2+ signalling and early embryonic patterning during zebrafish development. ***Clin Exp Pharmacol Physiol***, 34(9):897-904.

Wells, P.G.; Bhuller, Y.; Chen, C.S.; Jeng, W.; Kasapinovic, S.; Kennedy, J.C.; Kim, P.M.; Laposa, R.R.; McCallum, G.P.; Nicol, C.J.; Parman, T.; Wiley, M.J. and Wong, A.W. 2005. Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species. ***Toxicol Appl Pharmacol***, 207(2 Suppl):354-66.

Wulliman, M.F. and Knipp, S. 2000. Proliferation pattern changes in the zebrafish brain from embryonic through early postembryonic stages. ***Anat Embryol (Berl)***, 202(5):385-400.

Yamamoto, M.; Clark, J.D.; Pastor, J.V.; Gurnani, P.; Nandi, A.; Kurosu, H.; Miyoshi, M.; Ogawa, Y.; Castrillon, D.H.; Rosenblatt, K.P. and Kuro-o, M. 2005. Regulation of oxidative stress by the anti-aging hormone klotho. ***J Biol Chem***, 280(45): 38029-38034.

Yin, Y.; Stephen, C.W.; Luciani, M.G. and Fåhraeus, R. 2002. p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. ***Nat Cell Biol***, 4(6):462-7.

Zaccagnini, G.; Martelli, F.; Fasanaro, P.; Magenta, A.; Gaetano, C.; Di Carlo, A.; Biglioli, P.; Giorgio, M.; Martin-Padura, I.; Pelicci, P.G. and Capogrossi, M.C. 2004. p66ShcA modulates tissue response to hindlimb ischemia. ***Circulation***, 109(23):2917-23.

Zhou, S.; Chen, H.Z.; Wan, Y.Z.; Zhang, Q.J.; Wei, Y.S.; Huang, S.; Liu, J.J.; Lu, Y.B.; Zhang, Z.Q.; Yang, R.F.; Zhang, R.; Cai, H.; Liu, D.P. and Liang, C.C. 2011. Repression of P66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction. ***Circ Res***, 109(6):639-48.

PART II

List of published papers

Priami Chiara, De Michele Giulia, Cotelli Franco, Cellerino Alessandro, Giorgio Marco, Pelicci Pier Giuseppe and Migliaccio Enrica.

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

Aging and Disease, April 2015. Volume 6, Number 2, pp 95-108. IF 2016: 4.65

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 (H_2O_2 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].

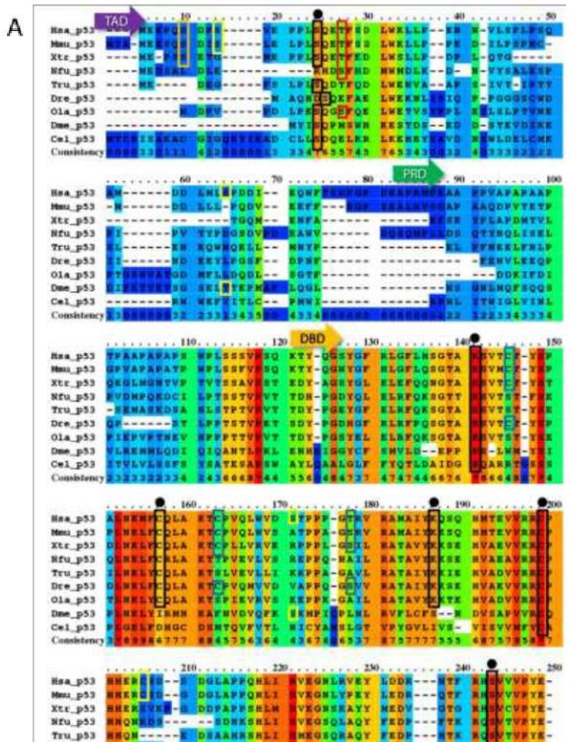
p53 (TRP53) is universally known for its role in tumor suppression, but its role in aging is still unclear. Analyzing the physiological functions of p53, it seems that this protein is both the major defense against cancer and the road that leads to aging. In this view, aging is considered an unavoidable 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].



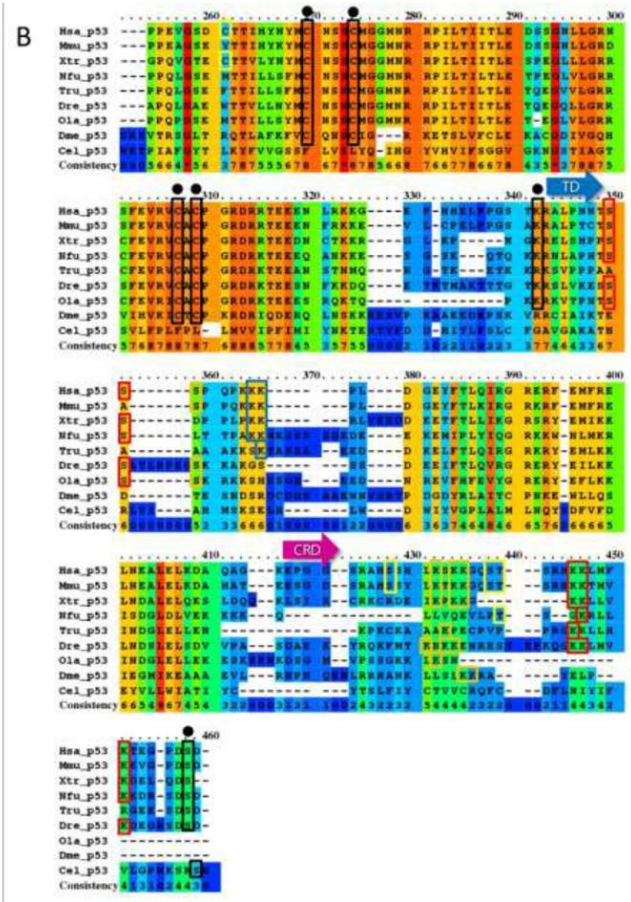


Figure 1. Multiple sequence alignment of p53 of different species. Predicted p53 orthologs were searched using online resources (UCSC Genome Browser, Ensembl Genome Browser and Nothobranchius furzeri transcriptome browser found at https://gen100.imb-jena.de/EST2UNI/nfintb/) and aligned using the online 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)

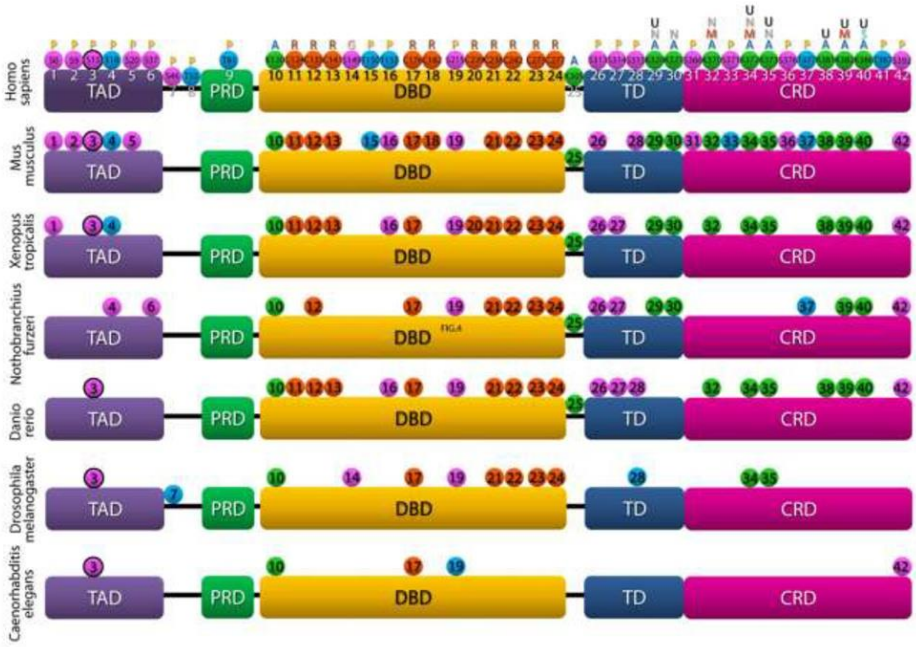


Figure 2. Modular organization of p53 orthologs of different vertebrate and invertebrate species. TAD, violet: Transactivation Domain; PRD, green: Proline Rich Domain; DBD, yellow: DNA Binding Domain, TD, dark blue: Tetramerization domain; CRD, fuchsia: C-terminal Regulatory Domain. Post-translational 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.

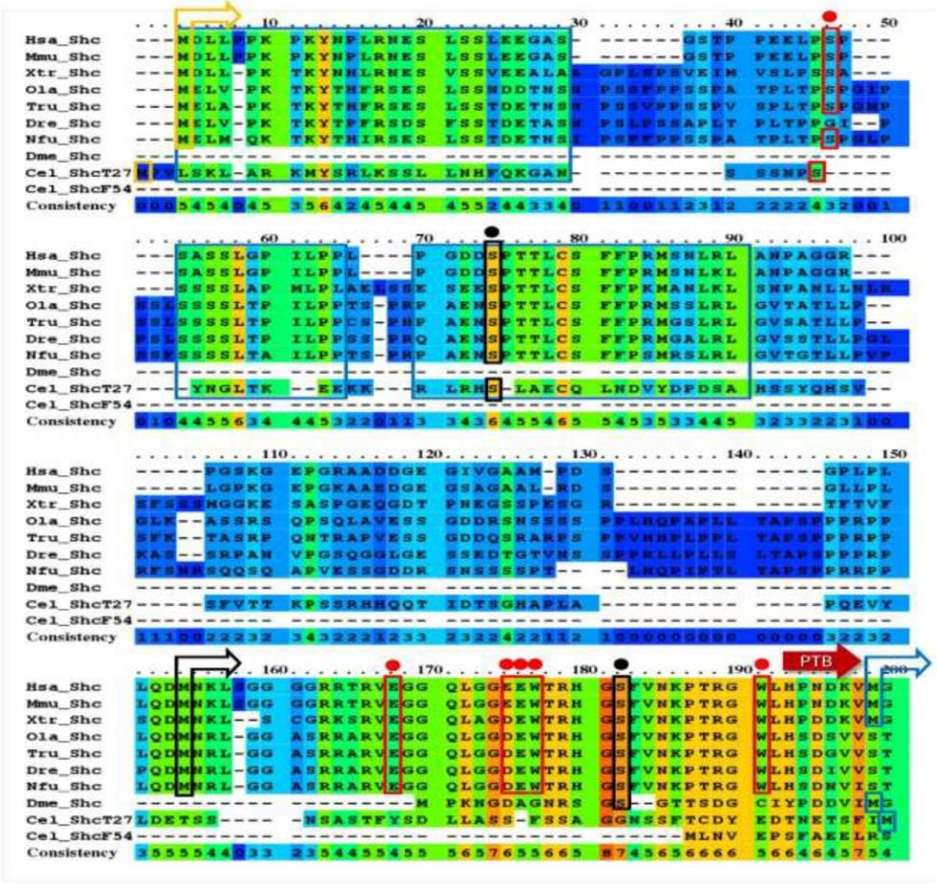
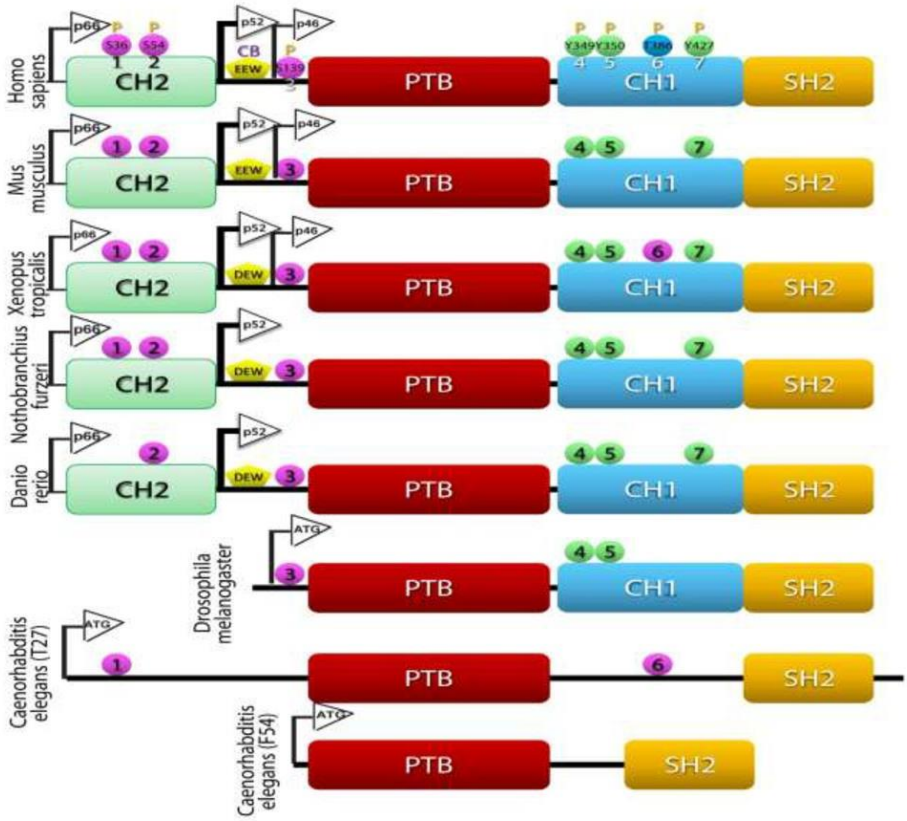


Figure 3. Multiple sequence alignment of p66Shc CH2 domain of different species. Predicted p66Shc orthologs were searched using online resources (UCSC Genome Browser, Ensembl Genome Browser and Nothobranchius furzeri transcriptome browser found at https://gen100.imb-jena.de/EST2UNI/nfintb/) and aligned 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

PART III

Manuscript in preparation

CRISPR/Cas9-mediated knock-out of Δ40p53 isoform in zebrafish

Priami C[1,2], Ruscitto F[2], De Michele G[2], Carra S[1], Deflorian G[3], Cellerino A[4], Giorgio M[2], Pelicci PG[2], Migliaccio E[2,*] and Cotelli F[1,*].

[1]University of Milan, Department of Biosciences, Via Celoria 26, 20133 Milan, Italy; [2]European Institute of Oncology, Department of Experimental Oncology, IFOM-IEO Campus, Via Adamello 16, 20139 Milan, Italy; [3]FIRC Institute of Molecular Oncology, IFOM-IEO Campus, Via Adamello 16, 20139 Milan, Italy; [4]Scuola Normale Superiore, Bio@SNS, Department of Neurosciences, Piazza dei Cavalieri 7, 56126 Pisa, Italy; *: equal contribution.

Summary

Δ40p53 is a short isoform of p53 lacking the first TAD and MDM2 binding domain. Δ40p53 is involved in stress response, in particular upon protein damage, and it forms tetramers with p53, orchestrating its transcriptional activity. Zebrafish gives the unique opportunity to generate a knock-out model of Δ40p53, since the targeted mutation of Δ40p53 ATG in mice impairs the stability of p53 transcript, leading to p53 partial loss of function. We report here the CRISPR/Cas9-mediated generation of three $\Delta40p53^{-/-}$ stable lines. $\Delta40p53^{-/-}$ embryos are not counterselected and do not show developmental abnormalities. We demonstrate that, in our model, the integrity of full-length p53 mRNA is maintained, as p53 mRNA levels throughout development. So far, this is the first attempt of knocking-out a specific isoform of a gene: with our findings, we propose the use of CRISPR/Cas9 technology to efficiently editing splicing isoforms without altering the expression of the other mRNAs transcribed from the same *locus*.

Our $\Delta 40p53^{-/-}$ model represents a valuable tool to dissect *in vivo p53* transcriptional activity in response to different stress agents and physiological development.

Introduction

Genome editing via the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology has given a new boost to zebrafish (*Danio rerio*) as a model to investigate vertebrate physiology and human pathologies. So far, targeted mutagenesis in zebrafish was only possible using Zinc-Finger Nucleases (ZFNs) or Transcription Activator-Like Effector Nucleases (TALENs). These two systems have several disadvantages (high costs, design limitations, procedure difficulties), so that site-directed genome editing in zebrafish has remained prohibitive for many laboratories (Hruscha *et al*., 2013). By contrast, the CRISPR/Cas9 system turned out to be extremely versatile, inexpensive and fast. Since its first applications in 2013 (Hwang *et al*., 2013a; Chang *et al*., 2013), several improvements to CRISPR/Cas9 technique have been made: (i) the use of Cas9 protein (also codon-optimized for zebrafish), instead of Cas9 mRNA, increases its mutagenic activity (Jao *et al*., 2013; Gagnon *et al*., 2014); (ii) co-injection of a donor plasmid with the short guide RNA (sgRNA) and the Cas9 mRNA results in site-directed knock-in of DNA cassettes into the genome, by homology-independent double-strand break (DSB) repair pathways (Auer *et al*., 2014; Kimura *et al*., 2014; Hisano *et al*., 2015); (iii) the use of a CRISPR/Cas9 vector system allows tissue-specific gene knock-out (De Santis *et al*., 2016). Here, we report the first application of the CRISPR/Cas9 technology to specifically knock-out a splicing isoform of a gene: the Δ40p53 isoform of p53. We also demonstrate that the small INDELs induced by Cas9 cleavage and Non-Homologous End-Joining

(NHEJ) repair do not produce aberrant splicing events and do not alter mRNA levels of the other p53 isoforms.

Known for being the "guardian of the genome", p53 is the most frequently mutated protein in human cancer (Vogelstein *et al*., 2010). p53 is active mainly in cell cycle control. Different levels of stress and damages to macromolecules can trigger different p53-dependent transcriptional responses that lead to transient cell cycle arrest, apoptosis and cell senescence (Bargonetti and Manfredi, 2002). However, it is still unclear how a single gene can orchestrate such a wide range of different pathways in response to a plethora of signals. This complexity can be explained by the finding that the p53 gene encodes several p53 isoforms through the use of internal promoters, internal ribosomal entry site (IRES) and alternative splicing. These mechanisms are responsible for the production of the 12 p53 isoforms already identified in humans, that are distinguishable for the presence of different domains and specifically regulated (Khoury and Bourdon, 2011).

In this study, we focus on a short isoform of p53, named Δ40p53, encoded by the p53 gene. In mammals, the canonical p53 transcript encodes Δ40p53 thanks to an IRES located in exon 4. This isoform lacks the first TAD, but retains the second TAD and all the p53 functional domains, maintaining the ability to form oligomers with the canonical p53 protein. Together with the first TAD, Δ40p53 lacks the MDM2 binding domain: it has been demonstrated that Δ40p53 is unable to complex with MDM2 avoiding ubiquitination and degradation (Courtois *et al*., 2002; Yin *et al*., 2002; Ungewitter and Scrable, 2010).

The Δ40p53 isoform determines p53 functions in stress response, aging and development. It is known that Δ40p53, thanks to its intact oligomerization domain, has the ability to form oligomers with p53,

determining its localization (Ghosh *et al*., 2004) and fine-tuning its transcriptional activity: (i) endoplasmic reticulum (ER) stress promotes the formation of Δ40p53/p53 oligomers leading to G2 arrest of the cell cycle (Bourougaa *et al*., 2010) (ii) the human Δ40p53 ortholog accumulates in response to genotoxic stress enhancing p21 expression (Powell *et al*., 2008); (iii) Δ40p53, in concert with p53 and via the activation of the aging protein $p66^{Shc}$, induces cell cycle arrest at G2/M transition specifically upon oxidative stress-induced cellular damage (Gambino *et al*., 2013). The formation of oligomers between Δ40p53 and p53 depends on protein levels of these two isoforms. Since Δ40p53 lacks the MDM2 binding domain, Δ40p53/p53 heterotetramers are more stable than p53 homotetramers, resulting in the modulation of p53 transcriptional activity (Ungewitter and Scrable, 2010).

It has been observed that Δ40p53 might play also a role in physiological development: in mESCs derived from wild-type mice, p53 and Δ40p53 levels are high and decrease together with the loss of pluripotency. High levels of Δ40p53 are observed in embryos immediately after implantation. Therefore, Δ40p53 has been considered an embryonic isoform of p53 (Ungewitter and Scrable, 2010). However, the role of Δ40p53 in normal somatic tissues is far from being elucidated *in vivo*.

So far, only full-length p53 and the short isoform Δ113p53 have been extensively investigated in zebrafish (Langheinrich *et al*., 2002; Berghmans *et al*., 2005; Lee *et al*., 2008; Guo *et al*., 2010; Gong *et al*., 2015). However, it has been discovered that a Δ40p53 ortholog is present in zebrafish. Davidson and colleagues isolated, through 3' RACE PCR, a 1.7kb transcript produced by alternative splicing of the p53 mRNA. This 1.7kb transcript shows an in-frame STOP codon that does not allow full-length p53 translation. It also shows a downstream ATG that encodes an N-terminal p53

isoform lacking the first TAD, as mammalian Δ40p53, replaced by an alternative N-terminal amino-acid sequence with no similarity with other zebrafish proteins. The microinjection of Δ40p53 mRNA in wild-type embryos is associated with severe developmental alterations and embryonic lethality. Interestingly, the same phenotype is obtained with the microinjection of human Δ40p53 ortholog. On the contrary, microinjection of Δ40p53 mRNA in $p53^{M214K}$ embryos (harbouring a dominant negative mutation of p53; Berghmans *et al*., 2005) does not lead to any morphological alteration, suggesting that, as previously shown in mammals, the Δ40p53 isoform acts only in cooperation with p53. Moreover, Δ40p53 expression increases after treatment with ionizing radiation. As its mammalian ortholog, Δ40p53 drives the overexpression of p21 in concert with p53 (Davidson *et al*., 2010). In conclusion, this work shows that zebrafish Δ40p53 recapitulates mammalian Δ40p53 functions: nevertheless, further efforts are necessary to investigate the role of Δ40p53 in development, under normal conditions and upon different stresses.

Remarkably, zebrafish gives the unique opportunity to generate a knock-out model of Δ40p53. Indeed, $\Delta40p53^{-/-}$ mice are not available, because murine Δ40p53 is produced by an ATG contained in p53 exon 4. The targeted mutation of Δ40p53 ATG impairs the stability of p53 transcript (Melis *et al*., 2011), leading to p53 partial loss of function. Considering this, mice do not represent a good model to investigate effects of Δ40p53 ablation.

We report here the generation and preliminary characterization of the first $\Delta40p53^{-/-}$ model, produced in zebrafish, that lacks specifically this isoform. Our $\Delta40p53^{-/-}$ has the unique advantage of retaining the intact full-length p53 mRNA and expression levels.

Materials and Methods

Zebrafish strains and maintenance

Zebrafish (*Danio rerio*) from wild-type AB and Δ40p53$^{-/-}$ strains were maintained and bred at the Zebrafish facility of the FIRC Institute of Molecular Oncology accordingly to the national guidelines (Italian decree "4 March 2014, n° 26"). All the experimental procedures were approved by the internal OPBA (Organismo Preposto al Benessere Animale) and the Italian Ministry of Health (Project n° 1021/16).

Target site selection

Target site was selected within the specific Δ40p53 sequence in p53 intron 2. The target site matches the sequence 5'G-N_{18}-NGG and has been checked for specificity in the zebrafish genome using the online tool "Optimized CRSPR Design Target Finder", developed by Dr Zhang laboratory at Massachusetts Institute of Technology (crispr.mit.edu:8079/; zebrafish genome reference: danRer7).

sgRNA template generation and transcription

In order to generate a template for sgRNA transcription, we used the DR274 plasmid (Addgene, #42250; Hwang *et al*., 2013a) and the cloning-free method already described in Burg *et al.*, 2016. A first PCR was performed using M13 forward primer (M13Fw, Burg *et al.*, 2016, Supplementary material) and a target-specific reverse primer ("CustomRv", Supplementary material), containing the T7 promoter sequence, the target sequence without the PAM where the initial T is substituted with a G to achieve the best performance by T7 polymerase, and a 3' sequence that anneals perfectly to DR274 plasmid. A 25 cycle PCR was run as follows: 15ng of plasmid template, 2U of Phusion High Fidelity DNA Polymerase

(NEB), 0.2mM dNTPs and 0.5µM each primer with an annealing temperature of 59°C. The 321bp amplicon was then purified with the Gel Extraction Kit (QIAGEN), according to the manufacturer instructions. The second PCR was performed using T7 forward primer ("T7Fw", Burg *et al.*, 2016, Supplementary material) and the sgRNA reverse primer ("sgRNARv", Burg *et al.*, 2016, Supplementary material); a 25 cycle PCR was run using 45ng of the previews amplicon as a template and an annealing temperature of 53°C. The 120bp amplicon was purified by QIAquick PCR Purification Kit (QIAGEN). The sgRNA was transcribed (2h at 37°C) using the MEGAshortScript (Ambion) according to the manufacturer instructions. The sgRNA was then DNAse treated (15' at 37°C with TURBO DNAse, Ambion) and precipitated with ammonium acetate/ethanol, according to the procedure described in Hruscha and Schmidt, 2014.

Cas9 protein expression and purification

The Cas9 protein was produced and purified by G. Ossolengo (Pepservice, Cogentech SCARL). Cas9 was expressed in bacteria as Cas9-MBP-His6 fusion protein using the pMJ806 plasmid (Addgene, #39312; Jinek *et al*., 2012) and purified by Immobilized Metal Affinity Chromatography (IMAC). The Cas9 was then separated from its fusion partners MBP-His6 by cleavage with the TEV enzyme followed by IMAC and re-purified with Ion Exchange Chromatography.

Microinjection and initial screening

Zebrafish zygotes were collected and microinjected through the chorion under an Olympus SZX9 using the Picospritzer III microinjector (Parker Instrumentation); microinjection needles were prepared pulling GC100F-10 glass capillaries (Harvard Apparatus) with a P-97 puller (Sutter Instrument).

Each zygote was microinjected directly into the single cell with 2nl of the following microinjection mix, that has been previously incubated 5 minutes at room temperature: 1mg/ml Cas9 protein, 50ng/µl sgRNA, 300mM NaCl, 1mg/ml dextran rhodamine (Sigma-Aldrich) in RNAse free water; thus, each embryo received 2ng of Cas9 protein and 100pg of sgRNA. In total, 300 embryos born from 5 AB couples were microinjected; 100 embryos were not microinjected and used as negative controls to evaluate lethality at 24hpf. 72hpf embryos were collected for single embryo gDNA extraction.

gDNA extraction and T7 assay

Single embryos or pools of 10 embryos were lysed over night at 55°C in lysis buffer (10mM Tris HCL pH=8, 1mM EDTA, 0.3% Tween-20, 0.3% NP-40) + 1mg/ml Proteinase K. gDNA was purified by phenol/chloroform extraction and precipitated with ammonium acetate/ethanol. 100ng of gDNA were then used as template to amplify the genomic region surrounding the Δ40p53 target site with specific primers ("Δ40g", Supplementary material). A touchdown PCR was run from an annealing temperature of 63°C to 57°C for 35 cycles. Then the PCR products was denatured/renatured to allow the formation of heteroduplex (mutated/wild-type PCR products) as follows: 95°C 10', -2°C/s to 85°C, -0.3°C/s to 25°C. After denaturation/renaturation, 500ng of the PCR product was digested with 2.5U of T7 endonuclease I (NEB) for 25' at 37°C. The formation of the heteroduplexes cleaved by T7 endonuclease was then assessed by electrophoresis on 2% agarose gel.

Genotyping of heterozygous fish

Adult fish born from the mating of wild-type and chimeric fish were genotyped by gDNA extraction from a 2mm portion of the caudal fin. gDNA extraction was performed as previously described. Amplification of the

genomic region surrounding the Cas9 target site and subsequent T7 assay was performed as previously described. PCR amplicons from fish resulted positive to T7 assay were cloned into the pCR 2.1-TOPO vector (Invitrogen) following the manufacturer instructions. Six clones for each fish were then sequenced and electropherograms analysed with Chromas lite software (Technelysium) to identify INDEL mutations. For the chosen $\Delta 40p53^{-/-}$ line, an assay was designed to genotype fish by a single PCR, where the two forward primers anneal specifically to the wild-type or the mutated allele ("wt" and "mut", Supplementary material).

Evaluation of full-length p53 expression

Three pools of 20 48hpf $\Delta 40p53^{-/-}$ embryos and two pools of respective siblings for each line were used for total RNA extraction with Qiazol Reagent (QIAGEN) according to the manufacturer instructions. After extraction, 30µg of total RNA were treated with 6U of TURBO DNase (Ambion) for 30' at 37°C and then column purified (Quick-RNA MiniPrep kit, Zymo Research). 1µg of total RNA was then retrotranscribed 1h at 42°C for cDNA synthesis using 0.5µg of a primer mix consisting in oligodT and random primers (Invitrogen) in 3:1 ratio, 160U of ImProm-II Reverse Transcriptase (Promega), 3mM $MgCl_2$, 0.3mM dNTPs and 20U of RNAse Inhibitor (NEB). Full-length p53 mRNA levels and presence of aberrant p53 transcripts were then investigated by RT-PCR using specific primer for full-length p53 ("FLp53", Table1) and β-actin ("βact", Table1), used as housekeeping gene. A 30-cycle touchdown PCR (annealing temperature decreasing from 63°C to 54°C) was run as follows: ~200ng of cDNA as template, 0.2µM each primer, 1.5mM $MgCl_2$, 0.3mM dNTPs and 2.5U of Taq polymerase (Qiagen).

Evaluation of p53, Δ40p53 and Δ113p53 expression during development

Three pools of 30-50 $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ embryos for each developmental stage were used for total RNA extraction as previously described. cDNA was then synthetized using 0.5µg of oligodT primers only. To investigate p53 expression, a two-step qPCR was run using ~100ng of cDNA as template, 0.2µM each primer and Fast SYBR Green Master Mix 2X (Applied Biosystems) in a total volume of 20µl. qPCR was run as follows: 2' at 95°C, then 3'' at 95°C and 30'' at 60°C for 40 cycles. p53 was discriminated from other isoforms using specific couples of primers ("qp53", Supplementary material); eEF1α-1a was used as housekeeping gene (HK). Results are presented as the ratio between as the ratio between $AvgCt_{HK}$ and $AvgCt_{p53}$.

In order to investigate Δ40p53 and Δ113p53 expression during development, we considered one pool of $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings for each developmental stage. We performed a preliminary semiquantitative RT-PCR using ~100ng of cDNA as template, 0.2 µM each primer, 0.3mM dNTPs, 1.5mM $MgCl_2$, 0.5U Taq Polymerase (Qiagen). RT-PCR was run as follows: 3' at 94°C, 1' at 94°C, 1' at 60°C, 30'' at 72°C for 30 cycles, 10' at 72°C. 5ul of each PCR reaction was run on 2% agarose gel. eEF1α-1a was used as HK. Primers for Δ40p53 and Δ113p53 are specific for each isoform ("qΔ40" and "qΔ113", Supplementary material).

Evaluation of transcriptional response to R-roscovitine treatment

As a pilot experiment, 50 $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings at gastrulation stage (6hpf) were treated with R-roscovitine (Calbiochem). R-roscovitine was dissolved in DMSO and then diluted in E3 water to a final concentration of 50µM or 100µM. The final concentration of DMSO was 0.1%; 0.1% DMSO in E3 water was used as control treatment. Before treatment,

embryos were dechorionated 5' at 37°C with 2mg/ml Pronase (Sigma-Aldrich) in E3 water and then rinsed in E3 water. R-roscovitine treatment lasted 16h. Embryos were collected at 24hpf and immediately frozen. RNA extraction and cDNA synthesis were performed as previously described. qPCR for p53, Δ40p53, Δ113p53, p21 and $p66^{Shc}$ was run as previously described. In this case, we used top1 as housekeeping gene. Primers for each gene are listed in the Supplementary material. The $2^{(-\Delta\Delta CT)}$ method was used for relative quantification of gene expression; values relative to untreated $\Delta40p53^{+/+}$ siblings were used as calibrators.

Results

Δ40p53, an N-terminally truncated isoform of p53, is produced in zebrafish by an alternative splicing mechanism. The full-length p53 ATG is located in exon 2: when the downstream intron is partially retained (as described in Davidson *et al.*, 2010), an in-frame STOP codon leads to premature end of p53 translation. The downstream Δ40p53 ATG, contained in the retained intron, is used for Δ40p53 translation: as a result, this isoform shows a unique N-terminal domain, but it lacks the first p53 transactivation domain (TAD) and shares all the other functional domains with p53 (Figure 1.A).

Selective ablation of the Δ40p53 isoform in zebrafish

In order to selectively knock-out this isoform without altering full-length p53 coding sequence (CDS), we looked for a Cas9 target site within the specific Δ40p53 CDS located in p53 intron 2. We found a Cas9 target site that matches the sequence 5'TG-N_{18}-NGG; this has been checked for specificity in the zebrafish genome (danRer7) using the online tool

"Optimized CRSPR Design Target Finder" (crispr.mit.edu:8079/; Dr Zhang lab - MIT). This target sequence is extremely close to Δ40p53 ATG (Figure 1.A). Since it has been shown that 5'GG- and 5'AG- are preferentially accepted as initiating bases for T7 RNA polymerase (Hwang *et al*., 2013a), we replaced the 5'T- with a 5'G- to generate the template for *in vitro* transcription of the sgRNA. This substitution is not expected to reduce efficiency or specificity of the Cas9 cleavage since the mismatch in position 1 of the target sequence is well tolerated by Cas9 (Anderson *et al*., 2015). We generated the sgRNA through a cloning-independent method that requires a custom oligonucleotide (containing the target sequence without the PAM) and the DR274 plasmid (Hwang *et al*., 2013a) as a template. Nested PCR was used to generate the template for *in vitro* transcription of the sgRNA.

A representation of the pipeline used to generate the three $\Delta40p53^{-/-}$ lines is represented in Figure 1.B. Briefly, we performed an initial screening on microinjected embryos to confirm that we successfully induced mutations within the Δ40p53 unique sequence (Figure 1.B.b). Then, we raised mosaic fish to sexual maturity and crossed them with wild-type animals (Figure 1.B.c). Following this, we screened the offspring to identify "carrier" mosaic fish, capable of transmitting the mutated allele to their offspring (Figure 1.B.d). Carriers were then crossed again with wild-type to generate $\Delta40p53^{+/-}$ fish: adult $\Delta40p53^{+/-}$ were then genotyped to characterize the mutated allele (Figure 1.B.e); heterozygous fish harbouring the same mutation were bred to generate monoallelic knock-out animals (Figure 1.B.f).

In detail, Cas9 protein and sgRNA were microinjected with a molar ratio of 2:1 into zebrafish zygotes directly into the one-cell. Survival of microinjected and control embryos was scored at 24hpf (Figure 1.B.a). We

observed no significant differences in mortality between microinjected and control embryos: 247/300 microinjected embryos (82.3%) and 84/100 control embryos (84%) were alive at 24hpf. No abnormalities were observed in microinjected embryos: thus, we conclude that the doses of Cas9 protein and sgRNA used are well tolerated by zebrafish embryos and do not affect early development. The efficiency of our protocol was tested at 72hpf: 10 microinjected and 4 control embryos were sacrificed for single embryo gDNA extraction and subsequent T7 assay. 10/10 microinjected embryos were positive to T7 assay; no false positive was detected in control embryos (Figure 2.A). Thus, we estimated that 100% of microinjected embryos could be considered mosaic, harbouring one or more mutations in a fraction of their somatic or germ cells. When mosaics are crossed with wild-type, only fish that harbour mutations in the germline are able to generate heterozygous embryos. These are considered "carriers". To identify carriers, we crossed 10 adult mosaic with wild-type fish. We then sacrificed one pool of 10 48hpf embryos born from each couple for T7 assay: considering that one couple resulted infertile, we found that 6/9 mosaic fish transmit the mutation to their offspring. Thus, we estimated that a 60% of heritability has been achieved (Figure 2.B). The offspring born from carrier mosaics was raised to adulthood. At the age of 2 months, adults were genotyped to characterize INDEL mutations: $\Delta 40p53^{+/-}$ fish were discriminated from wild-type by T7 assay: out of 30 animals born from 3 different mosaic fish, 17 resulted heterozygous, according to the expected frequency of 50%. We then characterize the INDEL mutations of $\Delta 40p53^{+/-}$ fish by molecular cloning and sequencing of PCR amplicons of the genomic region surrounding the Cas9 target site. We identified 9 different INDELS: fish born from the same mosaic parent can harbour different mutations, as previously observed

(Hwang *et al*., 2013b). A list of the mutations obtained is presented in Table 1.

We chose three different mutations to generate three monoallelic $\Delta 40p53^{-/-}$ lines: line 1 harbour a deletion of 11bp and an insertion of 4bp that determines a premature STOP codon occurring 12bp downstream Δ40p53 ATG; line 2 and 3 harbour a deletion of 7bp and an insertion of 5bp, respectively, that both cause a frameshift in Δ40p53 CDS. Offspring from the mating of $\Delta 40p53^{+/-}$ fish characterized by the same mutation resulted viable in all the three lines; at least 3 matings per couple were analysed and no developmental abnormalities was detected in the offspring of $\Delta 40p53^{+/-}$ fish. Lethality was assessed in the offspring of $\Delta 40p53^{+/-}$ fish and respective $\Delta 40p53^{+/+}$ siblings: no significant difference was scored for at least 3 matings per couple (data not shown). 20 embryos born from each heterozygous mating were collected for genotyping, in order to verify the presence of mutants: $\Delta 40p53^{-/-}$ embryos were present at the expected frequency (data not shown).

p53 mRNA integrity and levels are maintained in $\Delta 40p53^{-/-}$ embryos

Our goal is to selectively knock-out Δ40p53 without altering p53 expression levels. This is not feasible in mice, because murine Δ40p53 is produced by an ATG contained in p53 exon 4. The targeted mutation of Δ40p53 ATG impairs the stability of p53 transcript, leading to p53 partial loss of function (Melis *et al*., 2011). Considering that we altered intron 2 of p53 sequence, our first issue was to investigate the presence of aberrantly spliced p53 mRNAs. To reveal them, three pools of 20 48hpf $\Delta 40p53^{-/-}$ embryos and two pools of respective $\Delta 40p53^{+/+}$ siblings for each line were used for total RNA extraction and cDNA synthesis. Both $\Delta 40p53^{-/-}$ and $\Delta 40p53^{+/+}$ embryos used for this and the following experiments are the

offspring of $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ couples, born from $\Delta40p53^{+/-}$ fish, so that the contribution of WT Δ40p53 inherited by heterozygous females is excluded. We amplified full-length p53 cDNA: Fw primer is located in p53 exon 2, upstream to the mutated intron 2, while Rv primer is located on p53 STOP codon. We detected only one band in PCR-amplified cDNA from $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ sibling embryos of each line. Sequencing of PCR amplicons confirmed that only correctly spliced full-length p53 cDNA has been amplified. Levels of p53 expression were roughly quantified using β-actin as a control: p53 expression resulted unaltered between $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ sibling embryos of each line and among lines (Figure 2.C).

In line 1, a STOP codon occurs 12bp downstream Δ40p53 ATG and a 4aa peptide is produced: since in the other 2 lines a longer peptide of ~100aa, with possible unexpected effects, is produced, we chose line 1 for further investigations. We asked whether p53 mRNA levels are maintained between $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings during development. p53 expression was quantified by Real-Time RT-PCR at 9 developmental stages: late mid-blastula transition (when embryo counts 1000 cells), mid-gastrulation (50% epiboly), early somitogenesis (when embryo counts 1-3 somites), late somitogenesis (15-18 somites), 24hpf, 48hpf, 72hpf, 96hpf and 120hpf. As Figure 2.D shows, p53 mRNA levels result (i) comparable between $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ sibling embryos and (ii) higher at 1000-cell stage, decreasing from gastrulation and staying stable until 120hpf (Figure 2.D).

In conclusion, our $\Delta40p53^{-/-}$ model shows the unique opportunity to study the role of Δ40p53 in a context of intact p53 expression.

Δ40p53 and Δ113p53 expression is regulated during development and mRNA levels are maintained in Δ40p53$^{-/-}$ embryos

We asked whether the absence of Δ40p53 affects the expression of Δ40p53 itself and the other short isoform of p53, Δ113p53, that has been already characterized in zebrafish (Chen and Peng, 2009) as the ortholog of the human Δ133p53 isoform. We performed a preliminary experiment through semiquantitative RT-PCR on a pool of Δ40p53$^{-/-}$ and Δ40p53$^{+/+}$ embryos collected at the 9 already mentioned developmental stages. We found that: (i) mRNA levels of both the p53 isoforms are comparable between Δ40p53$^{-/-}$ and Δ40p53$^{+/+}$ embryos; (ii) Δ40p53 expression is higher at late mid-blastula transition and gastrulation stages, decreasing rapidly from early somitogenesis until 120hpf, when it becomes barely detectable; (iii) Δ113p53 expression is low at late mid-blastula transition, reaching a peak at gastrulation and decreasing slightly until 120hpf (Figure 3.A). These results suggest that expression levels of the short isoform Δ113p53 are unaltered in our model.

The CDK inhibitor R-roscovitine induces Δ40p53 expression

Since our aim is to investigate the role of Δ40p53 in the orchestration of p53-dependent transcriptional response to different stress agents, we performed a pilot experiment using the CDK inhibitor R-roscovitine. This drug has been already proved to be a potent p53-activating agent in zebrafish: the experimental conditions for its use are well set up in this model organism (Langheinrich *et al*., 2002, Lee *et al*., 2008, Guo *et al*., 2010). Δ40p53$^{-/-}$ and Δ40p53$^{+/+}$ embryos at mid-gastrulation stage (6hpf, when Δ40p53 expression is higher - Figure 3.A) were treated over-night with 50μM and 100μM R-roscovitine, similarly to Langheinrich *et al*., 2002. Both Δ40p53$^{-/-}$ and Δ40p53$^{+/+}$ siblings showed, at 24hpf: (i) moderate spine

curvature upon 50µM R-roscovitine treatment; (ii) severe developmental retardation and extended necrosis upon 100µM R-roscovitine treatment (data not shown).

We asked (i) whether Δ40p53 expression is triggered by R-roscovitine, which is already known to increase Δ113p53 expression (Guo *et al*., 2010), and (ii) whether Δ40p53 has a role in the transcriptional response to R-roscovitine. Therefore, we analysed changes in mRNA levels of *p53*, *Δ40p53*, *Δ113p53*, *p21* and $p66^{Shc}$ genes, by Real-Time RT-PCR. Our pilot experiment shows that: (i) Δ40p53 expression is induced by R-roscovitine in a dose-dependent manner; (ii) the increase in Δ40p53 expression is higher in $\Delta40p53^{+/+}$ siblings; (iii) p53 mRNA levels are not altered by R-roscovitine, as previously reported (Guo *et al*., 2010); (iii) p21 expression is poorly activated by R-roscovitine, but its activation resulted higher in $\Delta40p53^{-/-}$ embryos treated at the lower dose; (iv) Δ113p53 mRNA levels dramatically increases upon treatment in both $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings; (v) $p66^{Shc}$ expression does not vary in $\Delta40p53^{+/+}$ siblings, but it increases in $\Delta40p53^{-/-}$ embryos in a dose-dependent manner (Figure 3.B/C). This pilot experiment shows that R-roscovitine is able to trigger Δ40p53 expression and that the absence of Δ40p53 might influence the transcriptional response to this agent.

Discussion

The N-terminally truncated isoform of p53, named Δ40p53, is involved in stress response in mammals (Bourougaa *et al*., 2010; Powell *et al*., 2008; Gambino *et al*., 2013) and zebrafish (Davidson *et al*., 2010). It has also been reported a role for Δ40p53 in development, since this isoform is highly expressed in embryonic stem cells (ESCs), where it controls the switch from pluripotency to differentiation (Ungewitter and Scrable, 2010). To deeply investigate the role of Δ40p53 in normal development and in stress response,

we generated the first knock-out model of Δ40p53 in zebrafish. $\Delta40p53^{-/-}$ mice are not available, because murine Δ40p53 is produced by an ATG contained in p53 exon 4. The targeted mutation of Δ40p53 ATG impairs the stability of p53 transcript, leading to p53 partial loss of function (Melis *et al*., 2011). For this reason, and for its extensive use in developmental biology studies, we chose zebrafish as a model. In this organism, Δ40p53 ortholog recapitulates several Δ40p53 functions previously investigated in mammals (Davidson *et al*., 2010).

We took advantage of a Cas9 target site located in the Δ40p53-unique sequence (Figure 1.A) to generate three different monoallelic $\Delta40p53^{-/-}$ lines (Table 1). These lines differ for the mutation that disrupts Δ40p53 coding sequence (CDS): line 1 harbours a deletion of 11bp and an insertion of 4bp that determines a premature STOP codon occurring 12bp downstream Δ40p53 ATG; line 2 and 3 harbour a deletion of 7bp and an insertion of 5bp, respectively, that cause a frameshift in Δ40p53 CDS. In each line, $\Delta40p53^{-/-}$ embryos are viable, develop normally and they are not counterselected in the offspring born from $\Delta40p53^{+/-}$ fish. This was expected, as both $p53^{-/-}$ ($p53^{M214K}$, harbouring a dominant negative mutation of p53; Berghmans *et al*., 2005) and $\Delta113p53^{-/-}$ (Gong *et al*., 2015) grow normally under standard conditions. Both $p53^{-/-}$ and $\Delta113p53^{-/-}$ embryos show instead reduced transcriptional response to stress: upon γ-radiation, $p53^{M214K}$ embryos fail to undergo apoptosis, while $\Delta113p53^{-/-}$ are unable to repair DNA double strand breaks.

Since (i) CRISPR/Cas9 technology has been shown to occasionally cause aberrant splicing variants in targeted genes (Kapahnke *et al*., 2016) and (ii) mutations occurring in introns can lead to aberrantly-spliced mRNA production (Busslinger *et al*., 1981; Jiang *et al*., 2000; Davit-Spraul *et al*., 2014), we asked whether the small INDELs produced in p53 intron 2 cause

aberrant splicing events of full-length p53 mRNA. We demonstrated that in our three lines, only the correctly-spliced p53 mRNA is produced (Figure 2.C). These data indicate that any further analyses will be not compromised by the presence of unwanted "p53-like" products.

We then chose line 1 for further investigations, because in this case a 4aa-long peptide is produced, that is not expected to show unwanted or toxic effects. In order to attribute every phenotypic or molecular trait observed to the only lack of Δ40p53, we investigated p53 mRNA levels in $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings at 9 different developmental stages, from late mid-blastula transition, when embryo counts 1000 cells, to 120hpf. Our results demonstrate that p53 mRNA levels are comparable between $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings and that the absence of Δ40p53 does not act on p53 expression, at least in normal conditions. Moreover, p53 results highly expressed at 1000-cell stage; after gastrulation, its mRNA levels decrease and stay stable until 120hpf (Figure 2.D). This last finding is in agree with literature data: p53 expression is higher at very early developmental stages in mouse (Ungewitter and Scrable, 2010) and xenopus (Tchang *et al*., 1993) embryos.

Δ40p53 is considered an embryonic isoform of p53, beingly mostly expressed immediately after implantation in the mouse embryo (Ungewitter and Scrable, 2010). Therefore, we asked how Δ40p53 is regulated during zebrafish development. We also analysed Δ113p53 mRNA levels in $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings, since the lack of Δ40p53 might affect transcriptional levels of Δ113p53. This isoform, in fact, is transcribed by its own promoter located in p53 intron 5 (Chen and Peng, 2009), and it is known that, in both human and zebrafish, p53 itself regulates the expression of this short isoform (Aoubala *et al*., 2011; Guo *et al*., 2010). We obtained preliminary data by semiquantitative RT-PCR showing that Δ40p53 is

mostly expressed until gastrulation stage. On the contrary, Δ113p53 expression is very low at 1000-cell stage, it reaches a peak at gastrulation and decreases slightly until 120hpf. Moreover, mRNA levels of these two isoforms are comparable between $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings (Figure 3.A). These findings suggest that: (i) the two short p53 isoforms might play different roles in embryonic development; (ii) their mRNA levels are not altered by the absence of Δ40p53, at least in normal conditions. However, these data have to be confirmed by Real-Time RT-PCR assays and western blot, in order to demonstrate that expression of the three p53 isoforms is not altered also at a protein level. Finally, the fact that Δ40p53 mRNA levels are maintained in the $\Delta40p53^{-/-}$ line is not surprising. The nonsense-mediated decay (NMD) mechanism, which is responsible for the degradation of mRNAs harbouring premature non-sense mutations, is conserved in zebrafish (Wittkopp *et al.*, 2009), but it has been reported that only some CRISPR/Cas9-mediated frameshift mutations lead to a reduced level of targeted-gene mRNA (Li *et al.*, 2014; Mehrabian *et al.*, 2014). Most importantly, it has been demonstrated that mRNA harbouring premature STOP codons located in proximity to the initiation codon escape NMD (Perrin-Vidoz *et al.*, 2002; Inacio *et al.*, 2004). Given that the premature STOP codon harboured by the $\Delta40p53^{-/-}$ line is located only 9 nucleotides downstream to the ATG, the mutated Δ40p53 mRNA is likely to escape NMD.

The major advantage of our $\Delta40p53^{-/-}$ model resides in the possibility of investigating the role of Δ40p53 in stress response *in vivo*. Zebrafish embryos are a rapid tool to test a wide range of different stress agents. As a pilot assay, we decided to treat $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings with R-roscovitine, a cycline-dependent kinase inhibitor that preferentially inhibits CDK2, CDK7 and CDK9. R-roscovitine is a potent p53 activating agent,

being responsible for inhibition of MDM2 expression (Lu *et al*., 2011). We chose R-roscovitine because it has been extensively used by several groups to induce p53-dependent response in zebrafish. It is known that an over-night treatment with 50µM R-roscovitine induces: (i) developmental abnormalities (Langheinrich *et al*., 2002); (ii) p53 stabilization (Lee *et al*., 2008); (iii) overexpression of several p53 target genes, including *Δ113p53*, *p21* and *puma* (Guo *et al*., 2010). Moreover, R-roscovitine triggers mitochondrial apoptosis: this drug induces a post-translational modification of p53 that modulates its transcriptional activity, leading to mitochondrial membrane depolarization and releasing of cytochrome c (Wasierska-Gadek *et al.*, 2005).

We obtained preliminary data by treating $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings at gastrulation stage with 50µM and 100µM R-roscovitine and analysing changes in mRNA levels of *p53*, *Δ40p53*, *Δ113p53*, *p21* and $p66^{Shc}$ by qPCR. We included $p66^{Shc}$ since, interestingly, it takes part in p53-dependent apoptosis upon different stress (Migliaccio *et al*., 1999; Gambino *et al*., 2013). $p66^{Shc}$ triggers mitochondrial swelling through cytochrome c oxidation (Giorgio *et al.*, 2005), as R-roscovitine does by modulating p53 transcriptional activity. Furthermore, recent evidence suggests that p53 might directly enhance $p66^{Shc}$ expression by binding to its promoter upon treatment with the cell cycle progression inhibitor cyclophosphamide (Xiong *et al.*, 2016).

Our pilot experiment shows that Δ40p53 expression is induced by R-roscovitine: in particular, Δ40p53 appears to be strongly increased by treatment with the higher dose. Δ40p53 expression is higher in $\Delta40p53^{+/+}$ then in $\Delta40p53^{-/-}$ embryos: this might be due to the absence of Δ40p53 protein and the lack of a hypothetic positive-feedback mechanism involved in Δ40p53 transcriptional regulation. p53 mRNA levels are stable, while

Δ113p53 expression dramatically increases, as previously shown (Guo *et al*., 2010) in both $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ embryos. p21 expression does not increase in a dose-dependent manner: this might be due to the fact that the higher R-roscovitine dose is associated with a very severe phenotype and, thus, with apoptosis and not cell senescence. $p66^{Shc}$ expression does not vary in $\Delta40p53^{+/+}$ sibling, but surprisingly it increases in $\Delta40p53^{-/-}$ embryos in a dose-dependent manner. These preliminary data suggest the intriguing hypothesis that the absence of phenotypic differences between treated $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings might be the result of a mechanism of genetic compensation. Since Δ40p53 is upregulated by R-roscovitine treatment, it might be involved in the transcriptional response to this stress agent. The absence of Δ40p53 might be compensated by the upregulation of other p53-related pro-senescence or pro-apoptotic factors, such $p66^{Shc}$.

Δ40p53 has been shown to be involved, *in vitro,* in the transcriptional regulation of a mitotic signature whose downregulation triggers cell cycle arrest specifically upon protein damage (Bourougaa *et al*., 2010; Gambino *et al*., 2013). We will evaluate *in vivo*: (i) the contribution of Δ40p53 in response to protein damage; (ii) whether Δ40p53 is involved also in DNA damage response and (iii) whether and how the transcriptional response to protein and DNA damage differs in terms of genes involved in a $\Delta40p53^{-/-}$ context. To address our questions, $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ siblings will be treated with known p53-activating agents, such as UV (Zhao *et al*., 2012) or doxorubicin (Henriques *et al*., 2013), to induce primarily DNA damage, and with H_2O_2 and or the proteasomal inhibitor MG132 (Bretaud *et al*., 2007), to induce primarily protein damage. The transcriptome of $\Delta40p53^{-/-}$ will be investigated by RNA-sequencing analysis.

Figure 1

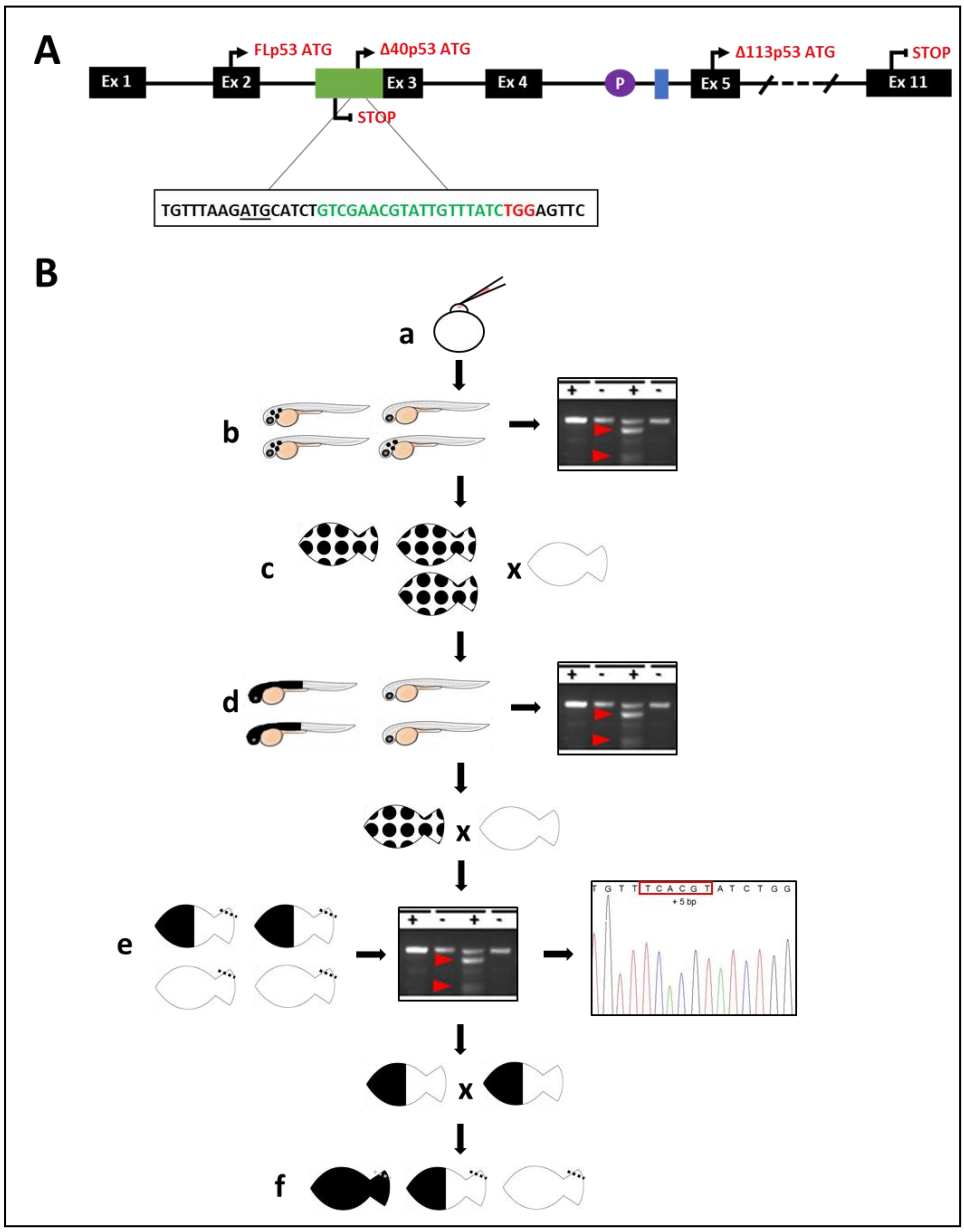

A
FLp53 ATG
Δ40p53 ATG
Δ113p53 ATG
STOP
Ex 1
Ex 2
Ex 3
Ex 4
P
Ex 5
Ex 11
STOP
TGTTTAAGATGCATCTGTCGAACGTATTGTTTATCTGGAGTTC
B
a
b
c
x
d
x
e
x
f

Figure 1. Pipeline used to generate the Δ40p53$^{-/-}$ lines. A. Zebrafish p53 genomic *locus* consists of 11 exons. Full-length p53 ATG is located in exon 2. When the following intron is partially retained (as described in Davidson *et al.*, 2010, green box), a STOP codon causes premature end of FLp53 translation. The downstream Δ40p53 ATG is used for Δ40p53 translation: this isoform has a unique N-terminal domain but lacks the first p53 transactivation domain. It shares all the other functional domains with p53. An internal promoter contained in the fourth intron (violet circle) drives the transcription of Δ113p53 isoform, whose 5'UTR is located in intron 4 (blue box) and in exon 5, where Δ113p53 ATG is located. All the p53 isoforms end with the STOP codon located in exon 11. To specifically knock-out the Δ40p53 isoform, we found a Cas9 target site (in green, PAM sequence in red). The target site is very close to Δ40p53 ATG (underlined): this determines the complete loss of function of the mutated Δ40p53 allele. **B.** Wild-type zebrafish embryos are microinjected with the Cas9 protein/sgRNA complex directly into the one-cell **(a)**. gDNA is extracted from single microinjected embryos to detect the presence of INDEL mutations via PCR amplification of the Δ40p53 *locus* and T7 assay **(b)**. Microinjected embryos are maintained until sexual maturity: mosaic fish harbour mutations in a fraction of their cells. Mutations are inheritable only if present in the germline of mosaic fish, that can be so considered «carriers». When mosaics are crossed with wild-type, only fish that harbour mutations in the germline are able to generate heterozygous embryos. To select carriers, pools of 10 embryos born from the mating of mosaic with wild-type fish are used for gDNA extraction, PCR amplification of the Δ40p53 *locus* and T7 assay **(c)**. Carrier mosaic fish are crossed again with wild-type and their offspring is raised to adulthood **(d)**. Fish born from the mating of carrier mosaics and wild-type are expected to be heterozygous with a frequency of 50%. They are subjected to gDNA extraction, PCR amplification of the Δ40p53 *locus* and T7 assay to identify heterozygosity. PCR amplicons from Δ40p53$^{+/-}$ fish are cloned and sequenced to characterize the mutations **(e)**. Δ40p53$^{+/-}$ fish harbouring the same mutation are bred. The resulting offspring consists of Δ40p53$^{+/+}$, Δ40p53$^{+/-}$ and Δ40p53$^{-/-}$ fish. Fish are raised to adulthood for genotyping. Knock-out fish harbouring the same mutation are bred to generate homozygous Δ40p53$^{-/-}$ lines **(f)**.

Figure 2

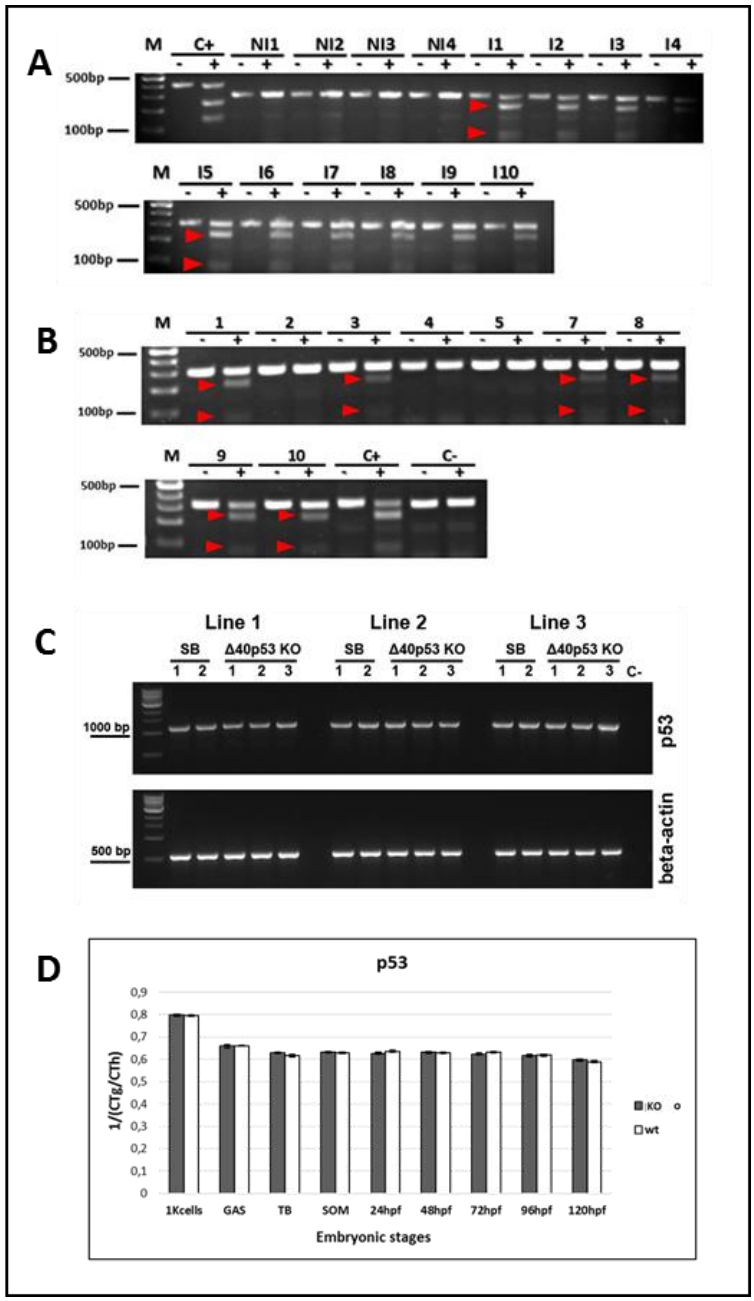

A
M
C+
NI1
NI2
NI3
NI4
I1
I2
I3
I4
500bp
100bp
M
I5
I6
I7
I8
I9
I10
500bp
100bp
B
M
1
2
3
4
5
7
8
500bp
100bp
M
9
10
C+
C-
500bp
100bp
C
Line 1
Line 2
Line 3
SB
Δ40p53 KO
1 2 1 2 3
C-
1000 bp
p53
500 bp
beta-actin
D
p53
1/(CTg/CTh)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
KO
wt
1Kcells
GAS
TB
SOM
24hpf
48hpf
72hpf
96hpf
120hpf
Embryonic stages

Figure 2. A. Efficiency of Cas9 specific cleavage upon microinjection of Cas9 protein/sgRNA: 10 embryos, that were microinjected directly into the one-cell with the Cas9 protein/sgRNA complex, and 4 control embryos were used for gDNA extraction. The genomic region surrounding the Cas9 target site was amplified by PCR and PCR product was denatured/renatured and digested with T7 endonuclease 1 (I: microinjected embryos; NI: control embryo; -: untreated PCR product; +: treated PCRproduct; C+: heterozygous gDNA was used as positive control). **B. Heritability of INDEL mutations.** 10 mosaic fish were crossed with wild-type and one pool of 10 embryos per couple was used for gDNA extraction. The genomic region surrounding the Cas9 target site was amplified by PCR and PCR product was denatured/renatured and digested with T7 endonuclease 1 (-: untreated PCR product; +: treated PCR product; C+ and C-: gDNA from mosaic and wild-type fish were used as positive and negative controls). **C. Full-length p53 expression in the three Δ40p53$^{-/-}$ lines.** Three pools of 20 48hpf Δ40p53$^{-/-}$ embryos and two pools of respective siblings for each line were used for total RNA extraction and cDNA reverse transcription. The presence of aberrant p53 transcripts were investigated by RT-PCR of full-length p53. β-actin was used to assess the quality of cDNA and to allow rough quantification of p53 mRNA levels. Only the band corresponding to the correctly spliced full-length p53 mRNA is present in both knock-outs and siblings of each line. mRNA levels of p53 are comparable between knock-outs and siblings and among the three lines. **D. p53 qPCR expression levels in line 1 at different developmental stages.** Three pools of 30-50 embryos for each developmental stage were used for total RNA extraction and cDNA reverse transcription. We considered 9 stages: late mid-blastula transition (1000-cell embryo), gastrulation (Ga, 50% epiboly), early somitogenesis (TB), late somitogenesis (So), 24hpf, 48hpf, 72hpf, 96hpf and 120hpf. Real-Time RT-PCR with p53-specific primers (that exclude the amplification of Δ40p53 and Δ113p53) shows that p53 mRNA levels are comparable between Δ40p53$^{-/-}$ and Δ40p53$^{+/+}$ siblings at each developmental stage.

Table 1

	Sequence	InDel	P.L.	N
WT	ATGCATCTGTCGAACGTATTGTTTATCTGGAGTTCTTTGTTTGAGCTTCA			
MT 1	ATGCATCTGTCGAACGTATTGTTTACGAACGTATCTGGAGTTCTTTGTTT	+8	107 aa	
MT 2	ATGCATCTGTCGAACGTATTGT--ACGTCGAACGTATCTGGAGTTCTTTG	+9	*in frame*	
MT 3	ATGCATCTGTCGAACGTATTGTCTGGGAGTTCTGGAGTTATCTGGAGTTC	+15	*in frame*	
MT 4	ATGCATCTGTCGAACGTATTGTTCGACAGATGCAATACGTATCTGGAGTT	+16	19 aa	
MT 5	ATGCATCTGTCGAACGTA-------TCTGGAGTTCTTTGTTTGAGCTTCA	-7	102 aa	[x2]
MT 6	ATGCATCTGTCGAACGTATTGTTTTATTGATCTGGAGTTCTTTGTTTGAG	+5	9 aa	[x2]
MT 7	ATGCATCTGT-------ATTGAACGTCTGGAGTTCTTTGTTTGAGCTTCA	-7	4 aa	[x3]
MT 8	ATGCATCTGTCGAACGTATTGTT----TGGAGTTCTTTGTTTGAGCTTCA	-4	103 aa	[x2]
MT 9	ATGCATCTGTCGAACGTATTGTTTCACGTATCTGGAGTTCTTTGTTTGAG	+5	106 aa	[x4]

Table 1. List of the INDEL mutations characterized in Δ40p53$^{+/-}$ fish. INDEL mutations were characterized by molecular cloning and following sequencing of the PCR products obtained by amplification of the Δ40p53 genomic region surrounding the Cas9 target site. Red dots: established Δ40p53$^{-/-}$ lines; red box: the Δ40p53$^{-/-}$ line used for studies on gene expression and stress response.

Figure 3

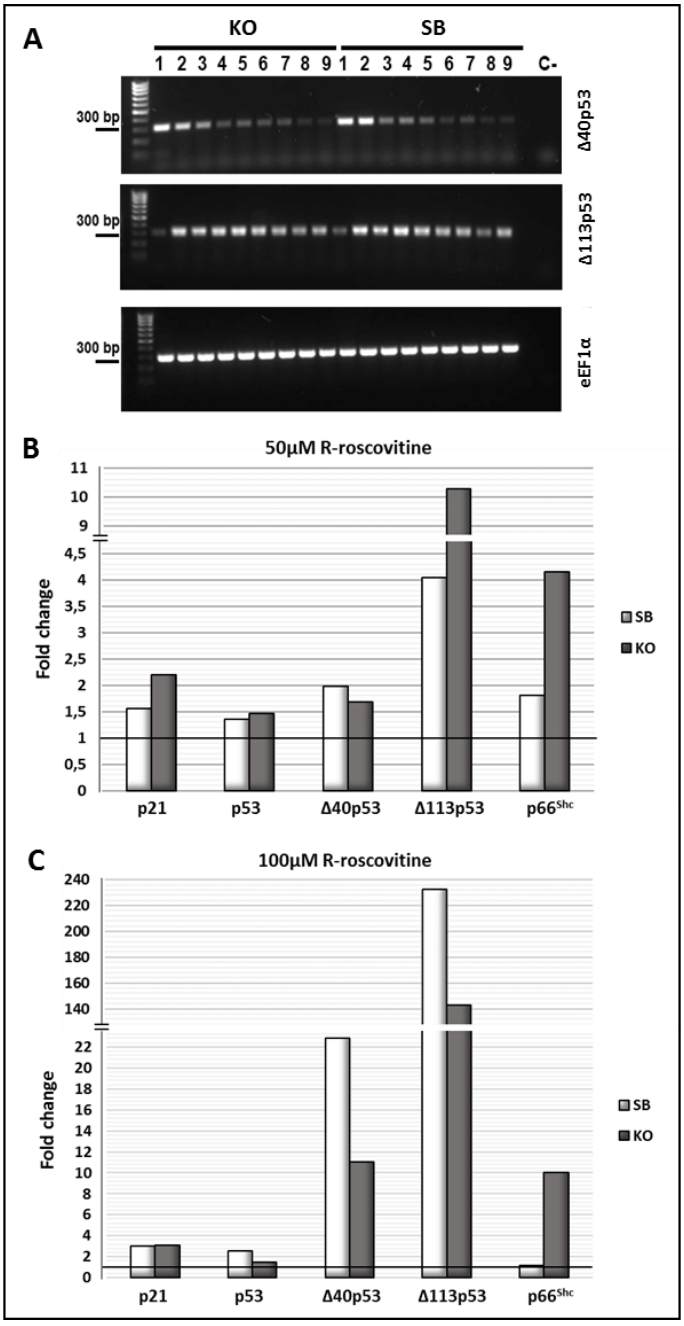

A
KO
SB
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 C-
300 bp
Δ40p53
300 bp
Δ113p53
300 bp
eEF1α
B
50μM R-roscovitine
Fold change
11
10
9
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
p21
p53
Δ40p53
Δ113p53
p66Shc
SB
KO
C
100μM R-roscovitine
Fold change
240
220
200
180
160
140
22
20
18
16
14
12
10
8
6
4
2
0
p21
p53
Δ40p53
Δ113p53
p66Shc
SB
KO

Figure 3. A. Δ40p53 and Δ113p53 mRNA levels in line 1 at different developmental stages. One pool of 30-50 embryos for each developmental stage was used for total RNA extraction and cDNA reverse transcription. We considered 9 stages. 1: late mid-blastula transition (1000-cell embryo); 2: gastrulation (50% epiboly); 3: early somitogenesis; 4: late somitogenesis; 5: 24hpf; 6: 48hpf; 7: 72hpf; 8: 96hpf; 9: 120hpf. Semiquantitative RT-PCR with primers specific for each isoform shows that mRNA levels of both Δ40p53 and Δ113p53 are comparable between $\Delta40p53^{-/-}$ and $\Delta40p53^{+/+}$ embryos. Δ40p53 expression rapidly decreases after gastrulation, while Δ113p53 is barely detectable at 1000-cell stage; its expression reaches a peak at gastrulation and then slightly decreases. **B. Real-Time RT-PCR fold changes of p53-target genes in $\Delta40p53^{-/-}$ embryos upon 50μM R-roscovitine treatment.** $\Delta40p53^{-/-}$ (KO) and $\Delta40p53^{+/+}$ (SB) embryos at gastrulation stage were treated over-night with 50μM R-roscovitine. They were collected at 24hpf for total RNA extraction and cDNA reverse transcription. Δ40p53 expression is triggered by R-roscovitine. Interestingly, $p66^{Shc}$ expression is activated only in $\Delta40p53^{-/-}$ embryos. As previously shown, p53 expression is stable, while Δ113p53 is strongly activated in both KO and SB embryos. *Topoisomerase 1* was used as housekeeping gene. The $2^{(-\Delta\Delta Ct)}$ method was used for relative quantification of gene expression. Values obtained for untreated SB embryos were used as calibrators for each gene. **C. Real-Time RT-PCR fold changes of p53-target genes in $\Delta40p53^{-/-}$ embryos upon 100μM R-roscovitine treatment.** $\Delta40p53^{-/-}$ (KO) and $\Delta40p53^{+/+}$ (SB) embryos at gastrulation stage were treated over-night with 100μM R-roscovitine and then collected as previously described. Δ40p53 expression is strongly induced by R-roscovitine and it results increased in $\Delta40p53^{+/+}$ embryos. Again, $p66^{Shc}$ expression results strongly activated only in $\Delta40p53^{-/-}$ embryos. p53 expression is stable, p21 expression is poorly increased, while Δ113p53 is dramatically activated in both KO and SB embryos. *Topoisomerase 1* was used as housekeeping gene. The $2^{(-\Delta\Delta Ct)}$ method was used for relative quantification of gene expression. Values obtained for untreated SB embryos were used as calibrators for each gene.

Supplementary material

Primer list & references

M13Fw	GTAAAACGACGGCCAGT	Burg *et al.*, 2016
CustomRv	CGCTAGCTAATACGACTCACTATAGGTCGAACGTATTGTTTATCGTTTTAGAGCTAGAAATAG	
T7Fw	GCTAGCTAATACGACTCACT	Burg *et al.*, 2016
sgRNARv	AAAAGCACCGACTCGGTG	Burg *et al.*, 2016
	Forward	**Reverse**
Δ40g	GTAAAACGACGGCCAGT	CGATCCCGGCAAGTACTC
wt	GCATCTGTCGAACGTATTGTTTA	CTGAGCCTAAATCCATGATCG
mut	TTAAGATGCATCTGTATTGAACG	CTTCTGAGACAATTGAAAAGGAAG
FLp53	GGTCGGCAAAATCAATTCTTG	ATCAGAGTCGCTTCTTCCTTC
βact	TGTTTTCCCCTCCATTGTTGG	TTCTCCTTGATGTCACGGAC
qp53	GGGAGAAGAATTTGATTATTCAGC	ATTCAGGTCCGGTGAATAAGTG
qΔ40	CTGGAGTTCTTTGTTTGAGCTTC	ATTCAGGTCCGGTGAATAAGTG
qΔ113	ATATCCTGGCGAACATTTGGAG	CATCTGCGGACCACTTCAG
qp21	GTTTCAGCTCTTGCAGAAGC	CGTTTCTGCTGCTTTTCCTG
qp66	CACCACCTTATGCTCCTTCTTC	TTCCGAGGACTCTCCCAAA
qEF1	ACATCAAGAAGATCGGCTACAA	CAGGCTTGAGGACACCAG
qTop1	GGACCATTCCCACAACGATTC	TGTGTTCGCTGTTGCTGTAC

References

Anderson, E.M.; Haupt, A.; Schiel, J.A.; Chou, E.; Machado, H.B.; Strezoska, Ž.; Lenger, S.; McClelland, S.; Birmingham, A.; Vermeulen, A. and Smith, A. 2015. Systematic analysis of CRISPR-Cas9 mismatch tolerance reveals low levels of off-target activity. ***J Biotechnol***, 211:56-65.

Aoubala, M.; Murray-Zmijewski, F.; Khoury, M.P.; Fernandes, K.; Perrier, S.; Bernard, H.; Prats, A.C.; Lane, D.P. and Bourdon, J.C. 2011. p53 directly transactivates Δ133p53α, regulating cell fate outcome in response to DNA damage. ***Cell Death Differ***, 18(2):248-58.

Auer, T.O.; Duroure, K.; De Cian, A.; Concordet, J.P. and Del Bene, F. 2014 Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. ***Genome Res***, 24(1):142-53.

Bargonetti, J. and Manfredi, J.J. 2002. Multiple roles of the tumor suppressor p53. ***Curr Opin Oncol***, 14(1):86-91.

Berghmans, S.; Murphey, R.D.; Wienholds, E.; Neuberg, D.; Kutok, J.L.; Fletcher, C.D.; Morris, J.P.; Liu, T.X.; Schulte-Merker, S.; Kanki, J.P.; Plasterk, R.; Zon, L.I. and Look, A.T. 2005. tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. ***Proc Natl Acad Sci USA***, 102(2):407-12.

Bourougaa, K.; Naski, N.; Boularan, C.; Mlynarczyk, C.; Candeias, M.M.; Marullo, S. and Fåhraeus, R. 2010. Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47. ***Mol Cell,*** 38(1):78-88.

Bretaud, S.; Allen, C.; Ingham, P.W. and Bandmann, O. 2007. p53-dependent neuronal cell death in a DJ-1-deficient zebrafish model of Parkinson's disease. ***J Neurochem***, 100(6):1626-35.

Burg L.; Zhang, K.; Bonawitz, T.; Grajevskaja, V.; Bellipanni, G.; Waring, R. and Balciunas D. 2016. Internal epitope tagging informed by relative lack of sequence conservation. ***Sci Rep***, 6:36986.

Busslinger, M.; Moschonas, N. and Flavell, R.A. 1981. Beta + thalassemia: aberrant splicing results from a single point mutation in an intron. ***Cell***, 27(2 Pt 1):289-98.

Chang, N.; Sun, C.; Gao, L.; Zhu, D.; Xu, X.; Zhu, X.; Xiong, J.W. and Xi, J.J. 2013. Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. ***Cell Res***, 23(4):465-72.

Chen, J. and Peng, J. 2009. p53 Isoform Delta113p53 in zebrafish. ***Zebrafish***, 6(4):389-95.

Courtois, S.; Verhaegh, G.; North, S.; Luciani, M.G.; Lassus, P.; Hibner, U.; Oren, M. and Hainaut P. 2002. DeltaN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. ***Oncogene***, 21(44):6722-8.

Davidson, W.R., Kari, C., Ren, Q., Daroczi, B., Dicker, A.P. and Rodeck, U. 2010. Differential regulation of p53 function by the N-terminal ΔNp53 and Δ113p53 isoforms in zebrafish embryos. ***BMC Dev Biol,*** 10:102.

Davit-Spraul, A.; Oliveira, C.; Gonzales, E.; Gaignard, P.; Thérond, P. and Jacquemin E. 2014. Liver transcript analysis reveals aberrant splicing due to silent and intronic variations in the ABCB11 gene. ***Mol Genet Metab***, 113(3):225-9.

De Santis, F.; Di Donato, V. and Del Bene, F. 2016. Clonal analysis of gene loss of function and tissue-specific gene deletion in zebrafish via CRISPR/Cas9 technology. ***Methods Cell Biol***, 135:171-88.

Gagnon, J.A.; Valen, E.; Thyme, S.B.; Huang, P.; Akhmetova, L.; Pauli, A.; Montague, T.G.; Zimmerman, S.; Richter, C. and Schier, A.F. 2014. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. ***PLoS One***, 9(5):e98186.

Gambino, V.; De Michele, G.; Venezia, O.; Migliaccio, P.; Dall'Olio, V.; Bernard, L.; Minardi, S.P.; Della Fazia, M.A.; Bartoli, D.; Servillo, G.; Alcalay, M.; Luzi, L.; Giorgio, M.; Scrable, H.; Pelicci, P.G. and Migliaccio, E. 2013. Oxidative stress activates a specific p53 transcriptional response that regulates cellular senescence and aging. ***Aging Cell,*** 12(3):435-45.

Ghosh, A.; Stewart, D. and Matlashewski, G. 2004. Regulation of human p53 activity and cell localization by alternative splicing. ***Mol Cell Biol,*** 24(18):7987-97.

Giorgio, M.; Migliaccio, E.; Orsini, F.; Paolucci, D.; Moroni, M.; Contursi, C.; Pelliccia, G.; Luzi, L.; Minucci, S.; Marcaccio, M.; Pinton, P.; Rizzuto, R.; Bernardi, P.; Paolucci, F. and Pelicci, P.G. 2005. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. ***Cell***, 122(2):221-33.

Gong, L.; Gong, H.; Pan, X.; Chang, C.; Ou, Z.; Ye, S.; Yin, L.; Yang, L.; Tao, T.; Zhang, Z.; Liu, C.; Lane, D.P.; Peng, J. and Chen, J. 2015. p53 isoform Δ113p53/Δ133p53 promotes DNA double-strand break repair to protect cell from death and senescence in response to DNA damage. ***Cell Res***, 25(3):351-69.

Guo, L.; Chua, J.; Vijayakumar, D.; Lee, K.C.; Lim, K.; Eng, H.; Ghadessy, F.; Coomber, D. and Lane, D.P. 2010. Detection of the 113p53 protein isoform: a p53-induced protein that feeds back on the p53 pathway to modulate the p53 response in zebrafish. ***Cell Cycle***, 9(10):1998-2007.

Henriques, C.M.; Carneiro, M.C.; Tenente, I.M.; Jacinto, A. and Ferreira, M.G. 2013. Telomerase is required for zebrafish lifespan. ***PLoS Genet***, 9(1):e1003214.

Hisano, Y.; Sakuma, T.; Nakade, S2.; Ohga, R.; Ota, S.; Okamoto, H.; Yamamoto, T. and Kawahara, A. 2015. Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. ***Sci Rep***, 5:8841.

Hruscha, A. and Schmid, B. 2014. Generation of zebrafish models by CRISPR /Cas9 genome editing. ***Methods Mol Biol***, 1254:341-50.

Hruscha, A.; Krawitz, P.; Rechenberg, A.; Heinrich, V.; Hecht, J.; Haass, C. and Schmid, B. 2013. Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. ***Development***, 140(24):4982-7.

Hwang, W.Y.; Fu, Y.; Reyon, D.; Maeder, M.L.; Kaini, P.; Sander, J.D.; Joung, J.K.; Peterson, R.T. and Yeh, J.R. 2013b. Heritable and precise zebrafish genome editing using a CRISPR-Cas system. ***PLoS One***, 8(7):e68708.

Hwang, W.Y.; Fu, Y.; Reyon, D.; Maeder, M.L.; Tsai, S.Q.; Sander, J.D.; Peterson, R.T.; Yeh, J.R. and Joung, J.K. 2013a. Efficient genome editing in zebrafish using a CRISPR-Cas system. ***Nat Biotechnology***, 31(3):227-9.

Inácio, A.; Silva, A.L.; Pinto, J.; Ji, X.; Morgado, A.; Almeida, F.; Faustino, P.; Lavinha, J.; Liebhaber, S.A. and Romão, L. 2004. Nonsense mutations in close proximity to the initiation codon fail to trigger full nonsense-mediated mRNA decay. ***J Biol Chem***, 279(31):32170-80.

Jao, L.E.; Wente, S.R. and Chen, W. 2013. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. ***Proc Natl Acad Sci USA***, 110(34):13904-9.

Jiang, Z.; Cote, J.; Kwon, J.M.; Goate, A.M. and Wu, J.Y. 2000. Aberrant splicing of tau pre-mRNA caused by intronic mutations associated with the inherited dementia frontotemporal dementia with parkinsonism linked to chromosome 17. ***Mol Cell Biol***, 20(11):4036-48.

Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A. and Charpentier, E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. ***Science***, 337(6096):816-21.

Kapahnke, M.; Banning, A. and Tikkanen R. 2016. Random Splicing of Several Exons Caused by a Single Base Change in the Target Exon of CRISPR/Cas9 Mediated Gene Knockout. ***Cells***, 5(4).

Khoury, M.P. and Bourdon, J.C. 2011. p53 Isoforms: An Intracellular Microprocessor? ***Genes Cancer***, 2(4):453-65.

Kimura, Y.; Hisano, Y.; Kawahara, A. and Higashijima, S. 2014. Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. ***Sci Rep***, 4:6545.

Langheinrich, U.; Hennen, E.; Stott, G. and Vacun, G. 2002. Zebrafish as a model organism for the identification and characterization of drugs and genes affecting p53 signaling. ***Curr Biol***, 12(23):2023-8.

Lee, K.C.; Goh, W.L.; Xu, M.; Kua, N.; Lunny, D.; Wong, J.S.; Coomber, D.; Vojtesek, B.; Lane, E.B. and Lane, D.P. 2008. Detection of the p53 response in zebrafish embryos using new monoclonal antibodies. ***Oncogene***, 27(5):629-40.

Li, K.; Wang, G.; Andersen, T.; Zhou, P. and Pu, W.T. 2014. Optimization of genome engineering approaches with the CRISPR/Cas9 system. ***PLoS One***, 9(8):e105779.

Lu, W.; Chen, L.; Peng, Y. and Chen, J. 2001. Activation of p53 by roscovitine-mediated suppression of MDM2 expression. ***Oncogene***, 20(25):3206-16.

Maier, B.; Gluba, W.; Bernier, B.; Turner, T.; Mohammad, K.; Guise, T.; Sutherland, A.; Thorner, M. and Scrable, H. 2004. Modulation of mammalian life span by the short isoform of p53. ***Genes Dev,*** 18(3):306-19.

Mehrabian, M.; Brethour, D.; MacIsaac, S.; Kim, J.K.; Gunawardana, C.G.; Wang, H. and Schmitt-Ulms, G. 2014. CRISPR-Cas9-based knockout

of the prion protein and its effect on the proteome. ***PLoS One***, 9(12):e114594.

Melis, J.P.; Hoogervorst, E.M.; van Oostrom, C.T.; Zwart, E.; Breit, T.M.; Pennings, J.L.; de Vries, A. and van Steeg, H. 2011. Genotoxic exposure: novel cause of selection for a functional ΔN-p53 isoform. ***Oncogene,*** 30(15):1764-72.

Migliaccio, E.; Giorgio, M.; Mele, S.; Pelicci, G.; Reboldi, P.; Pandolfi, P.P.; Lanfrancone, L. and Pelicci, P.G. 1999. The p66shc adaptor protein controls oxidative stress response and life span in mammals. ***Nature***, 402(6759): 309-313.

Perrin-Vidoz, L.; Sinilnikova, O.M.; Stoppa-Lyonnet, D.; Lenoir, G.M. and Mazoyer, S. 2002. The nonsense-mediated mRNA decay pathway triggers degradation of most BRCA1 mRNAs bearing premature termination codons. ***Hum Mol Genet***, 11(23):2805-14.

Powell, D.J.; Hrstka, R.; Candeias, M.; Bourougaa, K.; Vojtesek, B. and Fåhraeus R. 2008. Stress-dependent changes in the properties of p53 complexes by the alternative translation product p53/47. ***Cell cycle,*** 7(7):950-9.

Tchang, F.; Gusse, M.; Soussi, T. and Méchali, M. 1993. Stabilization and expression of high levels of p53 during early development in Xenopus laevis. ***Dev Biol***, 159(1):163-72.

Ungewitter, E. and Scrable, H. 2010. Delta40p53 controls the switch from pluripotency to differentiation by regulating IGF signaling in ESCs. ***Genes Dev,*** 24(21):2408-19.

Vogelstein, B.; Sur, S. and Prives, C. 2010. p53: The Most Frequently Altered Gene in Human Cancers. ***Nat Education***, 3(9):6

Wesierska-Gadek, J.; Gueorguieva, M. and Horky, M. 2005. Roscovitine-induced up-regulation of p53AIP1 protein precedes the onset of apoptosis in human MCF-7 breast cancer cells. ***Mol Cancer Ther***, 4(1):113-24.

Wittkopp, N.; Huntzinger, E.; Weiler, C.; Saulière, J.; Schmidt, S.; Sonawane, M and Izaurralde, E. 2009. Nonsense-mediated mRNA decay effectors are essential for zebrafish embryonic development and survival. ***Mol Cell Biol***, 29(13):3517-28.

Xiong, Y.; Liu, T.; Wang, S.; Chi, H.; Chen, C. and Zheng, J. 2016. Cyclophosphamide promotes the proliferation inhibition of mouse

ovarian granulosa cells and premature ovarian failure by activating the lncRNA-Meg3-p53-p66Shc pathway. ***Gene***, 596:1-8.

Yin, Y.; Stephen, C.W.; Luciani, M.G. and Fåhraeus, R. 2002. p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. ***Nat Cell Biol***, 4(6):462-7.

Zhao, X.; Wu, N.; Ding, L.; Liu, M.; Liu, H. and Lin, X. 2012. Zebrafish p53 protein enhances the translation of its own mRNA in response to UV irradiation and CPT treatment. ***FEBS Lett***, 586(8):1220-5.

In this second section of Part III, we show the findings obtained about two branches of this project, aimed at investigating the functional conservation of p53, p44 and $p66^{Shc}$ in *Nothobranchius furzeri* and revealing the role of $p66^{Shc}$ in embryonic development, taking advantage of *Danio rerio*. These data will be part of two short communications.

Preliminary investigation on the conservation of $p66^{Shc}$ and p53/p44 in *Nothobranchius furzeri*

One of our aims is to generate a genetic model of p44 and/or $p66^{Shc}$ overexpression or ablation in the short-living vertebrate *Nothobranchius furzeri*, in order to characterize the role of p44 in stress response in aging. Therefore, we first focused on investigating the functional conservation of p44 itself, and its partners p53 and $p66^{Shc}$, in this organism. Contrary to zebrafish, the developmental biology of this killifish has not been explored; transient manipulation of gene expression, whole-mount *in situ* hybridization and pharmaceutical treatment of embryos have not been implemented yet. Thus, we chose to take advantage of mammalian cell cultures derived from $p53^{-/-}$ and $p66^{Shc-/-}$ mice to study the functional conservation of *N. furzeri* orthologs of p44, p53 and $p66^{Shc}$ through heterologous complementation experiments.

1. Materials and Methods

1.1 *Nothobranchius furzeri* maintenance

Fish of the *Nothobranchius fuzeri* strain MZM 04/10 are maintained in a commercial system ("Stendalone", Tecniplast). Each male is maintained in a 3L tank and group of 3-4 females are maintained in 8L tanks. Water temperature is set at 26.5°C, pH at 6.5 and conductivity at 300µS. The circadian rhythm imposed is 14 hours of light and 10 hours of darkness. Adult fish are fed twice a day with frozen bloodworms (Ocean Nutrition).

Adult fish are bred twice a week: *N. furzeri* embryos are maintained in system water with 0.05% methylene blue for ~two weeks, then they are moved into humid peat moss until ready to be hatched. Embryos are hatched in small tanks with peat moss and system water at 8-10°C. After hatching, *Nothobranchius* larvae are fed twice a day with living brine shrimps (MBK Installations) until they are two-week old. Then, larvae are fed for one week with a mix of brine shrimps and chopped bloodworms and for two weeks, twice a day, with chopped bloodworms.

1.2 Molecular cloning of p66^{Shc}, p53 and p44

Total RNA was extracted from organs of male adult fish with Qiazol Reagent (QIAGEN) according to the manufacturer instruction. After extraction, 30µg of total RNA were treated with 6U of TURBO DNase (Ambion) for 30' at 37°C and then column purified (Quick-RNA MiniPrep kit, Zymo Research). 1µg of total RNA was then retrotranscribed 1h at 42°C for cDNA synthesis using 0.5µg of oligodT (Invitrogen), 160U of ImProm-II Reverse Transcriptase (Promega), 3mM MgCl2, 0.3mM dNTPs and 20U of RNAse Inhibitor (NEB). About 100ng of cDNA were used for RT-PCR to amplify full-length p66^{Shc}, p53 and p44 with the primers listed in the table below. A 30-cycle touchdown RT-PCR was run as follows: 2U of Phusion High Fidelity DNA Polymerase (NEB), 0.2mM dNTPs and 0.5µM each primer with an annealing temperature decreasing from 63°C to 55°C. Primer sequence was determined using the *N. furzeri* transcriptome browser (Petzold *et al.,* 2013; http://nfintb.leibniz-fli.de/nfintb/) searching for *N. furzeri shc1* and *p53* coding sequences. Primers to amplify the putative *N. furzeri* p44 ortholog were determined using the sequence of genomic *p53*

locus kindly provided by Dr Alessandro Cellerino. To allow subcloning into the retroviral pBABE-Puro:flag vector (Addgene #1764, previously modified with the insertion of a flag tag at the C-terminus of the multicloning site) we designed primer for restriction enzyme cloning. Addition of 3'-A overhangs to PCR products was performed using 1 U of Taq Polymerase (QIAGEN) for 30' at 72°C. Then PCR amplicons were ligated into the pCR 2.1-TOPO vector (Invitrogen) following the manufacturer instructions. Ligation reactions were used to transform TOP10 competent cells (Thermo Fisher Scientific) according to the manufacturer instructions. Plasmid DNA from 2ml of over-night mini-cultures was extracted with Nucleospin Plasmid Kit (Macherey-Nagel) and sequenced by Sanger sequencing. Sequences were then analysed using Chromas lite software (Technelysium).

cDNA	Forward primer	Reverse primer
Np66^{Shc}	CAGATACTTGAGTGTCCTTCC	GGCCTTGCGCTCCACTG
Np53	AGACAGAAGATATGGAGGACTCTG	CTAATCGCTGTCACTCCGGTC
Np44	GGCTGTTTGATTAATGCAAGT	CTAATCGCTGTCACTCCGGTC

N. furzeri p66^{Shc} cDNA was then subcloned into the pBABE-Puro:flag vector by exciding the insert via BamHI/HindIII restriction enzymes (NEB), gel extraction (QIAquick gel extraction kit) and O.N. ligation into the destination vector. *N. furzeri* p53 and p44 cDNA were subcloned into the pBABE-Puro:flag vector using BamHI/EcoRI restriction enzymes (NEB).

1.3 Cell culture, retroviral infection of $p66^{Shc-/-}$ and $p53^{-/-}$ MEFs and treatments

Mouse embryonic fibroblasts (MEFs) were isolated from 13.5 day-old $p66^{Shc-/-}$ or $p53^{-/-}$ embryos according to standard procedures. MEFs were cultured in DMEM supplemented with 10% foetal bovine serum, 100µg/ml streptomycin and penicillin, 2mM L-glutamine and incubated at 37°C, 5% CO_2 and 20% O_2. Retroviruses were produced by transfecting Phoenix-Eco cells with 10µg of pBABE-Puro:flag constructs. After 48h, supernatants were collected and filtered with a 0.45µm filter. Then 5ml of each supernatant was supplemented with 50µg of polybrene and added to early passage MEFs. Each supernatant was removed three hours later and a second cycle of infection was repeated. 48 hours after infection, selection was performed by adding 2µg/ml puromycin. Infected MEFs were maintained in selection for five days and then used for further experiments. To perform treatments, MEFs were washed twice with PBS and then incubated O.N. in DMEM medium containing 400µM H_2O_2 (Sigma-Aldrich) or 0.25µg/ml doxorubicin (Sigma-Aldrich).

1.4 Western blotting

Cells were lysed by scraping with 2ml of lysis buffer (50mM Tris pH8.0, 5mM EDTA, 150mM NaCl, 0.5% Nonidet P-40 and Mini Protease Inhibitor Cocktail – Sigma-Aldrich). Lysates were kept 30' on ice and centrifuged for 15' at 13000rpm and 4°C. Protein concentration in supernatants was determined with the Bio-Rad DC protein assay. 30µg of total protein lysate for each sample was supplemented with 5x Laemmli buffer (1.5M Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.01%

bromophenol blue 0,01%), boiled for 3', separated on SDS-polyacrylamide gel and then transferred at 100V for 1h and 15' to a 0.45µm nitrocellulose membrane. Blots were then incubated 1h in 5% non-fat dried milk in TBST. α-Shc1 polyclonal antibody (BD Transduction) was diluted 1:1000 and α-Flag M2 monoclonal antibody (Sigma-Aldrich) was diluted 1:2000 in milk and incubated O.N. at 4°C. Then blots were washed in TBST buffer and incubated 1h at room temperature with peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG (Amersham Biosciences GE Healthcare) diluted 1:10000 in milk. Chemiluminescence was detected with Clarity Western ECL Substrate (Bio-Rad).

1.5 Senescence assay on MEFs

Senescent MEFs were identified by their ability to positive-stain for acid β-galactosidase at pH 6. The Senescence β-Galactosidase Staining Kit (Cell Signalling) was used to reveal β-galactosidase activity according to the manufacturer instructions.

1.6 Real-Time RT-PCR

Untreated and treated MEFs were washed and scraped in PBS and briefly centrifuged. Pelleted cells were conserved at -80°C. Total RNA was extracted from MEFs with Qiazol Reagent (QIAGEN), according to the manufacturer instructions. After extraction, 30µg of total RNA were treated with 6U of TURBO DNase (Ambion) for 30' at 37°C and then column purified (Quick-RNA MiniPrep kit, Zymo Research). 1µg of total RNA was then retrotranscribed 1h at 42°C for cDNA synthesis using 0.5µg of a primer mix consisting in oligodT and random primers (Invitrogen) in 3:1 ratio, 160U of

ImProm-II Reverse Transcriptase (Promega), 3mM MgCl2, 0.3mM dNTPs and 20U of RNAse Inhibitor (NEB). Expression changes of the p53/p66^{Shc}-dependent G2/M transition genes were investigated using the TaqMan® Gene Sets technology, a high-throughput Real Time RT-PCR approach exploiting a custom-designed Applied Biosystems Micro Fluidic Card. A complete list of the p53/p66^{Shc}-dependent G2/M transition genes can be found in Gambino *et al.,* 2013. The Quantitative PCR Facility (Cogentech) performed the reactions in triplicate. The $2^{(-\Delta\Delta CT)}$ method was used for relative quantification of gene expression; values relative to untreated WT MEFs were used as calibrators.

2. Results

2.1 *Nothobranchius* p66^{Shc} is expressed in adult tissues

We used *Nothobranchius furzeri* transcriptome browser (Petzold *et al.,* 2013; http://nfintb.leibniz-fli.de/nfintb/) to identify the ortholog of *shc1*. Sequence analyses revealed that both the isoforms of *shc1* are present in this fish. To confirm that the p66^{Shc} isoform of *shc1* is expressed in adult tissues, we performed RT-PCR on *N. furzeri* brain, heart, intestine, liver and muscle (pools of 4 organs extracted from 4 adult males). We found that a PCR product of the expected length has been amplified in all the organs and tissues analysed (Figure 1.A) Molecular cloning of PCR products and sequence analyses revealed that this amplicon corresponds to the *N. furzeri* ortholog of p66^{Shc} (Np66^{Shc}) that shows conservation with mammalian p66^{Shc} (Priami *et al.,* 2015). We than asked whether this cDNA is functional: to confirm that a protein of the expected molecular weight is produced by utilization of the putative p66^{Shc} ATG, we subcloned Np66^{Shc} into the

retroviral pBABE-Puro:flag vector and infected mouse embryonic fibroblasts (MEFs) derived from $p66^{Shc-/-}$ mice with this construct. Western blot on protein lysates from $p66^{Shc-/-}$ MEFs using the α-Shc1 antibody that recognise mammalian Shc1 isoforms (Gambino *et al.,* 2013) shows that a 66KDa band is present in protein lysate from $p66^{Shc-/-}$ MEFs reconstituted with $Np66^{Shc}$ cDNA (Figure 1.B).

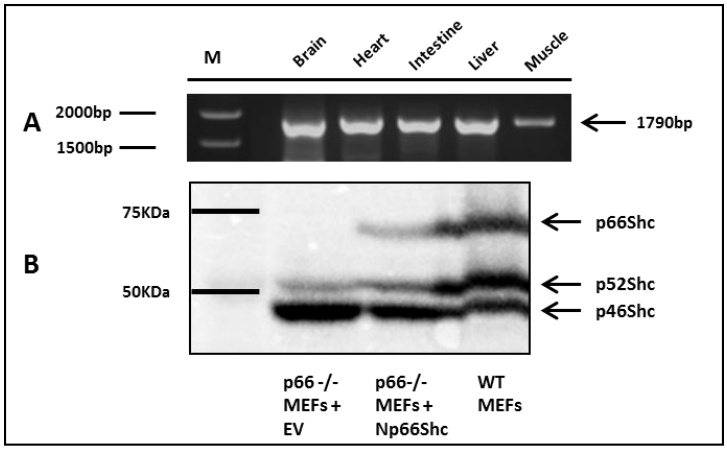


Figure 1. *Nothobranchius* $p66^{Shc}$ is expressed in adult tissues. (A) RT-PCR on total RNA extracted from pools of organs and tissues extracted from 4 adult males. The expected ~1800bp band, corresponding to the putative $p66^{Shc}$ ortholog cDNA, is present for all the tissues. **(B)** Western blot with α-Shc1 antibody on protein lysates of WT MEFs and $p66^{Shc-/-}$ MEFs reconstituted with empty vector (+EV) and *Nothobranchius* $p66^{Shc}$ (+$Np66^{Shc}$). A protein of the expected 66KDa molecular weight is present in the protein lysate of $p66^{Shc-/-}$ MEFs reconstituted with $Np66^{Shc}$.

2.2 *Nothobranchius* p53 and p44 partially recapitulate mammalian p53/p44 functions in oxidative stress response

We identified the putative p44 ortholog by sequence analysis of the *Nothobranchius furzeri p53* genomic *locus*. The mechanism we propose for p44 production in *N. furzeri* is the alternative splicing of *p53* intron 2: in this

intron, we found an in-frame STOP codon that leads to premature interruption of p53 translation. Partially overlapped to this STOP codon, an ATG, in-frame with the downstream *p53* exons, is present. The utilization of this ATG leads to the production of a N-terminal isoform of p53, where the first transactivation domain (TAD) is substituted with a different N-terminal aminoacidic sequence. This mechanism has been described also by Davidson and colleagues for the production of the p44 ortholog in zebrafish (Davidson *et al.,* 2010). Notably, N-terminal p44-specific aminoacidic sequences of *N. furzeri* and zebrafish do not show significant homology (Figure 2), suggesting that the p44-specific N-terminal domain present in these two teleost fish species might not have an impact on p44 function. Using specific primers, we were able to amplify by RT-PCR the full-length cDNA sequence of the putative p44 *N. furzeri* ortholog, which resulted expressed at low levels in several adult tissues (brain, heart, intestine and testes; Figure 3.A). Sequence analysis confirmed our predictions on p44 cDNA structure, which shares all the p53 exons from exon 3 and has a unique N-terminal sequence.

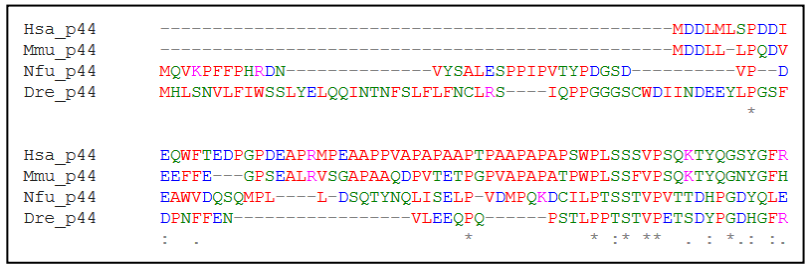


Figure 2. Multiple alignment of the N-terminal domains of human, mouse, *Nothobranchius* and zebrafish p44 protein. Human (Hsa), mouse (Mmu), Nothobranchius (Nfu) and zebrafish (Dre) p44 have been aligned using Clustal Omega: *Nothobranchius* and zebrafish p44-specific amino-acidic sequences do not show significant homology.

We asked whether *N. furzeri* p44 differs from p53 in its functions, in particular in response to oxidative stress (OS). The lack of a commercial antibody working on *N. furzeri* p53/p44 did not allow us to detect the endogenous protein. To address our question, we cloned both *Nothobranchius* p53 (Np53) and p44 (Np44) cDNA into the pBABE-Puro:flag retroviral vector, to take advantage of the flag tag for protein detection. We performed heterologous complementation experiments in MEFs derived from $p53^{-/-}$ mice and reconstituted with Np53 or Np44. Western blots with the α-flag antibody on protein lysates from Phoenix-Eco cells, used for retroviral packaging, and $p53^{-/-}$ MEFs reconstituted with fish proteins show that both Np53 and Np44 are expressed by mammalian cells (Figure 3.B). To investigate whether fish proteins are able to enter the mammalian machinery and to induce stress response, we treated $p53^{-/-}$ MEFs O.N. with 400μM hydrogen peroxide (to induce OS damage to macromolecules) or 0.25μg/ml doxorubicin (to induce specifically genotoxic stress - GS). We then investigated cell senescence upon treatments after seven days of recovery in standard medium by senescence-associated β-galactosidase staining. Results of this preliminary experiment show that (i) reconstitution with empty pBABE-Puro:flag vector is not associated with increased cell senescence upon treatments; (ii) reconstitution with Np53 resulted in a dramatic induction of cell senescence upon treatments but also at basal conditions; (iii) reconstitution with Np44 resulted in increased cell senescence only upon treatments and in particular upon OS (Figure 3.C).

Moreover, we investigated whether Np53 and Np44 are able to recapitulate mammalian p53 and p44 functions in terms of transcriptional regulation to stress response: we performed Real Time RT-PCR on total RNA

extracted from wild-type MEFs and $p53^{-/-}$ MEFs reconstituted with fish proteins and treated with H_2O_2 or doxorubicin. We analysed expression changes of p21 and 31 genes which transcriptional regulation depends on p53. These 31 genes are involved in the G2/M transition phase of the cell cycle and they are downregulated specifically upon OS in MEFs. This downregulation is increased in MEFs derived from Tgp44 mice, which overexpress the p44 isoform of p53 (Gambino *et al.,* 2013). Results of this preliminary experiment show that: (i) G2/M genes are downregulated in wild-type MEFs upon both the treatments as expected (Gambino *et al.,* 2013); (ii) G2/M genes are not downregulated upon treatments in $p53^{-/-}$ MEFs reconstituted with empty vector or Np53; (iii) reconstitution with Np44 is associated with the downregulation of G2/M genes upon treatment with H_2O_2 but not doxorubicin (Figure 3.D). Moreover, *p21* results upregulated in wild-type MEFs and in $p53^{-/-}$ MEFs reconstituted with Np53 upon both the treatments. Reconstitution with Np44 leads to mild *p21* upregulation only upon treatment with doxorubicin (Figure 3.E). Overall, this preliminary experiment suggests that Np53 is able to induce cell senescence upon both OS and GS via *p21* upregulation, but not downregulation of G2/M transition genes. On the contrary, Np44 induces cell senescence upon OS via downregulation of G2/M genes.

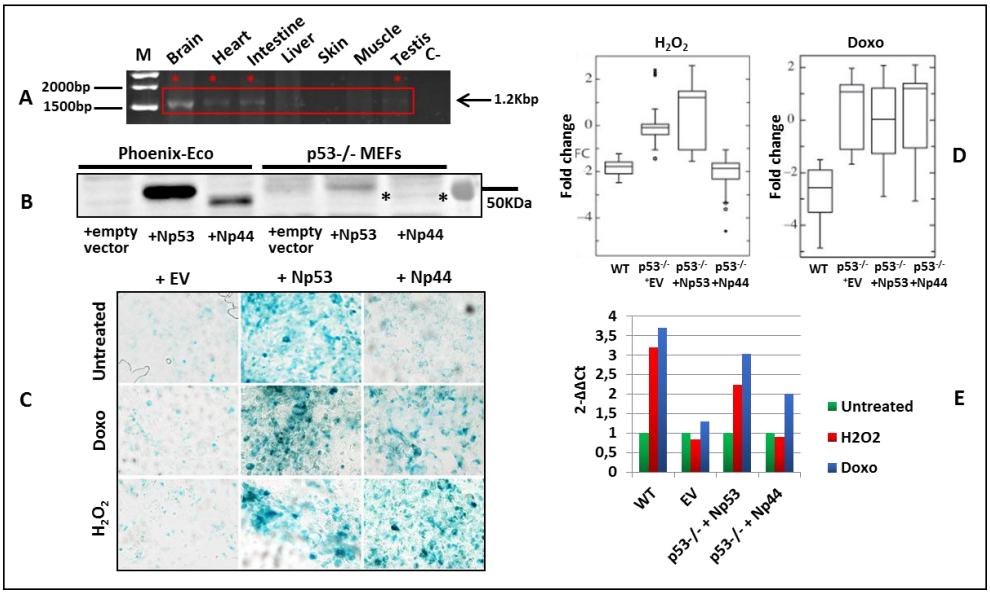


Figure 3. ***Nothobranchius*** **p53 and p44 partially recapitulate mammalian functions. (A)** RT-PCR on total RNA extracted from pools of organs and tissues of 4 adult males. The expected ~1200bp band, corresponding to the putative p44 ortholog cDNA is present in brain, heart, liver and testes (red asterisks). **(B)** Western blot with α-flag antibody on protein lysates of Phoenix-Eco and $p53^{-/-}$ MEFs reconstituted with empty vector (+EV), Np53 or Np44. Proteins of the expected ~50KDa and ~44KDa molecular weight are present in lysates from cells reconstituted with the retroviral constructs containing Np53 or Np44 cDNA. **(C)** Senescence-associated β-galactosidase activity in $p53^{-/-}$ MEFs reconstituted with EV, Np53 or Np44 and treated with H_2O_2 or doxorubicin. Np53 induces cell senescence also at basal conditions, while Np44 induces cell senescence only upon stress and in particular upon OS. **(D)** Box plot representation of Real-Time RT-PCR fold changes of 31 p53/p66Shc-dependent G2/M transition genes in WT MEFs and $p53^{-/-}$ MEFs reconstituted with EV, Np53 or Np44 and treated with H_2O_2 or doxorubicin. Np53 does not induce downregulation of G2/M genes while Np44 induces their downregulation only upon OS. **(E)** Real-Time RT-PCR fold-changes of p21 in WT MEFs and $p53^{-/-}$ MEFs reconstituted with empty vector EV, Np53 or Np44 and treated with H_2O_2 or doxorubicin. Np53 induces p21 upregulation upon both the treatments, while Np44 induces mild p21 upregulation only upon treatment with doxorubicin.

3. Discussion

$p66^{Shc}$ is the longest isoform encoded by the *shc1 locus*. The shortest isoforms, $p52^{Shc}$ and $p46^{Shc}$ are involved in Ras signalling. These two isoforms are phosphorylated by active tyrosine kinase receptors and drive the MAPK (Mitogen-Activated Protein Kinase) cascade and thus cell proliferation (Pelicci *et al.,* 1992). The third isoform, $p66^{Shc}$, is translated from a different mRNA. $p66^{Shc}$ contains the entire $p52^{Shc}$/$p46^{Shc}$ sequence plus an additional amino-terminal region (Ventura *et al*., 2002). $p66^{Shc}$, contrary to $p52^{Shc}$/$p46^{Shc}$, does not contribute to stimulate cell proliferation (Migliaccio *et al*., 1997). $p66^{Shc}$ is involved in oxidative stress response (OS): OS activates $p66^{Shc}$ that directly promotes apoptosis (Giorgio *et al.,* 2005). The $p66^{Shc-/-}$ mouse model, generated in 1999, is a genetic model of healthy aging. It has been demonstrated that the deletion of $p66^{Shc}$ improves general health in the aging mouse, being implicated in increased resistance to OS-induced apoptosis (Migliaccio *et al*., 1999). $p66^{Shc}$ deletion also ameliorates of the phenotype of different mouse models of human diseases, such as (i) acute ischemia (Zaccagnini *et al*., 2004), (ii) diabetes (Menini *et al*., 2006), (iii) age-dependent decrease of endothelial functions (Francia *et al*., 2004), (iv) hepatic steatosis (Koch *et al*., 2008) and (v) Alzheimer disease (Derungs *et al*., 2016). Nevertheless, all the molecular mechanisms underlying $p66^{Shc}$ functions in aging are far of being deeply understood. Our aim is to further investigate $p66^{Shc}$ genetics taking advantage of the short-living vertebrate *N. furzeri*. This teleost fish is an emerging model in which the generation of transgenic lines (Valenzano *et al*., 2011; Allard *et al*., 2013) and CRISPR/Cas9-mediated knock-out lines

(Harel *et al.,* 2015) are feasible, as well as screenings on compounds affecting lifespan and healthspan (Valenzano *et al*., 2006).

We used the *N. furzeri* transcriptome browser (Petzold *et al.,* 2013; http://nfintb.leibniz-fli.de/nfintb/) to identify the ortholog of *shc1*. We were able to clone the *N. furzeri* ortholog of $p66^{Shc}$ ($Np66^{Shc}$) and proved that it is expressed in several adult tissues (Figure 1.A). Our sequence analyses show that the aminoacidic domains important for $p66^{Shc}$ to exert its functions are conserved in *N. furzeri*. This is the basis for further functional analyses: heterologous complementation experiments in $p66^{Shc-/-}$ mammalian cells will be useful to elucidate whether $Np66^{Shc}$ is able to initiate OS response, as in mammals. Further investigations include *in vivo* analyses of $Np66^{Shc}$ expression changes under different environmental conditions or stresses and the generation of a genetic model of $Np66^{Shc}$ overexpression or ablation.

The tumour suppressor p53 is involved, as $p66^{Shc}$, in lifespan and aging modulation: when p53 activity in enhanced, but the mechanisms that ensure a normal regulation of its turn-over are maintained, p53 promotes longevity. On the contrary, when p53 is constitutively active, it promotes aging without an increase in cancer susceptibility. Therefore, the p53-dependent transcriptional response is fine-tuned to avoid proliferation of damaged, potentially cancerous, cells but also to avoid the loss of stem cell compartments and preserve tissue homeostasis (Feng *et al*., 2011; Reinhardt and Schumacher, 2012).

p44 is a short isoform of p53 that has been proved to orchestrate p53 functions. p44 lacks the first transactivation domain (TAD), but retains the

second TAD and all the p53 functional domains (Khoury and Bourdon, 2011). p44, thanks to its intact oligomerization domain, has the ability to form oligomers with p53, determining its localization and fine-tuning its transcriptional activity: (i) endoplasmic reticulum (ER) stress promotes the formation of p44/p53 oligomers leading to G2 arrest of the cell cycle that cannot be induced by p53 alone (Bourougaa *et al*., 2010); (ii) the human p44 ortholog accumulates in response to genotoxic stress enhancing p21 expression (Powell *et al*., 2008); (iii) p44 is responsible for the downregulation of a specific mitotic signature of ~200 G2/M transition genes in response to OS. Downregulation of these genes is p53/p66^{Shc}-dependent and enhanced when p44 is overexpressed (Gambino *et al.,* 2013).

Interestingly, p44 is involved in modulating p53 functions not only in response to stresses, but also in aging. The Tgp44 mouse overexpresses the p44 isoform and maintains unaltered full-length p53 expression. These mice show premature signs of aging: as early as 4-month old, they show osteoporosis, kyphosis, infertility and reduced body size, and die within the first year of age, without developing cancer (Maier *et al*., 2004). These findings show a correlation between OS response and aging, being both p44 and p66^{Shc} two aging proteins involved in promoting cell cycle arrest upon OS.

As for p66^{Shc}, our aim is to take advantage of *N. furzeri* to investigate the mechanisms through which p44 orchestrates p53 functions in determining stress response and aging processes. In zebrafish, the p44 isoform is produced by an alternative splicing mechanism, contrary to mammals,

where p44 is produced by alternative initiation of translation (Davidson *et al.,* 2010). We identified the putative p44 ortholog by sequence analysis of the *N. furzeri p53* genomic *locus*. The mechanism we propose for p44 production in *N. furzeri* is the same as zebrafish: the alternative splicing of *p53* intron 2. The utilization of an intronic ATG, in-frame with the downstream p53 exons, leads to the production of a N-terminally truncated isoform of p53, where the first TAD is lost. We demonstrated that the full-length cDNA sequence of the putative Np44 ortholog is expressed at low levels in several *N. furzeri* adult tissues (brain, heart, intestine and testes; Figure 1.A).

Our preliminary results of heterologous complementation experiments in $p53^{-/-}$ MEFs reconstituted with either Np53 or Np44 show that: (i) both Np53 and Np44 are able to enter the mammalian transcriptional regulation machinery; (ii) Np53 induces cell senescence upon OS and genotoxic stress (GS), but also at basal conditions; (iii) Np53 alone is not able to downregulate the 31 G2/M genes but it induces the upregulation of *p21* upon both OS and GS; (iv) Np44 alone is able to induce the downregulation of G2/M genes only upon OS and a mild upregulation of p21 upon GS (Figure 3). These data suggest that future experiments have to be planned in order to modulate Np53 expression in $p53^{-/-}$ MEFs: in fact, Np53 is able to induce cell cycle arrest also at basal conditions. This makes the other results difficult to interpret. On the other hand, Np44 is able to induce the downregulation of G2/M genes only upon OS. In mammals, p44 is implicated in OS response: this fact suggests that Np44 might recapitulate mammalian p44 functions in orchestrating the p53-dependent transcriptional response to different kind of stressors. *In vivo* experiments

in *N. furzeri* are necessary to clarify whether Np44 is indeed activated by OS and, for example, in which tissues is preferentially expressed upon OS and at basal conditions. Moreover, the alternative splicing mechanism through which Np44 is produced gives the chance to selectively knock-out Np44 in this organism, without interfering with Np53 sequence. Indeed, it has been observed in mice that a point mutation introduced to mutagenize the p44 ATG leads to a dramatic reduction of the p53 mRNA levels due to its instability (Melis *et al*., 2011). Considering this, a genetic model of selective p44 ablation is not feasible in mice. The production of a $p44^{-/-}$ model in *N. furzeri* will reveal how the p44 isoform modulates stress response *in vivo*. Finally, being *N. furzeri* the shortest-living vertebrate model organism, a $p44^{-/-}$ model will be also fundamental to deeply investigate the role of p44 in aging and lifespan modulation.

4. References

Allard, J.B.; Kamei, H. and Duan, C. 2013. Inducible transgenic expression in the short-lived fish Nothobranchius furzeri. ***J Fish Biol,*** 82(5):1733-8.

Bourougaa, K.; Naski, N.; Boularan, C.; Mlynarczyk, C.; Candeias, M.M.; Marullo, S. and Fåhraeus, R. 2010. Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47. ***Mol Cell,*** 38(1):78-88.

Davidson, W.R., Kari, C., Ren, Q., Daroczi, B., Dicker, A.P. and Rodeck, U. 2010. Differential regulation of p53 function by the N-terminal ΔNp53 and Δ113p53 isoforms in zebrafish embryos. ***BMD Dev Biol,*** 10:102.

Derungs, R; Camici, G.G.; Spescha, R.D.; Welt, T.; Tackenberg, C.; Späni, C.; Wirth, F.; Grimm, A.; Eckert, A.; Nitsch, R.M. and Kulic, L. 2016. Genetic ablation of the p66Shc adaptor protein reverses cognitive deficits and improves mitochondrial function in an APP transgenic mouse model of Alzheimer's disease. ***Mol Psychiatry***, 22(4):605-614.

Feng, Z.; Lin, M. and Wu, R. 2011. The Regulation of Aging and Longevity: A New and Complex Role of p53. ***Genes Cancer***, 2(4): 443-452.

Francia, P.; delli Gatti, C.; Bachschmid, M.; Martin-Padura, I.; Savoia, C.; Migliaccio, E.; Pelicci, P.G.; Schiavoni, M.; Lüscher, T.F.; Volpe, M. and Cosentino F. 2004. Deletion of p66shc gene protects against age-related endothelial dysfunction. ***Circulation***, 110(18):2889-95.

Gambino, V.; De Michele, G.; Venezia, O.; Migliaccio, P.; Dall'Olio, V.; Bernard, L.; Minardi, S.P.; Della Fazia, M.A.; Bartoli, D.; Servillo, G.; Alcalay, M.; Luzi, L.; Giorgio, M.; Scrable, H.; Pelicci, P.G. and Migliaccio, E. 2013. Oxidative stress activates a specific p53 transcriptional response that regulates cellular senescence and aging. ***Aging Cell,*** 12(3):435-45.

Giorgio, M.; Migliaccio, E.; Orsini, F.; Paolucci, D.; Moroni, M.; Contursi, C.; Pelliccia, G.; Luzi, L.; Minucci, S.; Marcaccio, M.; Pinton, P.; Rizzuto, R.; Bernardi, P.; Paolucci, F. and Pelicci, P.G. 2005. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. ***Cell***, 122(2):221-33.

Harel, I.; Benayoun, B.A.; Machado, B.; Singh, P.P.; Hu, C.K.; Pech, M.F.; Valenzano, D.R.; Zhang, E.; Sharp, S.C.; Artandi, S.E. and Brunet, A. 2015. A platform for rapid exploration of aging and diseases in a naturally short-lived vertebrate. ***Cell,*** 160(5):1013-26.

Khoury, M.P. and Bourdon, J.C. 2011. p53 Isoforms: An Intracellular Microprocessor? ***Genes Cancer***, 2(4):453-65.

Koch, O.R.; Fusco, S.; Ranieri, S.C.; Maulucci, G.; Palozza, P.; Larocca, L.M.; Cravero, A.A.; Farre', S.M.; De Spirito, M.; Galeotti, T. and Pani, G. 2008. Role of the life span determinant P66(shcA) in ethanol-induced liver damage. ***Lab Invest***, 88(7):750-60.

Maier, B.; Gluba, W.; Bernier, B.; Turner, T.; Mohammad, K.; Guise, T.; Sutherland, A.; Thorner, M. and Scrable, H. 2004. Modulation of mammalian life span by the short isoform of p53. ***Genes Dev,*** 18(3):306-19.

Melis, J.P.; Hoogervorst, E.M.; van Oostrom, C.T.; Zwart, E.; Breit, T.M.; Pennings, J.L.; de Vries, A. and van Steeg, H. 2011. Genotoxic exposure: novel cause of selection for a functional ΔN-p53 isoform. ***Oncogene,*** 30(15):1764-72.

Menini, S.; Amadio, L.; Oddi, G.; Ricci, C.; Pesce, C.; Pugliese, F.; Giorgio, M.; Migliaccio, E.; Pelicci, P.; Iacobini, C. and Pugliese, G. 2006. Deletion of p66Shc longevity gene protects against experimental diabetic glomerulopathy by preventing diabetes-induced oxidative stress. ***Diabetes***, 55(6):1642-50.

Migliaccio, E.; Giorgio, M.; Mele, S.; Pelicci, G.; Reboldi, P.; Pandolfi, P.P.; Lanfrancone, L. and Pelicci, P.G. 1999. The p66shc adaptor protein controls oxidative stress response and life span in mammals. ***Nature***, 402(6759): 309-313.

Migliaccio, E.; Mele, S.; Salcini, A.E.; Pelicci, G.; Lai, K.M.; Superti-Furga, G.; Pawson, T.; Di Fiore, P.P.; Lanfrancone, L. and Pelicci, P.G. 1997. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. ***EMBO J***, 16(4): 706-716.

Pelicci, G.; Lanfrancone L.; Grignani, F.; McGlade, J.; Cavallo, F.; Forni, G.; Nicoletti, I.; Grignani, F.; Pawson, T. and Pelicci, P.G. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. ***Cell***, 70(1):93-104.

Petzold, A.; Reichwald, K.; Groth, M.; Taudien, S.; Hartmann, N.; Priebe, S.; Shagin, D.; Englert, C. and Platzer, M. 2013. The transcript catalogue of the short-lived fish Nothobranchius furzeri provides insights into age-dependent changes of mRNA levels. ***BMC Genomics,*** 14:185.

Powell, D.J.; Hrstka, R.; Candeias, M.; Bourougaa, K.; Vojtesek, B. and Fåhraeus R. 2008. Stress-dependent changes in the properties of p53 complexes by the alternative translation product p53/47. ***Cell cycle,*** 7(7):950-9.

Priami, C.; De Michele, G.; Cotelli, F.; Cellerino, A.; Giorgio, M.; Pelicci, P.G. and Migliaccio, E. 2015. Modelling the p53/p66Shc Aging Pathway in the Shortest Living Vertebrate Nothobranchius Furzeri. ***Aging Dis,*** 6(2):95-108.

Reinhardt, H.C. and Schumacher, B. 2012. The p53 network: cellular and systemic DNA damage responses in aging and cancer. ***Trends Genet***, 28(3):128-36.

Valenzano, D.R.; Sharp, S. and Brunet, A. 2011. Transposon-Mediated Transgenesis in the Short-Lived African Killifish Nothobranchius furzeri, a Vertebrate Model for Aging. ***G3 (Bethesda),*** 1(7):531-8.

Valenzano, D.R.; Terzibasi, E.; Genade, T.; Cattaneo, A.; Domenici, L. and Cellerino, A. 2006. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. ***Curr Biol,*** 16(3):296-300.

Ventura, A.; Luzi, L.; Pacini, S.; Baldari, C.T. and Pelicci, P.G. 2002. The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. ***J Biol Chem***, 277(25):22370-6.

Zaccagnini, G.; Martelli, F.; Fasanaro, P.; Magenta, A.; Gaetano, C.; Di Carlo, A.; Biglioli, P.; Giorgio, M.; Martin-Padura, I.; Pelicci, P.G. and Capogrossi, M.C. 2004. p66ShcA modulates tissue response to hindlimb ischemia. ***Circulation***, 109(23):2917-23.

Regulation of $p66^{Shc}$/$p52^{Shc}$ and p53/p44 expression in the developing zebrafish embryo

$p66^{Shc}$ is involved in oxidative stress (OS) response and aging, while its role in in embryonic development has never been investigated. However, transcriptional response to OS is involved in orchestrating the balance between proliferation and differentiation during embryogenesis. Moreover, p53 and its short isoform p44 (the partners that cooperate with $p66^{Shc}$ in the transcriptional network induced by OS) play a role in early mouse embryogenesis. Thus, we asked whether $p66^{Shc}$ takes part to developmental processes, taking advantage of *Danio rerio* (zebrafish). Whole-mount *in situ* hybridization of $p66^{Shc}$ and qPCR analyses show that $p66^{Shc}$ expression is spatially restricted to the embryonic cephalic area and it is regulated over time.

1. Materials and Methods

1.1 *Danio rerio* maintenance

Adult zebrafish are maintained in a commercial system (Aquatic Habitat) with a density of 0.2L per adult zebrafish; water temperature is set at 28.5°C, pH at 7 and conductivity at 500µS. The circadian rhythm imposed is 14 hours of light and 10 hours of darkness. Fish are fed three times a day with living brine shrimps and standard dry food (MBK Installations). Juvenile zebrafish (from 6 days to three weeks) are fed with dry brine shrimps (JBL) and standard dry food (Zeigler). Zebrafish embryos are maintained at 28°C

in “E3” water (50mM NaCl, 0.17mMKCl, 0.33mM CaCl, 0.33mM $MgSO_4$, 0.05% methylene blue).

1.2 Synthesis of Digoxigenin (DIG)-labelled RNA probes for zebrafish p66S^{hc}, p52^{Shc}, p53 and p44.

Total RNA was extracted from a pool of 50 48hpf zebrafish embryos with Qiazol Reagent (QIAGEN) according to the manufacturer instructions. After extraction, 30µg of total RNA were treated with 6U of TURBO DNase (Ambion) for 30’ at 37°C and then column purified (Quick-RNA MiniPrep kit, Zymo Research). 1µg of total RNA was then retrotranscribed 1h at 42°C for cDNA synthesis using 0.5µg of oligodT (Invitrogen), 160U of ImProm-II Reverse Transcriptase (Promega), 3mM MgCl2, 0.3mM dNTPs and 20U of RNAse Inhibitor (NEB). About 100ng of cDNA were used for RT-PCR to amplify template sequences of p66^{Shc}, p52^{Shc}, p53 and p44 with the primers listed in the table below. A 30-cycle touchdown RT-PCR was run as follows: 2U of Phusion High Fidelity DNA Polymerase (NEB), 0.2mM dNTPs and 0.5µM each primer with an annealing temperature decreasing from 64°C to 57°C. PCR products of the expected length were then extracted from 0.8% agarose gel using QIAquick Gel Extraction Kit (QIAGEN), according to manufacturer instructions. Addition of 3’-A overhangs to gel-extracted PCR products was performed using 1 U of Taq Polymerase (QIAGEN) 30’ at 72°C. Then PCR amplicons were ligated at 16°C overnight into the pGEM-T easy vector (Promega), which harbours both SP6 and T7 promoter sequences.

Probe	Forward primer	Reverse primer	Ampl. length
Zp66^{Shc}	GTATGACAACAGCAGAAAACTTG	CTGTGGGGGACGAGGA	683bp
Zp52^{Shc}	GGCAGAACCGAAGAGAAAAG	ATATACCTGACAGTGTAGGAGAC	343bp
Zp53	GGTCGGCAAAATCAATTCTTG	GGTCGGCAAAATCAATTCTTG	217bp
Zp44	ATGTCAGGTTGCTATAATGTACC	CGATCCCGGCAAGTACTC	221bp

Ligation reactions were used to transform TOP10 competent cells (Thermo Fisher Scientific) according to the manufacturer instructions. Plasmid DNA from 2ml of overnight mini-cultures was extracted with Nucleospin Plasmid Kit (Macherey-Nagel) and sequenced by Sanger sequencing. Sequences were then analysed with Chromas lite software (Technelysium) to determine insert orientation. 5µg of plasmid DNA for each construct was then linearized 4h at 37°C with NcoI or SalI restriction enzymes (New England Biolabs). Digested plasmid DNA was then checked for complete linearization on agarose gel and purified with QIAquick PCR purification kit (QIAGEN).

For each target mRNA, both the antisense and the sense probe were produced. *In vitro* transcription was performed 2h at 37°C as follows: 1µg of each linearized plasmid as template, 10mM DTT (Promega), DIG-RNA Labelling mix (Roche), 30U of T7 or SP6 RNA polymerase (Promega). *In vitro* transcription reactions were then DNAse treated (15' at 37°C with 4U of Turbo DNAse, Ambion), and precipitated with ammonium acetate/ethanol. RNA DIG-labelled probes were then checked for integrity on agarose gel.

1.3 Whole-mount *in situ* hybridization on zebrafish embryos

Zebrafish embryos at early developmental stages were manually dechorionated and, after 24hpf, they were treated with 0.0003% phenylthiourea to avoid pigmentation. Embryos were fixed 2h at room temperature with 4% paraformaldehyde (Sigma-Aldrich) in PBS. Fixed embryos were then dehydrated in 25%-50%-75% methanol/PBS solutions and conserved in methanol at -20°C.

For whole-mount *in situ* hybridization, embryos were rehydrated in 75%-50%-25% methanol/PBS solutions and then washed four times 5' in PBS.

Whole-mount *in situ* hybridization was performed as described by Thisse and Thisse, 2008. Briefly, after rehydration, embryos were permeabilized by digestion with 10µg/mL proteinase K in PBT (PBS + 1% Tween-20) and post-fixed in 4% PFA for 20' at room temperature. Embryos were then washed in PBS and incubated for 3h at 65°C in hybridization mix. Following pre-hybridization, embryos were incubated over-night at 65°C in hybridization mix containing 1ng/µL sense or antisense probe. After washing in hybridization mix/SSC and PBT, embryos were incubated in blocking solution (2% sheep serum + 2% BSA in PBT) 1h at room temperature and then incubated over-night at 4°C with alkaline-phosphatase conjugated anti-digoxigenin antibody (Roche, diluted 1:3000 in blocking solution).

Embryos were then extensively washed in PBT and stained with NBT/BCIP (Promega), diluted in staining buffer (100mM Tris-HCl pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20) following manufacturer

instructions. Images of stained embryos were acquired on a Leica M205 FA microscope with a Leica DFC 450C camera.

1.4 Real-Time RT-PCR of $p66^{Shc}$ and $p52^{Shc}$

Total RNA was extracted from 3 pools of 30-100 zebrafish embryos at different developmental stages with Qiazol Reagen (QIAGEN), according to manufacturer instructions. After extraction, 30µg of total RNA were treated with 6U of TURBO DNase (Ambion) for 30' at 37°C and then column purified (Quick-RNA MiniPrep kit, Zymo Research). 1µg of total RNA was then retrotranscribed 1h at 42°C for cDNA synthesis using 0.5µg of a primer mix consisting in oligodT and random primers (Invitrogen) in 3:1 ratio, 160U of ImProm-II Reverse Transcriptase (Promega), 3mM MgCl2, 0.3mM dNTPs and 20U of RNAse Inhibitor (NEB). A two-step qPCR was run using ~100ng of cDNA as template, 0.2µM each primer and Fast SYBR Green Master Mix 2X (Applied Biosystems) in a total volume of 20µl. qPCR was run as follows: 2' at 95°C, then 3'' at 95°C and 30'' at 60°C for 40 cycles. $p66^{Shc}$ and $p52^{Shc}$ were discriminated each other using specific couples of primers, listed below. eEF1A-1a was used as housekeeping gene. Data are presented as the mean ± standard error. Gastrulation stage was used as internal calibrator.

Gene	Forward primer	Reverse primer	Ampl. length
$Zp66^{Shc}$	CACCACCTTATGCTCCTTCTTC	TTCCGAGGACTCTCCCAAA	139bp
$Zp52^{Shc}$	AGTGTCTTCCAATGGAGTATGTG	CATGCCGTGTCCACTCATC	105bp
ZEF1a	CGGTACTACTCTTCTTGATGCC	CAGGCTTGAGGACACCAG	152bp

2. Results

2.1 p66^{Shc} mRNA levels increase at later developmental stages

It has been shown in zebrafish that p52^{Shc}, the shorter isoform of the *shc1* gene, is involved in embryonic angiogenesis (Sweet *et al.*, 2012). On the contrary, the role of the p66^{Shc} isoform is still unknown. Therefore, to gain new insights on p66^{Shc} functions, we investigated for the first time how p66^{Shc}, the longest isoform of *shc1*, is regulated over time during zebrafish development. We extracted total RNA from three pools of zebrafish embryos at different developmental stages and we quantified mRNA levels of p66^{Shc} through Real-Time RT-PCR. We considered: 50% epiboly (when the zebrafish organiser is visible), early somitogenesis (1-3 somites), late somitogenesis (15-18 somites), 24hpf (hours post fertilization), 48hpf, 72hpf (when organogenesis is complete and the embryo hatches), 96hpf and 120hpf (Kimmel *et al.,* 1995). Gastrulation stage was used as internal calibrator. We also discriminated p66^{Shc} from p52^{Shc}, thanks to the design of primers that specifically amplify p66^{Shc} or p52^{Shc}: primers for p66^{Shc} are designed on the sequence that is unique to this isoform and previously determined (Priami *et al.,* 2015), while primers for p52^{Shc} are designed on the 5'UTR specific to this isoform and on exon 2 of its coding sequence (Figure 1). We used *eEF1α-1a* (eukaryotic elongation factor 1alpha-1a) as housekeeping gene, as its expression levels are stable during zebrafish development (McCurley and Callard, 2008). We observed that (i) mRNA levels of p66^{Shc} are very low at early developmental stages, then they dramatically increase, reaching a peak at 48hpf-72hpf; (ii) mRNA levels of p52^{Shc} are stable over time (Figure 2).

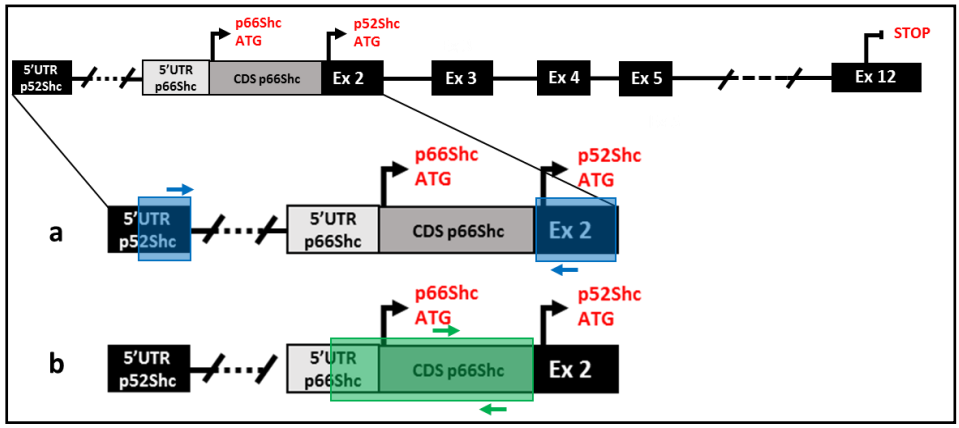


Figure 1. The *shc1 locus* in zebrafish. p66^{Shc} has a unique N-terminal domain and shares its amino-acidic sequence with p52^{Shc} from exon 2. **a)** The p52^{Shc} probe (blue box) is designed on p52^{Shc} 5'UTR/exon 2, to exclude a non-specific hybridization with p66^{Shc} mRNA. qPCR primers for p52^{Shc} (blue arrows) are also designed to exclude amplification of p66^{Shc} cDNA. **b)** The p66^{Shc} probe (green box) and p66^{Shc} qPCR primers (green arrows) are designed on the *shc1* sequence unique to p66^{Shc} to exclude non-specific hybridization with p52^{Shc} mRNA and amplification of p52^{Shc} cDNA.

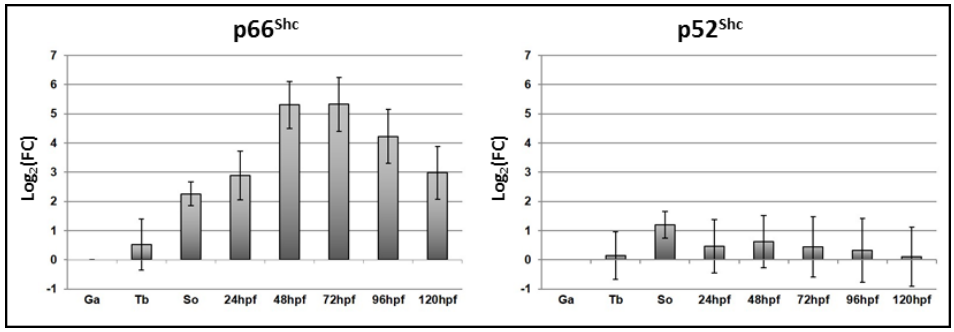


Figure 2. Expression regulation of p66^{Shc} and p52^{Shc} in zebrafish development. Log$_2$(Fold changes) of p66^{Shc} and p52^{Shc} at different developmental stages (Ga: gastrulation; TB: early somitogenesis; So: late somitogenesis; 24hpf; 48hpf; 72hpf; 96hpf; 120hpf). Real-Time RT-PCR was performed with specific couples of primers to discriminate the two isoforms (Figure 1). p66^{Shc} expression reaches a peak between 48hpf and 72hpf, then slightly decreases. p52^{Shc} expression is stable during development. Data are present as the mean ± standard error of three biological replicates. Ef1α-1a was used as housekeeping gene and gastrulation stage as internal calibrator.

2.2 $p66^{Shc}$ expression is restricted to the cephalic area in zebrafish development

We asked whether $p66^{Shc}$ expression is not only temporally, but also spatially regulated in zebrafish development. In order to determine $p66^{Shc}$ expression domain and to distinguish it from $p52^{Shc}$, we designed specific probes to discriminate the two isoforms through *in situ* hybridization: the probe for $p66^{Shc}$ is designed on the sequence unique to $p66^{Shc}$ and the probe for $p52^{Shc}$ is designed on 5'UTR unique to this isoform and on the first exon of its coding sequence (Figure 1). We determined, through *in situ* hybridization, $p66^{Shc}$ expression domain at five developmental stages: late somitogenesis (15-18 somites), 24hpf, 48hpf, 72hpf and 96hpf. We found that $p66^{Shc}$ expression is ubiquitous in the 15-18 somite-embryo, being more concentrated in the eye and the trigeminal placodes (recognizable as the two lateral patches at the lateral edge of the neuroepithelium) that will originate the trigeminal ganglia (Andermann *et al.,* 2002). At 24hpf, $p66^{Shc}$ expression is still ubiquitous, but concentrated in the brain, in the trigeminal placodes and in Rohon-Beard neurons, transient mechanoreceptors located dorsally in the spinal cord, that undergo programmed cell death after 24hpf (Reyes *et al.,* 2004; Figure 3.A). At 48hpf, $p66^{Shc}$ expression is localized (i) in the brain and in particular in the optic tectum, (ii) in the ciliary marginal zone of the retina, where retinal stem cells and retinal progenitor cells are located (Moshiri *et al.,* 2004) and (iii) in the trigeminal ganglion, now positioned ventrally to the brain between the eyes and the otic vescicles, where the nuclei of the touch sensory neurons of the fifth cranial nerve are located (Kimmel *et al.*, 1995; Figure 3.B).

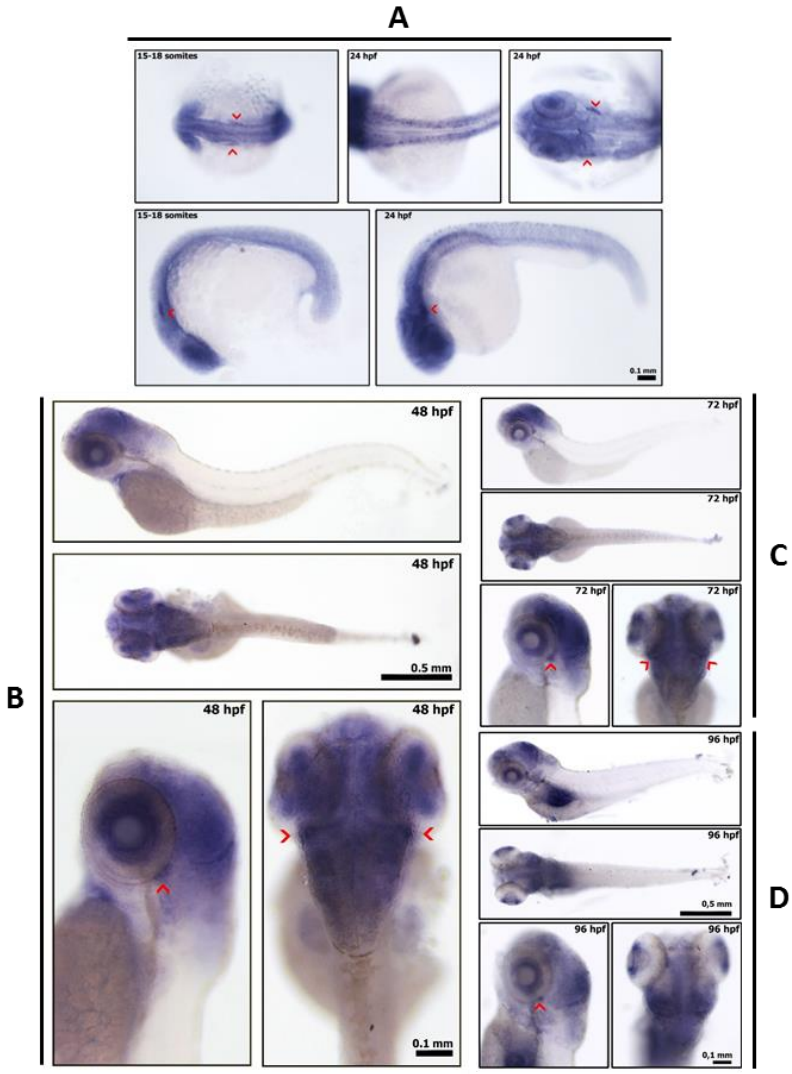


Figure 3. p66^{Shc} expression pattern in zebrafish development. p66^{Shc} expression is ubiquitously distributed in late somitogenesis and at 24hpf **(A)**, being more concentrated in the eye and the trigeminal placodes (red arrows). At 24hpf it is also evident in the Rohon-Beard neurons. At 48hpf **(B)** p66^{Shc} is expressed in the brain and in particular in the optic tectum, in the ciliary marginal zone of the retina, and in the trigeminal ganglion (red arrows). At 72hpf **(C)** and 96hpf **(D)**, p66^{Shc} expression is still restricted to the cephalic area and concentrated in the optic tectum, in the ciliary marginal zone of the retina and in the trigeminal ganglion (red arrows).

At 72hpf and 96hpf, $p66^{Shc}$ expression is still restricted to the cephalic area and concentrated in the optic tectum, in the ciliary marginal zone of the retina and in the trigeminal ganglion (Figure 3.C/D.). We also analysed $p52^{Shc}$ expression at 24hpf and 48hpf through *in situ* hybridization and we found that, at least at these developmental stages, $p52^{Shc}$ expression is not spatially restricted, being ubiquitously distributed in the embryo (Figure 4).

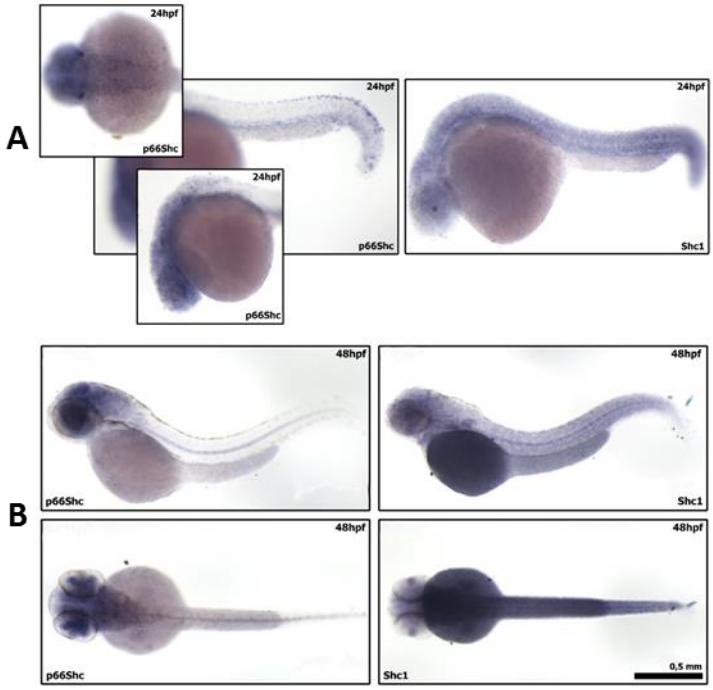


Figure 4. $p66^{Shc}$ and $p52^{Shc}$ expression patterns. Through whole-mount *in situ* hybridization we discriminated the expression patterns of the two isoforms of the *shc1* gene. At 24hpf **(A)** and 48hpf **(B)**, $p52^{Shc}$ is ubiquitously expressed, contrary to $p66^{Shc}$.

2.3 p53 and p44 share the same expression pattern.

p44 is a splicing isoform of p53 that is mostly expressed in the early mammalian embryo, when it is involved in the preservation of pluripotency: for this reason, it has been described as an embryonic isoform of p53 (Ungewitter and Scrable, 2010). We decided to investigate whether p44 and p53 have different expression domains in the developing zebrafish embryo. To address this question, we generated specific probes for p53 and p44: we took advantage of the sequence that is unique to p44 to design a short probe on the intron 2/exon 3-4 of p53 *locus*, while the p53 probe was designed across 5'UTR/exon 4 (Figure 5). We used this assay to discriminate p53 expression domains from p44 through whole-mount *in situ* hybridization. In the 48hpf zebrafish embryo, p53 resulted expressed (i) in the midbrain-hindbrain boundary, an area of persistent cell proliferation until 72hpf (Wulliman and Knipp, 2000); (ii) in the retina; (iii) in the fin buds, another area of active cell proliferation (Nomura *et al.,* 2006); (iv) in the pharyngeal arches, which will form gills and jaws; (v) in the otic vesicles and (vi) in the hatching glands. At the same developmental stage, whole-mount *in situ* hybridization with the p44-specific probe shows that this isoform shares the same expression pattern of p53 (Figure 6).

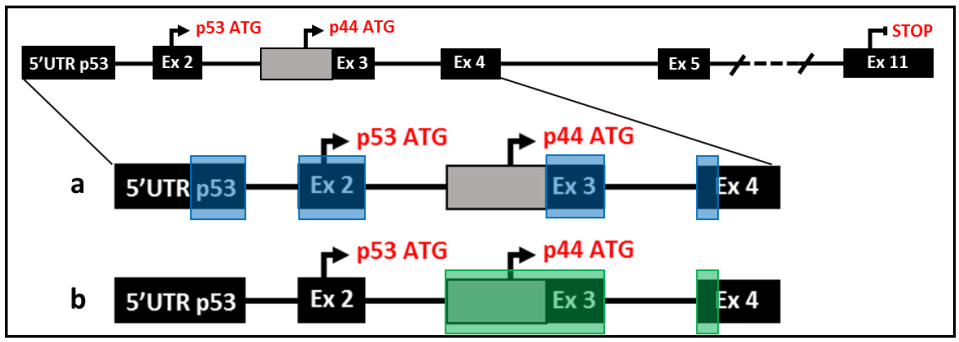


Figure 5. The *p53 locus* in zebrafish. p44 is a splicing isoform of p53. **a)** The p53 probe (blue box) is designed on p53 5'UTR and on exons 2-4 to exclude a non-specific hybridization with p44 mRNA. **b)** The p44 probe (green box) is designed on the *p53* sequence unique to p44 and on exons 3-4 to exclude non-specific hybridization with p53 mRNA.

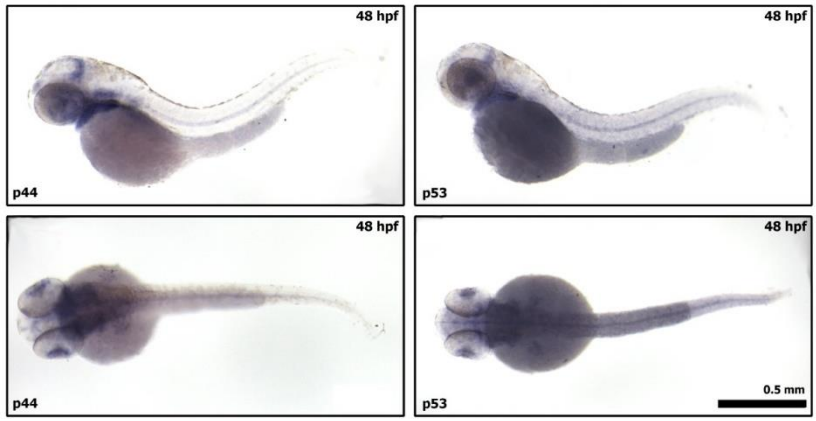


Figure 6. p53 and p44 expression patterns at 48hpf. Whole-mount *in situ* hybridization of p53 and p44 probes on 48hpf zebrafish embryos shows that these isoforms share the same expression domains. p53 and p44 are both expressed (i) in the midbrain-hindbrain boundary; (ii) in the retina; (iii) in the fin buds; (iv) in the pharyngeal arches; (v) in the otic vesicles and (vi) in the hatching glands.

3. Discussion

$p66^{Shc}$ is the longest isoform encoded by the *shc1 locus*. The shortest isoforms, $p52^{Shc}$ and $p46^{Shc}$ are involved in Ras signalling. These two isoforms are phosphorylated by active tyrosine kinase receptors and drive the MAPK (Mitogen-Activated Protein Kinase) cascade (Pelicci *et al*., 1992). The third isoform, $p66^{Shc}$, is translated from a different mRNA. $p66^{Shc}$ contains the entire $p52^{Shc}$/$p46^{Shc}$ sequence plus an additional amino-terminal region (Ventura *et al*., 2002). $p66^{Shc}$ is not involved in Ras signalling, being unable to activate the MAPK cascade. Therefore, $p66^{Shc}$, contrary to $p52^{Shc}$/$p46^{Shc}$ does not contribute to stimulate proliferation (Migliaccio *et al*., 1997). $p66^{Shc}$ is involved in oxidative stress response (OS): OS activates $p66^{Shc}$ and promotes apoptosis (Giorgio *et al.,* 2005). The $p66^{Shc-/-}$ mouse model, generated in 1999, is a genetic model of healthy aging. It has been demonstrated that the deletion of $p66^{Shc}$ improves general health in the aging mouse and that it is associated with an elongation in lifespan, although this datum is still controversial (Migliaccio *et al*., 1999; Ramsey *et al*., 2014). The fact that the ablation of $p66^{Shc}$ is associated with an amelioration of the aging phenotype in laboratory mice and in mouse models of human aging-associated diseases implies that $p66^{Shc}$ has been evolutionary selected for its positive functions exerted in development and/or reproductive phase of life. On the other hand, it is evident as well that $p66^{Shc}$ is not necessary in embryogenesis, because $p66^{Shc-/-}$ mice are viable and $p66^{Shc-/-}$ embryos are not counterselected during gestation. For these reasons, few attentions have been focused in understanding the role of $p66^{Shc}$ in development. Although, differences in phenotypic effects obtained with gene knockdown and mutagenesis

highlighted that mechanisms of genetic compensation may explain why deleterious mutations have sometimes no morphological effects, while gene knockdown leads to developmental alterations: mutations in the genome can trigger up- or downregulation of genes related to the ablated one (Rossi *et al.*, 2015). This means that the ablated gene has not to be considered "necessary", but, nevertheless, it is "functional". This might be the case of $p66^{Shc}$, given that, as it was explained in Part I, it belongs to a family of genes. In fact, there are some evidence suggesting a role for $p66^{Shc}$ in neurogenesis. The expression regulation of the three isoforms of the *shcA* gene was investigated in 1997 through *in situ* hybridization in the brain of the developing mouse: all the three isoforms are exclusively expressed within the ventricular zone, the area were immature neuroblasts proliferate, being $p66^{Shc}$ the less represented isoform. Considering that $p66^{Shc}$, contrary to the other isoforms, is not involved in proliferation, these findings lead to the intriguing hypothesis that $p66^{Shc}$, in concert with $p52^{Shc}/p46^{Shc}$, has the role of orchestrating cell fate in neurogenesis (Conti *et al*., 1997). According with these observations, it has been found that in human and murine embryonic stem cells the overexpression of $p66^{Shc}$ anticipates the loss of pluripotency and induces the acquisition of a neuronal fate (Papadimou *et al*., 2009). These data suggest that $p66^{Shc}$ might have a role in development and in particular in neuronal differentiation.

In zebrafish, it has been shown that specific knockdown of $p52^{Shc}/p46^{Shc}$ leads to defects of intersegmental vessel sprouting angiogenesis (Sweet *et al*., 2012). The aging-associated isoform of Shc, $p66^{Shc}$, has never been investigated in fish. Through bioinformatic analysis it has emerged that the

shc locus in zebrafish shows conservation with mammals: in Ensemble genome browser is present a protein coding putative transcript for $p66^{Shc}$ (ENSDART00000098400). Ensemble prediction was used as a reference for molecular cloning of the $p66^{Shc}$ full length cDNA, demonstrating for the first time that this isoform is actually expressed in zebrafish and that the functional domains present in the N-terminal sequence unique to $p66^{Shc}$ are conserved between zebrafish and mammals (Priami *et al.,* 2015).

Our Real-Time RT-PCR and whole-mount *in situ* hybridization analyses on zebrafish embryos at different developmental stages show that the shorter and the longest isoforms of *shc1* are differently regulated in zebrafish development. $p52^{Shc}$ behaves as a housekeeping gene, as it does not show a temporal regulation from gastrulation to the fifth day of development. Its expression pattern is ubiquitous at 24hpf and 48hpf: this data suggests that $p52^{Shc}$, known for its role in MAPK signalling, might not have a role in tissue specification but in sustaining general cell proliferation. On the contrary, $p66^{Shc}$ is temporally regulated in zebrafish development: its mRNA levels are low at early developmental stages and increase at later stages, reaching a peak between 48hpf and 72hpf, during the organogenesis process, when areas of active cell proliferation become more and more restricted and the vast majority of cell types are differentiated (Kimmel *et al.,* 1995). After 72hpf $p66^{Shc}$ expression slightly decreases. Whole-mount *in situ* hybridization of $p66^{Shc}$ at different developmental stages, from late somitogenesis to 96hpf, shows that $p66^{Shc}$ expression is concentrated, until 24hpf, in neural crest-derived structures, such as the trigeminal placodes and Rohon-Beard neurons, transient mechanoreceptors located dorsally in the spinal cord, that undergo programmed cell death after 24hpf (Reyes *et*

al., 2004; Figure 3). After 48hpf, interestingly, $p66^{Shc}$ is expressed only in the cephalic area: (i) in the brain and in particular in the optic tectum, where neural progenitors are located (Ito *et al.,* 2010); (ii) in the ciliary marginal zone of the retina, where retinal stem cells and retinal progenitor cells are located (Moshiri *et al.,* 2004) and (iii) in the trigeminal ganglion, now positioned ventrally to the brain between the eyes and the otic vesicles, where the nuclei of the touch-sensory neurons of the trigeminal or fifth cranial nerve are located (Kimmel *et al.,* 1995; Figure 4). Our data suggests that $p66^{Shc}$ might be involved in brain development and in the specification of the neural crest-derived structures where it appears to be strongly expressed (trigeminal ganglia and Rohon-Beard neurons). This hypothesis is supported by the fact that (i) also in mammals, $p66^{Shc}$ is localized in the proliferating area of the developing brain, the ventricular zone (Conti *et al.,* 1997), (ii) in human and murine embryonic stem cells, the overexpression of $p66^{Shc}$ induces the acquisition of a neuronal fate (Papadimou *et al*., 2009). Experiments of double *in situ* hybridization of $p66^{Shc}$ together with neural stem cell markers (e.g. *musashi-1*; Sakakibara *et al.,* 1996) and neural crest markers (e.g. *islet-1*; Inoue *et al.,* 1994) will confirm that $p66^{Shc}$ is localized in these cell types.

In this study, we also analysed p53 and p44 expression patterns during zebrafish development through *in situ* hybridization. p44 is a N-terminally truncated isoform that lacks the first transcription activation domain (TAD), but retains the second TAD and all the p53 functional domains, maintaining the ability to form oligomers with the canonical p53 protein. In mammals, it has been shown that the p44 isoform determines p53 functions: it is known that p44, thanks to its intact oligomerization domain, has the ability to form

oligomers with p53, determining its localization and fine-tuning its transcriptional activity, in particular in stress response (Ghosh *et al*., 2004; Bourougaa *et al*., 2010; Powell *et al*., 2008). p44 plays also an important role in development: p44 has been considered an embryonic isoform of p53, whose expression is high in pluripotent stem cells and decrease as differentiation occurs. The main function of p44 in the early stages of embryogenesis is to suppress p53 activity by preventing the trans-suppression of factors such as Nanog and IGF-1R, to preserve pluripotency. When the levels of p44 decrease, the negative regulation of p53 decreases as well and embryonic stem cells begin to differentiate (Ungewitter and Scrable, 2010).

The existence of a p44 ortholog in zebrafish has been shown by Davidson and colleagues in 2010: the microinjection of p44 mRNA in wild-type embryos is associated with severe developmental abnormalities. On the contrary, microinjection of p44 mRNA in $p53^{M214K}$ embryos does not lead to any morphological alteration, suggesting that, as previously shown in mammals, the p44 isoform acts only in cooperation with FLp53. Moreover, p44 overexpression is evident after treatment with ionizing radiation: as in mammals, in zebrafish this isoform is overexpressed in response to stress (Davidson *et al*., 2010). This work supports the idea that zebrafish p44 recapitulates mammalian p44 functions, but do not investigate the role of p44 in physiological conditions.

Here we show that p44 and p53 share the same expression patterns in 48hpf embryos. Both the isoforms are expressed: (i) in the midbrain-hindbrain boundary, an area of persistent cell proliferation until 72hpf (Wulliman and Knipp, 2000); (ii) in the retina; (iii) in the fin buds, another

area of active cell proliferation (Nomura *et al.,* 2006); (iv) in the pharyngeal arches, which will form gills and jaws; (v) in the otic vesicles and (vi) in the hatching glands. It is not surprising that p53 and p44 are expressed in the same areas, considering that (i) p44 has been described as a splicing isoform of p53 (Davidson *et al.,* 2010) and (ii) it is demonstrated that p44 has to interact with p53 to exert its functions (Maier *et al.,* 2004; Ungewitter and Scrable, 2010). Interestingly, both p53 and p44 are expressed in proliferating areas of the developing zebrafish embryo: this observation is consistent with previous data showing that p44 and p53 cooperate during embryogenesis in maintaining the balance between proliferation and differentiation (Ungewitter and Scrable, 2010). We recently generated a $p44^{-/-}$ line in zebrafish in which p53 expression is unaltered: the $p44^{-/-}$ line will be useful to elucidate the role of p44 in development, under normal and stressed conditions.

4. References

Andermann, P.; Ungos, J. and Raible, D.W. 2002. Neurogenin1 defines zebrafish cranial sensory ganglia precursors. Dev Biol, 251(1):45-58.

Bourougaa, K.; Naski, N.; Boularan, C.; Mlynarczyk, C.; Candeias, M.M.; Marullo, S. and Fåhraeus, R. 2010. Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47. ***Mol Cell,*** 38(1):78-88.

Conti, L.; De Fraja, C.; Gulisano, M.; Migliaccio, E.; Govoni, S. and Cattaneo, E. 1997. Expression and activation of SH2/PTB-containing ShcA adaptor protein reflects the pattern of neurogenesis in the mammalian brain. ***Proc Nat Acad Sci USA***, 94(15):8185-90.

Davidson, W.R., Kari, C., Ren, Q., Daroczi, B., Dicker, A.P. and Rodeck, U. 2010. Differential regulation of p53 function by the N-terminal ΔNp53 and Δ113p53 isoforms in zebrafish embryos. ***BMD Dev Biol,*** 10:102.

Ghosh, A.; Stewart, D. and Matlashewski, G. 2004. Regulation of human p53 activity and cell localization by alternative splicing. ***Mol Cell Biol,*** 24(18):7987-97.

Giorgio, M.; Migliaccio, E.; Orsini, F.; Paolucci, D.; Moroni, M.; Contursi, C.; Pelliccia, G.; Luzi, L.; Minucci, S.; Marcaccio, M.; Pinton, P.; Rizzuto, R.; Bernardi, P.; Paolucci, F. and Pelicci, P.G. 2005. Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. ***Cell***, 122(2):221-33.

Inoue, A.; Takahashi, M.; Hatta, K.; Hotta, Y. and Okamoto H. 1994. Developmental regulation of islet-1 mRNA expression during neuronal differentiation in embryonic zebrafish. ***Dev Dyn***, 199(1):1-11.

Ito, Y.; Tanaka, H.; Okamoto, H. and Ohshima, T. 2010. Characterization of neural stem cells and their progeny in the adult zebrafish optic tectum. ***Dev Biol***, 342(1):26-38.

Kimmel, C.B.; Ballard, W.W.; Kimmel, S.R.; Ullmann, B. and Schilling, T.F. 1995. Stages of embryonic development of the zebrafish. ***Dev Dyn,*** 203(3):253-310.

Maier, B.; Gluba, W.; Bernier, B.; Turner, T.; Mohammad, K.; Guise, T.; Sutherland, A.; Thorner, M. and Scrable, H. 2004. Modulation of mammalian life span by the short isoform of p53. ***Genes Dev,*** 18(3):306-19.

McCurley, A.T. and Callard, G.V. 2008. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. ***BMC Mol Biol,*** 9:102.

Migliaccio, E.; Giorgio, M.; Mele, S.; Pelicci, G.; Reboldi, P.; Pandolfi, P.P.; Lanfrancone, L. and Pelicci, P.G. 1999. The p66shc adaptor protein controls oxidative stress response and life span in mammals. ***Nature***, 402(6759): 309-313.

Migliaccio, E.; Mele, S.; Salcini, A.E.; Pelicci, G.; Lai, K.M.; Superti-Furga, G.; Pawson, T.; Di Fiore, P.P.; Lanfrancone, L. and Pelicci, P.G. 1997. Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. ***EMBO J***, 16(4): 706-716.

Moshiri, A.; Close, J. and Reh, T.A. 2004. Retinal stem cells and regeneration. Int J Dev Biol, 48(8-9):1003-14.

Nomura, R.; Kamei, E.; Hotta, Y.; Konishi, M.; Miyake, A. and Itoh N. 2006. Fgf16 is essential for pectoral fin bud formation in zebrafish. ***Biochem Biophys Res Commun,*** 347(1):340-6.

Papadimou, E.; Moiana, A.; Goffredo, D.; Koch, P.; Bertuzzi, S.; Brüstle, O.; Cattaneo, E. and Conti, L. 2009. p66(ShcA) adaptor molecule accelerates ES cell neural induction. ***Mol Cell Neurosci***, 41(1):74-84.

Pelicci, G.; Lanfrancone L.; Grignani, F.; McGlade, J.; Cavallo, F.; Forni, G.; Nicoletti, I.; Grignani, F.; Pawson, T. and Pelicci, P.G. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. ***Cell***, 70(1):93-104.

Powell, D.J.; Hrstka, R.; Candeias, M.; Bourougaa, K.; Vojtesek, B. and Fåhraeus R. 2008. Stress-dependent changes in the properties of p53 complexes by the alternative translation product p53/47. ***Cell cycle,*** 7(7):950-9.

Priami, C.; De Michele, G.; Cotelli, F.; Cellerino, A.; Giorgio, M.; Pelicci, P.G. and Migliaccio, E. 2015. Modelling the p53/p66Shc Aging Pathway in the Shortest Living Vertebrate Nothobranchius Furzeri. 2015. ***Aging dis,*** 6(2):95-108.

Ramsey, J.J.; Tran, D.; Giorgio, M.; Griffey, S.M.; Koehne, A.; Laing, S.T.; Taylor, S.L.; Kim, K.; Cortopassi, G.A.; Lloyd, K.C.; Hagopian, K.; Tomilov, A.A.; Migliaccio, E.; Pelicci, P.G. and McDonald, R.B. 2014. The influence of Shc proteins on life span in mice. ***J Gerontol A Biol Sci Med Sci***, 69(10):1177-85.

Reyes, R.; Haendel, M.; Grant, D.; Melancon, E. and Eisen, J.S. 2004. Slow degeneration of zebrafish Rohon-Beard neurons during programmed cell death. Dev Dyn, 229(1):30-41.

Rossi, A.; Kontarakis, Z.; Gerri, C.; Nolte, H.; Hölper, S.; Krüger, M. and Stainier, D.Y. 2015. Genetic compensation induced by deleterious mutations but not gene knockdowns. ***Nature***, 524(7564):230-3.

Sakakibara, S.; Imai, T.; Hamaguchi, K.; Okabe, M.; Aruga, J.; Nakajima, K.; Yasutomi, D.; Nagata, T.; Kurihara, Y.; Uesugi, S.; Miyata, T.; Ogawa, M.; Mikoshiba, K. and Okano, H. 1996. Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev Biol, 176(2):230-42.

Sweet, D.T.; Chen, Z.; Wiley, D.M.; Bautch, V.L. and Tzima, E. 2012. The adaptor protein Shc integrates growth factor and ECM signaling during postnatal angiogenesis. ***Blood,*** 119(8):1946-55.

Thisse, C. and Thisse, B. 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. ***Nat Prot,*** 3(1):59-69.

Ungewitter, E. and Scrable, H. 2010. Delta40p53 controls the switch from pluripotency to differentiation by regulating IGF signaling in ESCs. ***Genes Dev,*** 24(21):2408-19.

Ventura, A.; Luzi, L.; Pacini, S.; Baldari, C.T. and Pelicci, P.G. 2002. The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter. ***J Biol Chem***, 277(25):22370-6.

Wulliman, M.F. and Knipp, S. 2000. Proliferation pattern changes in the zebrafish brain from embryonic through early postembryonic stages. ***Anat Embryol (Berl),*** 202(5):385-400.

Acknowledgements

I would like to thank Prof. Franco Cotelli (University of Milan), Dr. Enrica Migliaccio (European Institute of Oncology) and Prof. Marco Muzi-Falconi (University of Milan) for mentoring and supervision of my research activity.

I am grateful to Federica Ruscitto for her exceptional contribution in the experimental work as a former MSc student of the University of Milan. I thank Prof. Pier Giuseppe Pelicci, Dr. Marco Giorgo (European Institute of Oncology) and Dr. Alessandro Cellerino (Scuola Normale Superiore di Pisa) for critical discussion.

I also thank Dr. Gianluca Deflorian (FIRC Institute of Molecular Oncology) for his fundamental technical help and Dr. Gianfranco Bellipanni (Temple University) for his contribution in designing the CRISPR/Cas9 strategy described in the “Manuscript in preparation”.

Finally, I would like to thank Prof. Giulio Pavesi (University of Milan) and Dr. Lucilla Luzi (European Institute of Oncology) for help with bioinformatic tools and all the members of Prof. Cotelli’s ZebrafishLab and Dr Migliaccio’s group for their advice.