Epigenetic characterization of skeletal muscle and heart tissue of the turquoise killifish *Nothobranchius furzeri*, a novel short-lived animal model for aging studies

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Datum der Disputation:

“Just keep swimming”

Dory (Finding Nemo, 2003)

Conventions regarding the spelling of genes, proteins and species

All genes and abbreviations thereof are written using lower case and *italic*; e.g. *DNA methyltransferases* or *dnmts*.

microRNAs are abbreviated with miR followed by a "-" and a designated number.

All protein abbreviations are written using capital letters; e.g. PCNA.

Names of species are written in *italic* and abbreviated with the first letter of genus and the name of the species; e.g. *H. sapiens* for *Homo sapiens*. In cases multiple species are mentioned or in front of a gene or microRNA, the species name is also abbreviated with the first two letters; e.g. *Hsa* for *Homo sapiens*.

Antibodies are named as proteins with an additional prefix "anti-".

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1 Introduction

- A brief overview on aging, causes and associated diseases

Multiple definitions of aging are being used in the scientific community. Despite differences, the majority of them agrees that the term aging describes a complex process of a decline of functionality e.g. a gradual loss of fitness and fecundity paired with an increase of mortality over a given period (Rose, 1991; Flatt, 2012; Rose *et al*., 2012). Therefore aging describes a multifaceted phenomenon that has various sources and outcomes. Interestingly, some aspects of aging seem genetically determined as indicated by studies that identified genes involved in the control of lifespan (Knauf *et al*., 2006; Pinton *et al*., 2007; Dërmaku-Sopjani *et al*., 2013; Priami *et al*., 2015) as well as pre-mature aging, often caused by genetic defects (Shumaker *et al*., 2006; Rossi *et al*., 2010; Karikkineth *et al*., 2017; Maierhofer *et al*., 2017). However, for humans, in addition to the loss of reproduction capacity and the loss of fitness, aging often represent a risk factor for overall frailty and important risk factors including many types of cancer (Hoeijmakers, 2009), Alzheimer's disease (Mosher and Wyss-Coray, 2014; Barnes, 2015) and cardiovascular disease (Strait and Lakatta, 2012; Lakatta, 2015). Aging induces many different changes on various levels that might be directly caused by the aging process or are a consequence thereof. It modulates function and structure, gene expression and signaling. While tissues of an individual's body may age at different degree and speed, the underlying mechanisms causing the multifactorial aging process are yet not fully understood. So far, multiple theories have been proposed and various sources have been identified that ultimately lead to the advancement of aging. There is growing evidence that damage and failure of maintenance on molecular and cellular levels accumulate and therefore trigger progression of aging on organismal level. Hence, to study the aging process is of utmost relevance in modern biomedical research where results promise a better life quality to the older people. Here within, molecular causes of aging will be introduced and selected ones will be described in more detail in the consecutive subtopics. Additionally, common aging-associated diseases and animal research models will be briefly outlined.

1.1 Molecular aspects of aging

By identifying nine hallmarks of aging, López-Otín *et al.* (2013) shed light on the cellular and molecular basis of this process (see Fig.1-1). The authors separated these hallmarks into three main groups: primary hallmarks marking the source of damage (I), antagonistic hallmarks in response to initial damage (II) and integrative hallmarks being the perpetrators of functional decline with age (III).

(I) Genomic instability accumulates over a lifetime as multiple threats harm the DNA continuously. Repair mechanisms may fail leading to an accumulation of damaged DNA. Errors during replications, as well as reactive oxygen species (ROS) such as hydrogen peroxide, superoxide or hydroxyl radicals from e.g. mitochondria or exogenous threats from chemical agents, are causing additional DNA damage and may lead to point mutations and translocations. Telomeres are the protective ends of chromosomes that shorten after each replication. Many cell types do not express telomerase reverse transcriptase (TERT), an enzyme responsible for elongation of telomeres at chromosome ends. Hence, a cell will stop dividing if telomeres reach a minimum length to maintain genomic integrity and cell cycle arrest and senescence are induced. The epigenetic alterations occurring during aging are also known as epigenetic drift and will be discussed in more detail below. Loss of protein homeostasis, also called proteostasis, is another hallmark of aging that is caused by declined function of the proteome network and by aggregation of misfolded proteins. This phenomenon can be found in age-associated diseases like Alzheimer's and Parkinson's disease.

(II) Deregulated nutrient sensing, amongst many other factors, leads to accelerated aging. By influencing the expression of nutrient sensing proteins lifespan can be modulated in several model organisms. Mitochondrial dysfunction is increasing with age and leads to oxidative stress. The efficiency of the respiratory chain is reduced while leakage of electrons occurs more often leading to diminished production of ATP. With age, the capability of clearance of ROS is reduced which leads to oxidative stress resulting in damaged protein and DNA. If a critical limit is reached, stable arrest of the cell cycle is induced: this process is known as cellular senescence and is accompanied by phenotypical changes. After a certain number of cell doublings also known as the Hayflick limit (Hayflick and Moorhead, 1961; Hayflick, 1965; Shay and Wright, 2000), the cells remain in a steady state due to telomere shortening and DNA damage preventing genetic errors being passed on descendants. Senescent cells secrete a typical mixture of pro-inflammatory cytokines and matrix metalloproteinases also known as "senescence-associated secretory phenotype" (SASP) (Rodier and Campisi, 2011; Muñoz-Espín and Serrano, 2014).

(III) Altogether, these incidents lead ultimately to stem cell exhaustion and altered cellular signaling determining an "aging phenotype". Stem cells are a valuable resource pool of cells that can divide and differentiate in multiple cell types but also have limited capacity for self-renewal; therefore they are important for tissue regeneration and maintenance. With increasing age, accumulation of damaged DNA and proteins takes place in these cells like in other cells as well; stem cells eventually become senescent or apoptosis is being induced when damage repair is no longer possible, hence leading to depletion or exhaustion of stem cells (Oh *et al.*, 2014). Cellular signaling includes different cascades and pathways that regulate communication between cells. During the aging process

deregulation may become evident at many levels: inflammatory signaling, lack of substrates and oxidative stress are just some examples that result in internal and external communication changes causing alterations at organismal levels (Almeida *et al.*, 2017).

Figure 1-1: The molecular Hallmarks of Aging (López-Otín *et al.*, 2013): nine hallmarks with distinct descriptions of changes during the aging process have been identified, which may influence each other causing further alterations and advance the aging phenotype. Sources of damage such as genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis lead response reaction like deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence ultimately causing stem cell exhaustion and altered intercellular communication (clock-wise description of depicted contributors to the aging process).

1.1.1 DNA damage

During cell cycle, but also in consequence of to environmental influences such as ultra-violet radiation, ROS or chemicals, different forms of DNA damage may occur of which the most severe are double-strand-breaks (DSBs) that require instant repair. Fortunately, throughout the cell cycle, dedicated checkpoints ensure proper DNA synthesis and initiate cell cycle arrest to allow for DNA repair (Shaltiel *et al.*, 2015). There are several forms of DNA damage responses (DDR) and distinct DNA repair mechanisms are activated depending on the type of damage: nucleotide mutation or damage of a single DNA strand can be repaired by nucleotide excision repair (NER) and base excision repair (BER), while double strand breaks are efficiently repaired by homologous recombination repair (HRR) and non-homologous end joining (NHEJ). For BER, DNA glycosylases recognize single altered DNA and remove them restoring the original unmodified nucleotide. Conversely, NER is activated if a larger stretch of DNA is damaged or modified, and the removal of the altered part is executed by excision nucleases in cooperation with DNA helicases. DNA polymerase and DNA ligases are involved in the subsequent reconstruction of the double strand. While during NHEJ the ends of broken DNA strands are re-ligated and thereby loss of nucleotides occurs, the HRR is somewhat more complicated as it involves the sister chromatid as a template. This repair mechanism might be more energy- and time-consuming, however, no mutations are introduced into the primary DNA sequence. If DDR is not possible, apoptosis is initiated to prevent the spreading of mutated DNA in dividing sister or descending cells (Roos and Kaina, 2013).

Specific epigenetic changes are associated with DNA damage. Upon occurrence of DSBs, for example, the histone 2A (H2A) variant H2AX becomes phosphorylated at the Serine 139 and is then termed γH2AX. γH2AX is involved in recruiting DNA repair proteins: hence, γH2AX has been shown to be a valid marker for DNA damage (Kuo and Yang, 2008). Once the DNA damage has been repaired γH2AX is de-phosphorylated.

1.1.2 Mitochondria and oxidative stress

Mitochondria are cellular organelles that can be found in almost every eukaryotic cell and provide adenosine tri-phosphate (ATP) via oxidative phosphorylation (OXPHOS) for the energy demand of the cell. Mitochondria are not only the main supplier of cellular energy by the respiratory chain, but also of other products such as amino acids and lipids. In addition to that, substrates from the conversion of energy and metabolism play an important role in methylation and acetylation. Of note, several metabolites derived from the tricarboxylic acid (TCA) cycle also catalyze important epigenetic reactions: e.g. α-ketoglutarate (αKG), derived by isocitrate dehydrogenase (IDH) is a co-substrate for demethylating enzymes like ten-eleven translocases (TETs) and jumonji C (JmjC) demethylases all

belonging to the large dioxygenase family (Kohli and Zhang, 2013). In recent years, specific mutated IDH forms have been identified as the source of the so-called oncometabolite hydroxy-ketoglutarate that inhibits TET function and that of other enzymes functionally depending on αKG (Xu *et al*., 2011). S-adenosylmethionine (SAM) is a methyl donor for histone methylases and DNA methyltransferases (Gut and Verdin, 2013). In addition, the acetyl CoA (ACoA) abundantly synthesized during glycolysis and TCA cycle is also crucial for the activity of Lysine acetylases being the most important source of acetyl-groups (Choudhary *et al*., 2014; Drazic *et al*., 2016). Furthermore, mitochondria are essential for calcium storage and signaling; in cardiovascular diseases often the impaired function of mitochondria leads to less efficient calcium handling with negative consequences on the output of the heart and thus to heart failure (Cartwright *et al.*, 2011; Eisner, 2014; Feridooni *et al.*, 2015).

Dysfunctional mitochondria are the main source for ROS that lead to oxidative stress and DNA damage. In this condition, aggregates of oxidized proteins may form and thereby accelerate the aging process (Gonzalez-Freire *et al.*, 2015). The contribution of ROS to the aging process has been postulated since the 1950s with the "free radical theory of aging" (Harman, 1956; 1992). Oxidative stress in fact describes an imbalance of free radicals and the impaired depletion thereof (Beckman and Ames, 1998; Balaban *et al*., 2005). During aging mitochondrial function is impaired due to multiple reasons, leading to oxidative stress which is present in many different pathophysiological conditions associated with aging-related processes like inflammation and senescence (Cencioni *et al.*, 2013; Sun *et al.*, 2016). In aged animals, accumulation of dysfunctional mitochondria occurs in many tissue types (Sun *et al.*, 2016). In fact, it has been reported that mitochondrial DNA (mtDNA) instability increases with age and is subject to reorganization; these processes take place in all organisms from lower eukaryotes to mammals (Szklarczyk *et al*., 2014; Pinto and Moraes, 2015). Aberrant mitochondrial function leads to a lack of energy supply. Hence, cells will need to switch metabolic pathways from beta-oxidation of fatty acids to glycolysis of simple carbohydrates to ensure proper cellular function. In cancer the switch from OXPHOS to glycolysis is well known as the Warburg effect. There has been evidence that during aging a switch of metabolites similar to oncometabolites is taking place generating the so-called gerometabolites (Menendez *et al.*, 2014), which may cause epigenetic changes similar to those occurring in tumors. Gerometabolites are small molecules such as nicotinamide adenine dinucleotide (NAD^+) which decreases with age and thereby leads to advancement of the aging process (Verdin, 2015). NAD^+ plays an important role for the degradation of HIF1α via Sirtuin1 (SIRT1). With low levels of NAD^+, pseudo-hypoxic environments are established and oncometabolites such as fumarate and succinate accumulate while αKG is reduced to R-2-hydroxyglutaric acid (2-HG), another driver of tumorigenesis (Raffaghello and Longo, 2017).

In vitro, conditions determining oxidative stress are mimicked in various ways: through the addition of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), ATP generation and phosphorylation are affected as it is an ionophore that interacts with the electron transport chain and uncouples the proton gradient. Also, by radiation or adding hydrogen peroxide to cell culture media, oxidative stress can be induced virtually in all cell types.

1.1.3 Cell cycle and proliferation

Cells proliferate by duplication and division, a procedure that is also known as cell cycle. This is a neatly controlled process with several checkpoints to ensure appropriate DNA replication and cell division. The production of germline cells via cell division is called meiosis, while mitosis is intended as the cell division of somatic cells. Cell division can be divided into four phases: gap1 (G1), synthesis (S), gap2 (G2) and mitosis (M). During G1 phase cells grow slowly and might undergo into a quiescent phase named G0, however, after passing the G1 check point cells enter the S phase in which DNA replication is performed at variable speed. In phase G2 the cell checks for errors (G2 checkpoint), continues to grow and prepares for mitosis. In fact, during G2 phase potential errors of DNA replication may be repaired to prevent mutations being passed on to the daughter cells. Mitosis leads to formation of two identical cells with a diploid genome, each containing two sets of chromosomes, while meiosis produces four haploid cells (gametes) with one set of chromosomes, respectively. Meiosis is divided into two phases: after chromosome duplication during S-phase, meiosis (I) leads to segregation of homolog chromosomes which is followed by meiosis (II), that results in segregation of sister chromatids (Alberts *et al.*, 2008).

During aging, proliferation and cell cycle of somatic cells may slow down for various reasons: the organism is fully grown and proliferation of cells occurs mainly for maintenance of intact tissue and function. The process of cell cycle can be stopped due to accumulation of DNA mutations and damage, which should not be passed on to daughter cells. After a certain number of cell divisions telomeres that protect the ends of chromosomes are becoming too short to ensure e.g. proper protection. In the case of cell cycle arrest due to short telomeres or DNA damage, cells become senescent. Senescent cells are irreversible arrested and do not proliferate any longer.

A small number of enzymes are involved in the tight control of cell cycle. Cyclins are binding to cyclin dependent kinases (CDKs) and thereby form a complex that phosphorylates a specific subset of proteins that are responsible for cell cycle progression, whilst regulating also other processes (Bendris *et al.*, 2015). During each phase of the cell cycle, specific cyclin-CDK complexes are activated (Fig.1-2A). Inhibitors of CDKs (CDKIs) lead to conformation changes and inactive cyclin-CDK

complexes. CDKIs are divided into two subgroups: the INK4 family and the KIP/CIP family (Fig.1-2A; red boxes).

The *ink4* locus consists of *p15*, *p16*, *p18* and *p19* that – upon accumulation - can lead to G1 arrest and cellular senescence (Simboeck *et al.*, 2011). In fact, high expression levels of *ink4* have been shown to be a biomarker for aging (Krishnamurthy *et al*., 2004). During tumorigenesis the gene products of INK4 locus are indeed often mutated or inactivated (Cánepa *et al.*, 2007). Knock-out studies with mice, for example, have shown that individual depletions of members of the INK4 family lead to spontaneous cancer formation when compared to wild-type control animals. In addition, the INK4 locus has been shown to be a target of polycomb mediated tri-methylation, which leads to gene silencing (Popov and Gil, 2010) (Fig.1-2B). Remarkably, in lower organisms, such as zebrafish and killifish, *p16* is not present. Nevertheless, different fish species are being used as animal models for cancer research. Screening for cancerogenic compounds and drugs for treatment are being performed as well as using transplantation of human tumor cells and xenografts to investigate a broad variety of cancer types such as thyroid and pancreatic cancer, leukemia and hepatocellular cancer (Blackburn and Langenau, 2014; Zhao *et al*., 2015; Astone *et al*., 2017). While zebrafish have a low incidence of neoplasia formation, spontaneous tumor formation has been reported for old killifish (Di Cicco *et al*., 2011). Besides fish, usually rodents are used for cancer studies and many different types of models are investigated for preclinical and basic research studies (Cheon and Orsulic, 2011; Day *et al.*, 2015). Interestingly, some species develop spontaneous tumors with age while others are tumor-free till old age (Pompei *et al*., 2001; Gorbunova *et al*., 2014).

The KIP/CIP family consists also of several members of which *p21* is the most prominent, that leads to cell cycle arrest and ultimately to cellular senescence. p21 also plays a role in DNA damage repair when binding to proliferating cell nuclear antigen (PCNA) (Karimian *et al.*, 2016). The expression of *pcna* is a hallmark of cell cycle and proliferation as it is located in the nucleus and interacts with polymerase δ at the replication fork (Moldovan *et al.*, 2007; Strzalka and Ziemienowicz, 2011).

The tumor repressor p53, which plays various roles during the aging process (Nicolai *et al.*, 2015), is also involved in control of cell cycle. Its activation upon oxidative stress or DNA damage can lead to cell cycle arrest and DNA repair or induction of apoptosis and thereby to protection against cancer and genomic instability (Efeyan and Serrano, 2007).

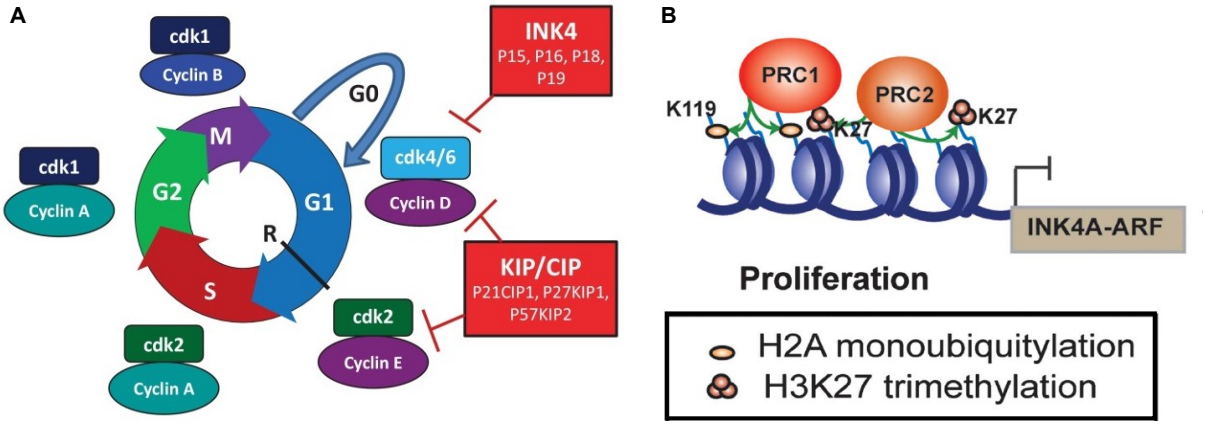

Figure 1-2: Cell cycle and its regulation. (A) Control of the cell cycle by cyclin-CDK complexes and inhibitors thereof (after Hardwick and Philpott 2014. Mitosis (M) is followed by a gap phase (G1) during which repair may take place. During the synthesis phase (S) DNA becomes replicated; afterwards a second gap phase (G2) is initiated. The cell may also enter a quiescent state (G0) and re-enter cell cycle at a later point. Specific cyclins and CDKs are responsible for advancing cycle during each phase. Inhibitors of the cyclin/CDK complex (red boxes) block the progression of cell cycle. (B) The genomic sequence of the INK4 locus is object to epigenetic modifications that lead to silencing of the genes (after Ribeiro *et al.* 2013): Tri-methylation of the locus is mediated by PRC1 and PRC2 complex and leads thereby to silencing of gene expression of genes of the INK4 locus, leading to aberrant cell cycle progression.

1.1.4 Epigenetic modifications during aging: an overview

Epigenetics comprise all information stratified in addition to the information of the DNA sequence and transmitted horizontally from cell to cell or vertically from ancestors to descendants. More specifically, epigenetics may be information for folding and transcription of DNA as well as post-translational modifications (PTM), which are attached to proteins by very specialized enzymes. Hence, epigenetics change the genome but do not involve changes in DNA sequence (Brunet and Berger, 2014). Many twin studies address the impact of epigenetic changes occurring to individuals with the same genetic onset, which might be due to different environmental exposure and/or diet (Bell and Spector, 2011). Depending on the need of each cell and each organ at a given time post-translational modifications may change while aging or in the case of diseases. Among many, at least three major subclasses of epigenetic components are important for this study: 1) Histone modifications, 2) DNA methylation and 3) non-coding RNA expression. Changes in these factors are made or regulated by specific enzymes and can be reversed or inhibited, which makes them an interesting targets for the development of drugs aiming at introducing changes in gene expression without altering the primary DNA sequence.

1.1.4.1 *Histones and their structural and functional role*

Depending on species the genome comprises 10^5 to 10^{12} base pairs (bp) per nuclei and due to space limitations in the nucleus not all DNA is always accessible. In fact, portions of the genome that are not needed at a given time point must be condensed and packed away (Fig.1-3A). In this context, two forms of chromatin are generally to be distinguished: i) heterochromatin that is the condensed form of chromatin inaccessible to transcriptional proteins. It is usually not actively transcribed or at very low rate, and ii) euchromatin with a relaxed structure often present in regions undergoing active transcription. The typical chromatin organization is determined by a chain of the nucleosomes. A nucleosome is made of 146 bp of DNA are wrapped around a histone core consisting two of each of the following histones: H2A, H2B, H3 and H4 (Matouk and Marsden, 2008). These eight histones build an octamer core protein with double-stranded DNA wrapped around. Nucleosomal DNA might be further condensed by coil-coil interactions (Luger *et al.*, 1997; Luger and Richmond, 1998). This complex structure is further stabilized by the interaction with extranucleosomal proteins including the "linker" histone 1 (H1), which aids the formation of condensed structures contributing together with the heterochromatin protein1 (HP1) to compacting the chromatin into 30 nm thick fibers (Robinson and Rhodes, 2006). In addition to the canonical histones also non-allelic variants of some histones are known that affect chromatin dynamics, genomic stability and DNA repair (Biterge and Schneider, 2014). They differ greatly in amino acid composition and sequence when compared to the canonical histones. Each histone has a tail of amino acids that protrudes out from the nucleosome and on which modifications such as acetylation, phosphorylation ubiquitination or methylation of certain amino acids can occur (Kouzarides, 2007; Bannister and Kouzarides, 2011). There are amino acids such as Lysine (K) that are more prone to modifications than others and in many cases up to three residues are attached to a specific amino acid. At the moment, more than 1000 different histone modifications have been documented, of which the functional or structural meaning is only partially or poorly understood (Pal and Tyler, 2016).

1.1.4.2 *Epigenetic enzymes involved in histone and DNA methylation and demethylation*

Enzymes involved in epigenetic processes can be classified into three different groups, the so-called epigenetic writers, readers and erasers (Fig.3C). Enzymes that introduce new histone modifications are also called epigenetic writers. Epigenetic readers recognize the histone mark and recruit multi-protein complexes which may modify gene expression with their enzymatic activity, while enzymes that are responsible for the removal of histone marks are known as epigenetic erasers. Here, selected examples for methylation of histones and DNA will be introduced in brief.

The repressive H3K27me3 is established by the Polycomb Repressive Complex (PRC) 2. There are two main polycomb complexes, a larger PRC1 and a highly conserved PRC2 that form the Polycomb Group (PcG). Silencing gene activity during development is the most prominent known function of H3K27me3 regulating gene expression (Di Croce and Helin, 2013). PcG, for example, targets genes important for cell differentiation and lineage commitment (Bracken *et al*., 2006, 2007). Subunits of the polycomb repressive complex 2 (PRC2) are responsible for methyl residue transfer: the complex consists of the four members polycomb subunit SUZ12 (SUZ12), embryonic ectoderm development (EED) and enhancer of zeste (EZH) 1 or 2, with 2 (EZH2) being the main actor. Although less characterized, Polycomb repressive complex 1 (PRC1) is also involved in histone methylation (Schuettengruber *et al*., 2007, 2017).

Removal of methyl groups – namely demethylation - of the H3K27me3 histone mark is mediated by lysine demethylases (KDMs) mainly KDM6a and KDM6b. Other repressive histone marks such as histone 3, lysine 9, tri-methylation (H3K9me3) and histone 4, lysine 20, tri-methylation (H4K20me3) have been investigated in this study and by others. These histone marks have also specific methyl transferases and demethylases that modify the Lysine-attached residues. The Lysine demethylases KDM6, with KDM6b being also known as UTX, are responsible for removal of the trimethylation mark. Ablation of *kdm6b* in *C. elegans* leads up to 30 % lifespan extension (Jin *et al.*, 2011; Maures *et al.*, 2011) by reducing *insulin-like receptor subunit β* (*daf2*) pathway genes and increasing daf16 (FOXO) translocation to the nucleus, eventually changing nutrient sensing and uptake.

Other groups of specialized enzymes are responsible for the transfer and removal of acetyl residues to and from Lysine. The histone acetyl transferases (HATs) add acetyl residues while histone de-acetylases (HDACs) are responsible for their removal (Fig.1-3B and C).

Modification of DNA is performed by specific enzyme families as well. There are three DNA methyltransferases (DNMTs) involved in DNA methylation process at the fifth carbon base of cytosine generating 5-methyl-cytosine (5mC) using S-adenosylmethionine (SAM) as methyl donor. DNMT1 is responsible for maintenance of methylation pattern after replication and mitosis, while DNMT3a and DNMT3b enable *de-novo* DNA methylation during development (Wu and Zhang, 2010).

On the opposite, Ten-Eleven translocation proteins (TETs) and Thymine DNA Glycosylase (TDG) are involved in the demethylation of DNA by stepwise oxidation of the 5-methylcytosine. During this process, TET proteins use molecular oxygen to catalyze decarboxylation of α-keto-glutarate which then generates a highly reactive intermediate that converts 5mC to 5-hydroxy-methyl cytosine (5hmC) (Kohli and Zhang, 2013). In an iterative fashion, 5hmC becomes further oxidized to 5 formyl-cytosine (5fmC) and 5-carboxy-cytosine (5caC) (Lu *et al.*, 2015). In addition to its de-methylating

function, TDG is playing an important role in the process of DNA repair as part of the base excision repair machinery (BER) (Weber *et al.*, 2016).

1.1.4.3 ***Histone modifications***

In general, methylation of histones is often associated with a more condensed, and therefore closed chromatin confirmation, which means that most likely gene expression is downregulated or switched off. Methylation of certain histone marks - such as H3K27me3 and H3K9me3 - is closely associated with methylation of DNA (Cedar and Bergman, 2009) and many sites of methylated DNA harbor also methylated histone residues. However, other methylation marks such as H3K4me3 and H3K27me1 are transcription activating signals. Histone 3, lysine 4, tri-methylation (H3K4me3), for example, is associated with an open chromatin due to its conformation. Meanwhile, acetylation of histones is rather associated with an open chromatin configuration and therefore an active gene expression, e.g. regions rich in histone 4, lysine 16 acetylation (H4K16ac), histone 3, lysine 9 acetylation (H3K9ac).

During aging histone modifications are subject to change in many ways affecting gene expression and genomic stability, DNA repair and replication. In humans, global histone methylation is decreased with age, while it might be accumulated at specific loci (Weidner and Wagner, 2014). This phenomenon may lead to a loss of heterochromatin and therefore to increased genome instability which is more prone to DNA damage and might favor disease development (O'Sullivan and Karlseder, 2012).

For this thesis, the focus was directed to histone modifications associated with closed chromatin conformations, more specifically to the tri-methylated form of histone 3, lysine 27 (H3K27me3). In many diseases global methylation pattern changes and in many types of cancer such as colorectal, bladder, endometrial or gastric cancer, the genome becomes hypermethylated due to aberrant expression of methyltransferases. Thereby, genes controlling cell cycle and proliferation such as the INK4 locus become methylated and are hence silenced and neoplasia and tumors may form. DNA damage and inflammation also have been reported to be associated with increased levels of H3K27me3. Senescence-associated heterochromatin foci (SAHF) are regions of the genome in which H3K9me3 are enriched with aging. During mammalian aging a global loss of histone methylation of histone3 K9 and K27 is observed, while only certain CpG islands might show increased methylation with age (López-Otín *et al.*, 2013). The INK/Arf locus harboring *p15*/*p16* tumor suppressors is repressed by H3K27 trimethylation in proliferating cells. However, KDM6b demethylates this locus during aging. Thereby, their cell cycle suppressing function is activated, and senescence is induced (Agger *et al.*, 2009; Agherbi *et al.*, 2009).

Loss of heterochromatin and decreased levels of H3K27me3 and H3K9me3 are observed in patients with the Hutchinson-Gilford progeria syndrome (HGPS) which are similar epigenetic alterations like those occurring during chronical aging (Shumaker *et al.*, 2006).

1.1.4.4 ***DNA Methylation***

DNA methylation describes the process of the addition of methyl residues (-CH3) to different types of nucleotides of which methylated cytosines are the most abundant ones in eukaryotes. So far several modifications of cytosine and adenine are known to convey information additional to the genomic sequence including 5-methyl-cytosine (5mC) and 5-hydroxymethyl-cytosine (5mhC), N4-methyl-cytosine (m4C), 6-methyl-adenine (6mA) and 6-carboxy-methyl-adenine (6cmA) (Heyn and Esteller, 2015). 5mC and 5hmC are among the best characterized DNA modifications. While 5mC is enriched at promoters of silenced genes, 5hmC can be primarily found at the promoter region of actively transcribed genes (Bachman *et al.*, 2014). DNA methylation and hydroxymethylation influence developmental stages as well as stages of disease (Greco *et al.*, 2016).

The methylation of cytosines (5mC) is an event that occurs inside and outside coding portions of the genome and is particularly evident in those regions also known as “CpG islands”, where cytosine and guanine are symmetrically present. The majority of CpG islands can be found in intergenic regions of highly repetitive DNA sequence elements, which are highly methylated in healthy somatic cells (Matouk and Marsden, 2008). 5mC is a stable DNA modification that often, although not exclusively, occurs during replication and that can be passed on to daughter cell ensuring the transmission of the methylation pattern (Bachmann and Bergmann, 2012). DNA methylation of cytosine in promoter regions is a way to silence a particular stretch of DNA and therefore represses gene expression by preventing the RNA polymerase to bind and thus leading to heterochromatin formation. During aging, a loss of global methylation in all tissues has been observed. Interestingly, an increase of DNA methylation is occurring mainly at CpG islands with age (Bell, CG *et al.*, 2012; Bell, JT *et al.*, 2012). The determination of the so-called “epigenetic clock”, in fact, is based on the methylation status of 353 CpG islands. It is possible to estimate human chronological age with an accuracy of about +/- 3.5 years by analyzing their methylation status (Horvath, 2013). In many diseases DNA methylation has been shown to be aberrantly divergent from the methylation patterns present in healthy individuals and is yet associated with accelerated epigenetic age (Horvath *et al*., 2016; Knight *et al*., 2016; Fries *et al*., 2017; Maierhofer *et al*., 2017).

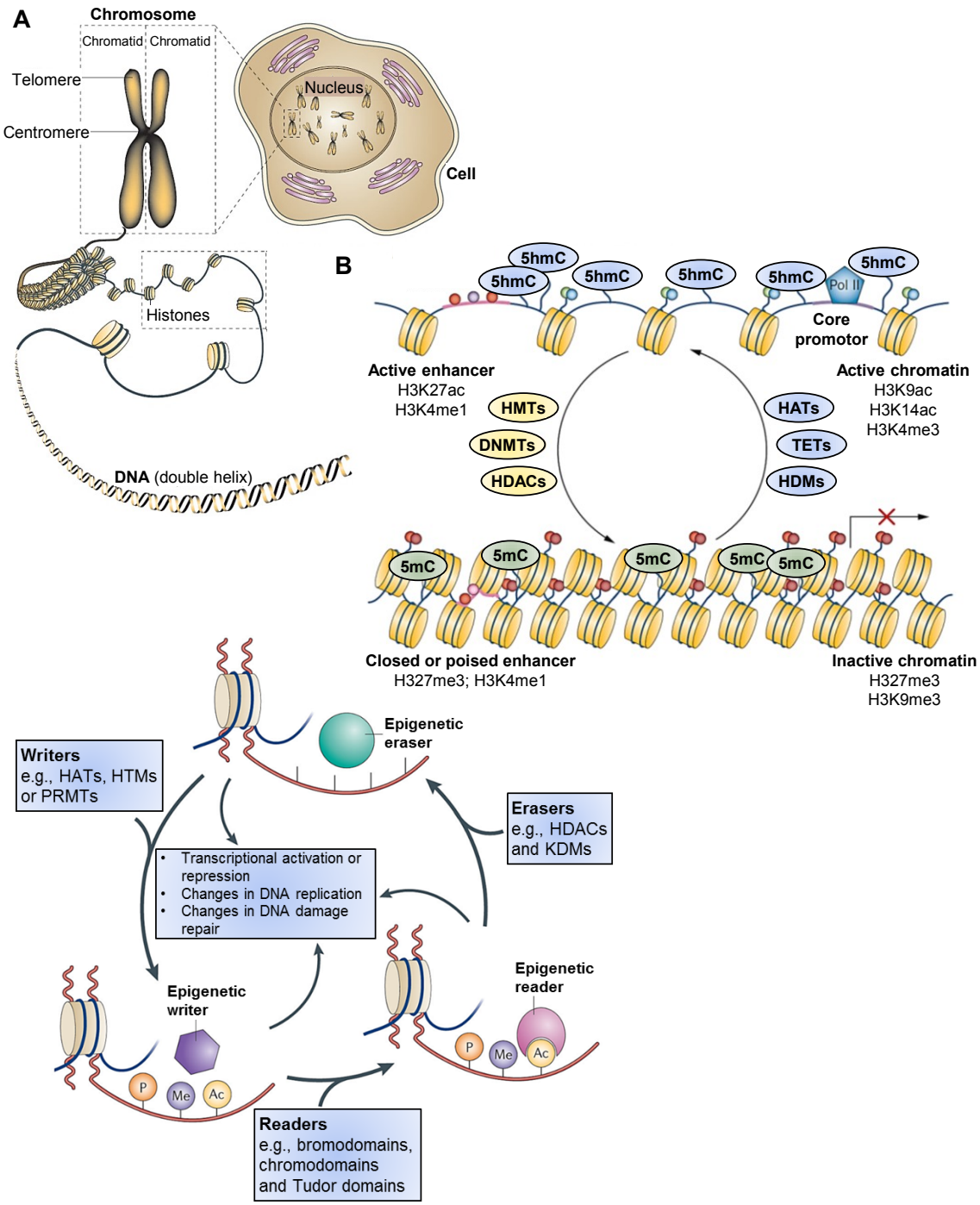

Figure 1-3: Epigenetic marks impact transcription : (A) In the nucleus chromosomes can be found that consist of DNA wrapped around histones forming nucleosomes (modified after Sparmann and Van Lohuizen, 2006). (B) Open chromatin (upper part) is generally marked by 5-hydroxy-methyl cytosine (5hmC) on DNA and polymerase (Pol II) that transcribes DNA into mRNA. Active histone marks such as H3K27ac, H3K9ac and H3K4me3 can be found. Histone methyl transferases (HMTs), DNA methyltransferases (DNMTs) and histone de-acetylases (HDACs) establish markers of heterochromatin. They are involved in forming closed chromatin conformation such as 5 methyl cytosine (5mC), H3K27me3, H3K9me3 and H3K4me1. Histone acetyl transferases (HATs), ten-eleven translocases (TETs) and histone demethylases (HDMs) are involved in reversing the closed chromatin towards an open euchromatin structure (modified after Greco and Condorelli, 2015). (C) Enzymes that establish marks are epigenetic writers; those, processing the information are epigenetic readers and those erasing the marks are erasers (modified after Falkenberg and Johnstone, 2014).

1.1.4.5 ***microRNAs***

While the majority of the genome is transcribed, only a small percentage of it encodes proteins and therefore non-coding RNAs (ncRNAs) represent the largest portion of transcripts. ncRNAs are classified according to their size. Long non-coding (lnc) RNAs are more than 200 nucleotides long, while small non-coding RNAs have less than 200 nucleotides (Kung *et al.*, 2013). microRNAs or miRNAs are small molecules of about 20-24 nucleotides of RNA that are highly conserved across species (Bartel, 2009, 2004), in humans about 1881 different miRNAs are annotated (Kozomara and Griffiths-Jones, 2014). Biogenesis of miRNA is a multistep process: In the nucleus, a primary transcript with a hairpin structure of the miRNA (pri-miR) is cleaved by Drosha into pre-miR and exported into the cytoplasm. The Dicer protein hydrolyzes the pre-miR into a duplex structure, which is then further processed into the mature miRNA structure by the Argonaut 2 (Ago2) protein and the RNA-induced silencing complex (RISC; Fig.1-4A).

miRNAs are able to regulate transcription and gene expression by binding to mRNA. The double-stranded RNA molecule will become degraded, which then leads to repression of expression of a particular gene. One miRNA may have many different targets that can be regulated and a gene can be targeted by several different miRNAs. Aberrant expression of miRNAs can be observed in many types of diseases, including cancer and cardiovascular afflictions (Gurha and Marian, 2013; Tuna *et al*., 2015; Costantino *et al*., 2016). However, not only disease may change expression, also aging and aging-associated implications lead to a miRNA expression pattern that differs from that of healthy, young individuals (Fig.1-4B) (Menghini *et al*., 2014; Lee *et al*., 2015; Seeger and Boon, 2015).

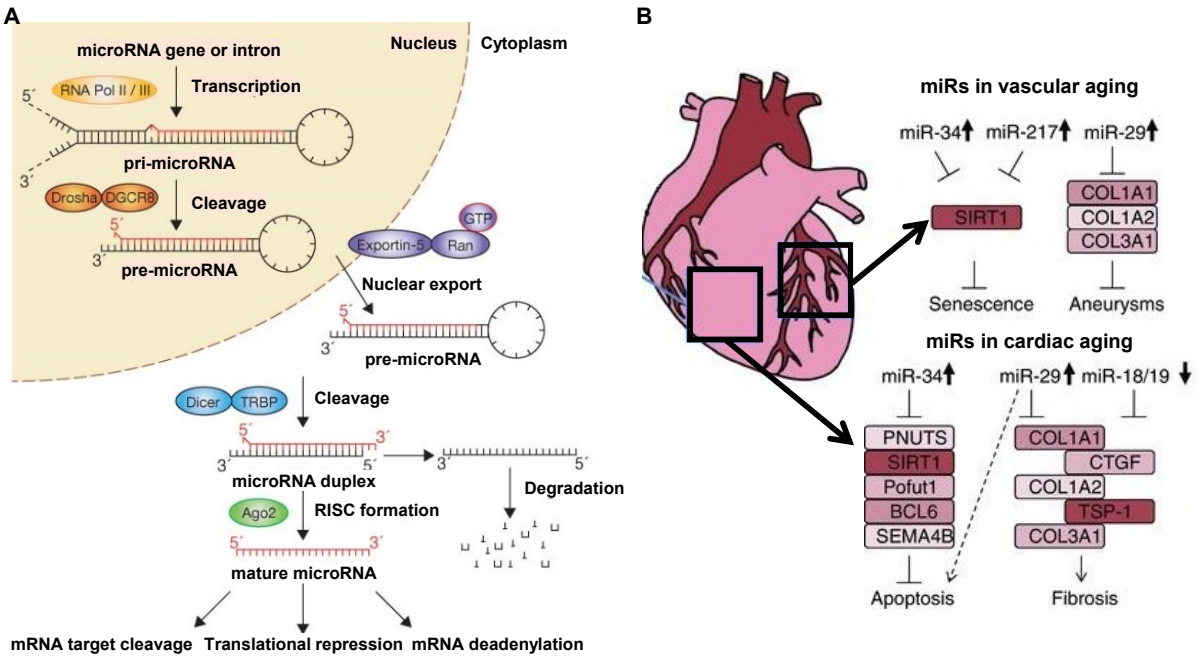

Figure 1-4: miRNAs play important roles in myocardial aging and disease : (A) Biosynthesis of microRNA: (modified after Winter *et al.* 2009). The microRNA gene becomes transcribed by RNA polymerase II/III. The hairpin structure of the pri-microRNA is cleaved into a pre-microRNA by Drosha and then exported into the cytoplasm. Further cleavage by Dicer leads to a microRNA duplex structure, which becomes a functional microRNA via maturation mediated by Ago2 and RISC. (B) Examples of miRNA in vascular and cardiac aging (modified after Seeger and Boon, 2015). A complex interplay of multiple microRNAs that are regulated by the aging process affects other cellular processes such as apoptosis, senescence and formation of aneurysms or fibrosis.

In the field of cardiovascular research, miR-29 is known for playing opposing roles when it comes to cardiac health and disease, hence it was subject to more detailed investigations described hereafter in this thesis. On one side it plays a critical role in development and growth regulation (Kamran *et al.*, 2015), on the other side it is involved in many types of diseases including heart disease and aneurysm formation (Boon and Dimmeler, 2011). It is known for being a negative regulator of fibrosis (Gurha and Marian, 2013), repressor of methyltransferases (Morita *et al.*, 2013) and counteracts miR-21 (Zanotti *et al.*, 2015). After myocardial infarction (MI), miR-29 is downregulated in the region of fibrotic scar, leading to an increased level of collagen (van Rooij *et al.*, 2008). High levels of miR-29 have been associated with disorganization of myofibrils (Slusarz and Pulakat, 2015). Interestingly, miR-29 accumulates with age (Boon *et al.*, 2011) and is likely to be involved in apoptosis of cardiomyocytes (Seeger and Boon, 2015). miR-29 has multiple targets, among those are enzymes involved in DNA methylation processes, namely DNMTs (Morita *et al.*, 2013) and TETs (Zhang *et al.*, 2013) as well as collagens (COLs) (Van Rooij and Olson, 2012; Zanotti *et al.*, 2015).

1.2 Aging challenges Western societies

Over the past few decades tremendous success has been reached by improving medical care and environmental factors. In many industrialized countries, people are becoming older and mid-lifespan increases with the population of elderly is growing significantly (Kanasi *et al.*, 2016). In Europe and many other countries maximum life expectancy at birth is increased considerably. While 39 % of all females born worldwide between 1950 and 1955 survived until the age of 80, 60 % of all females born between 2000 and 2005 are expected to survive until the age of 80. In the birth cohort of 1950-1955, 29 % of all males were expected to reach the age of 80 while in the cohort of 2000-2005 52 % of all males worldwide will reach the age of 80 (United Nations, 2015). The worldwide median age has been increasing over the last fifty years as well rising from 23.9 in 1950 to 29.6 in 2015, being for 2017 in Germany about 46.2 years and Europe 41.7 (United Nations, 2015). In Germany, as well as in other Western countries, life expectancy is on the rise, averaging for women at 82.8 years and men at 77.7 years (Pötzsch and Rößger, 2015). Moreover, life expectancy and therefore the percentage of elderly people are predicted to continue to be growing. In Europe, a quarter of the population is older than 60 years, worldwide 13 % of all humans are above the age of 60 and this age group continues to be growing faster than younger age cohorts and are expected to double by 2050 (United Nations, 2017). Hence, it is of major interest for many researchers to improve quality of life and thus enabling a healthy life as long as possible. The major goal is to extent the time spent in a disease-free condition, also described by the term healthspan.

Despite having different onsets, many conditions have a common denominator: aging. With increasing age, chronic diseases are becoming an important health and economic problem and represent relevant causes for death. In fact, nowadays, about 40.5 % of all female deaths are caused by cardiovascular diseases such as ischemia and hypertension, 4 % are estimated to be caused by cancer. About 31.8 % of all males are estimated to die from cardiovascular diseases and 9 % from cancer (WHO, 2014). Therefore, it is of great interest to investigate pathophysiological pathways relevant in these non-communicable aging-associated diseases with the aim of improving the health and life expectancy of elderly people worldwide. To achieve this goal, it becomes apparent that there is a growing demand for more aging research: i) To investigate changes in life quality occurring at many levels; ii) to understand better the mechanisms of aging; iii) to be able to decelerate the mechanisms of the aging process and fight diseases that occur primarily at old age aiming at a longer and healthy lifespan for people.

Meanwhile, aging as such is not determined to be a disease itself, only aging-associated diseases have been recognized by the Food and Drug Administration (FDA). However, efforts are being undertaken to reach such classification in order to ease the implementation of pharmaceutical

interventions and treatment regimens to extend lifespan. In the following, a brief overview of the most common aging-associated diseases is given.

1.3 Aging-associated diseases: selected aspects

Many diseases are associated with increased age or are more likely to occur at old age including such as cardiovascular diseases (Strait and Lakatta, 2012; Lakatta, 2015), Alzheimer's (Mosher and Wyss-Coray, 2014; Barnes, 2015) and any types of cancer (Hoeijmakers, 2009), while pre-mature aging is often caused by genetic defects.

1.3.1 Cardiovascular disease

The heart undergoes significant changes as an individual person ages. Slower contraction and incomplete relaxation lead to reduced contractile function. This can be observed also during exercising and an age-related cardiac hypertrophy while calcium signaling might be impaired (Feridooni *et al.*, 2015). An overall decline in functional capacity over time and increasing loss of cardiac myocytes may foster heart failure (Oxenham and Sharpe, 2003). Remarkably, cardiovascular diseases (CVD) are a major cause for death worldwide and aging is the most important risk factor for cardiovascular disease (WHO, 2014). Myocardial infarction (MI), ischemia, hypertension, congestive heart failure and lack of regeneration after injury are just few examples that many people are confronted with and suffer from. After MI, new extracellular matrix (ECM) and fibrotic tissue form which results in a stiffened infarcted area that is less contractile and less functional and constitutes scar tissue (Lighthouse and Small, 2016). Thereby a lack of oxygen availability may occur and risk for arrhythmia rises (Stratton and McKinsey, 2016). Cardiac fibrosis impairs heart function likewise: fibrogenesis is induced by transforming growth factor β (TGFβ) causing hypoxia and disturbed matrix-metalloproteases (MMPs) (Gramley *et al.*, 2009). Hypoxia describes a lack of oxygen supply that might be caused by atherosclerosis and ischemia leading to MI (Majmundar *et al*., 2010); one of its hallmarks is the stabilization of hypoxia inducible factor 1α (HIF1α) that is a master transcription factor regulating the response to hypoxic conditions by altering gene and miRNA expression (Greco *et al*., 2014)

Aged hearts show not only morphological but also specific molecular alterations when compared to that of younger ones, including a different gene expression pattern (Park *et al.*, 2012). Animal models, mostly rodents, which resemble certain types of cardiac disease, have been well established.

1.3.2 Neurodegenerative diseases

Loss of neuronal cells due to disruption of the proteostasis network is associated with many diverse forms of neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS). Many of them are caused by genetic mutations and protein misfolding as well as altered protein degradation. The biggest risk factor is aging and treatment options are limited yet (Reeve *et al*., 2014; Rodriguez *et al*., 2015). A common hallmark of all these diseases is aggregation of amyloid-rich protein plaques. The disease-associated genes involved may be heterogeneous; however, they trigger plaque formation by disturbing stress response pathways. In a healthy state, chaperones are a highly abundant and represent a diverse group of proteins that shelter posttranslational folding and refolding processes, ubiquitination and disaggregation of other proteins and protein complexes. These processes might be impaired causing loss of memory and mobility with advanced age or in disease state (Labbadia and Morimoto, 2015). Protein aggregates have been observed in many tissues of various aged model organisms and detection in histological probes is possible e.g. with staining for lipofuscin.

1.3.3 Cancer

Cancer is termed to be an expanding mass of proliferating cells that are able to invade surrounding tissue and may spread throughout the body. Most malignant primary tumors stem from only one diverging cell, that has accumulated multiple mutations during cell division or from toxicants (Alberts *et al.*, 2008). Even though cancer and aging appear very different, they share common features (López-Otín *et al.*, 2013). Cellular damage accumulates over time, leads during to cellular senescence and promotion of cellular proliferation and growth in cancerous tissue. Furthermore, these processes are accompanied by deregulated nutrient sensing and inflammation (Hanahan and Weinberg, 2011). Tumors of epithelial origin, carcinoma, are the most common types of cancer whereas sarcoma, that rise from connective tissue or muscle occur rarely (Ferlay *et al.*, 2015). Cancer occurrence is accompanied by changes of the epigenetic landscape. Hypermethylation in the promoter region of regulatory genes can be found in various cancer types (Belinsky, 2004; Park, 2010). Hence, targeting epigenetic pathways to combat cancer is under active investigation (Dawson and Kouzarides, 2012).

1.3.4 Premature aging

In humans, several forms of progeroid diseases are known in which genetic disorders accelerate aging. The patients suffer from premature aging that induces diseases that usually occur only at old age. Moreover, patients have a significantly reduced life expectancy compared to healthy individuals.

Defects of the nuclear envelope due to a mutation in the *laminA* gene are the cause of Hutchinson Gilford progeria syndrome (HGPS). Malfunction of DNA repair is the cause for Werner-Syndrome (WS) and Cockayne Syndrome (CS). Mutations in the sequence of the helicase *wrn* can lead to different forms of truncated or missense mRNA (Oshima and Hisama, 2014) and thereby delay cell cycle progression and base excision repair (BER) mechanisms (Rossi *et al.*, 2010). In CS, the transcription and transcription-coupled nucleotide excision repair (TC-NER) is impaired by mutations in *ercc6* or *ercc8* genes (Karikkineth *et al.*, 2017). A global loss of heterochromatin and genomic instability has been associated to HGPS and WS (Haithcock *et al*., 2005; Scaffidi and Misteli, 2006; Shumaker *et al*., 2006).

1.4 Model organisms for aging research

There has been a long-standing interest in delaying or even reverting aging. This particular field of research has been expanding ever since it has been shown that lifespan extension by genetic modification is possible in animal models (Kenyon *et al*., 1993). Apart from the most common animals used for research such as mice, rats and zebrafish, several different animal models are being used for aging research purposes. Depending on objective and scientific issue there is a broad variety of model organisms with long and rather short lifespan to choose from to address specific questions of aging processes. Hence, scientists are investigating all kind of species to answer questions on healthy aging and lifespan. As diverse as they seem they all have their limitations, advantages, and disadvantages which need to be considered carefully for the intended experiments and objectives. Hereafter, a brief overview highlighting selected examples will be given followed by a more detailed description of a new entrant to the circus.

1.4.1 Overview on established model organisms for aging research

Organisms like fungi such as *Podospora anserina* (Osiewacz, 2002; Osiewacz *et al.*, 2013) and budding yeast (Hallén, 2012; Kaeberlein, 2010) are used to answer fundamental mechanisms such as mitochondrial function and the mitochondria's role in the aging process. To investigate aging on an organismal level the filamentous ascomycete fungus *Podospora anserina* has been used as it displays a senescent phenotype with increasing chronological age depending on growth conditions and genetic onset. The model provided valuable insight on oxidative stress signaling and regulation of mitochondrial quality control (Osiewacz, 2011). *Podospora* was the first organism in which it had been demonstrated that mitochondrial DNA instability and rearrangement lead to senescence (Kück

et al., 1985). Several mechanisms involving respiratory chain and autophagy that increase lifespan in this model have been identified (Dufour *et al*., 2000; Knuppertz *et al*., 2014)

Chronological and replicative aging of yeast (*Saccharomyces cerevisiae*) is investigated by many fellow scientists (Longo *et al.*, 2012). The unicellular model can be studied in either of the two ways, loss of viability or loss of reproduction. It has been a model for studies on caloric restriction as well as for ROS production and mitochondrial damage (Osiewacz and Scheckhuber, 2006). Most prominently, overexpression of the *histone deacetylase sirtuin2* (*sir2*) prolongs lifespan by its activity in yeast. This protein is downregulated with age under control conditions (Dang *et al.*, 2009). Sirtuins are present also in mammals, mediating the effect of caloric restriction (Cantó and Auwerx, 2009) and protecting from cardiovascular disease (Cencioni *et al.*, 2015).

The nematode *Caenorhabditis elegans* is another model scientists use to study the mechanisms of aging. Being well-established since the 1960s, *C. elegans* was the first multicellular organism to be completely sequenced in 1998. Modulating the insulin-signaling pathway in *C. elegans* has been shown to increase lifespan: the insulin-like protein daf2 targets FOXO protein daf16, which is a key player in stress response. Inhibiting daf2 leads to activation of daf16 and thereby to activation of stress response genes like heat-shock proteins (HSP) (Gami and Wolkow, 2006). An individual overexpression of these genes has been shown to prolong lifespan as well.

In another model, the fruit fly *Drosophila melanogaster*, questions of aging are being addressed likewise, as it has the advantage of being highly reproductive active to enable large population sizes. *Drosophila* is a model for neuronal and stem cell research and the exhaustion of stem cells at old age (He and Jasper, 2014). Some aging phenotypes regarding heart (Wessells and Bodmer, 2007, 2004) and muscle aging (Demontis *et al.*, 2013) have been described as well as the influence of environmental changes (Klarsfeld and Rouyer, 1998) and infections (Bjedov *et al.*, 2010) concerning lifespan and increase of several molecular markers of aging in old *Drosophila*.

Vertebrates are more complex in their physiology and more closely related to humans and are mainly used for specific objectives of the aging process. The zebrafish *Danio rerio* lives up to 5 years, hence they are not the first choice for aging studies but are rather used for developmental studies. However, many genetic mutations of genes related to aging processes have been studies in this model and protocols are well established. The Japanese rice fish Medaka *Oryzias latipes* is another fish species that is being investigated. Biomarkers of aging have been observed in this particular animal model (Ding *et al.*, 2010). Meanwhile, axolotls and salamanders are mainly used for studies on limb and tissue regeneration (Russell *et al.*, 2017).

To address lifespan assays and feeding studies the animal model most closely related to humans, the rhesus monkey *Macaca mulatta* is used among other species such as mice. Non-human primates are seen as the most appropriate animals to investigate aging and aging-related diseases. In most cases, macaques are subject to studies. The National Institute of Health of the US (NIH) provided important insight on aging with long-termed studies with these models running over 30 years (Colman, 2017). Studies using non-human primates are addressing many neurological questions as well as questions regarding inflammatory diseases, lifespan and caloric restriction and much more (Didier *et al.*, 2016; Simmons, 2016). Because of intensive costs for maintenance and long duration of those experiments as well as animal experiments being viewed as ethically critical by many people, primates are for many investigators not the first choice of model animals despite being closely related to humans.

More commonly used are mice (*Mus musculus*) for experiments of aging research, as they represent an established animal model that can be genetically manipulated, the genome is well known and they have a relatively short generation time. Moreover, several mouse strains have been established mimicking different onsets of age-related diseases as mice may no develop certain aging-associated diseases naturally (Liao and Kennedy, 2014). A detailed database from Jackson Laboratories provides profound information on phenotype, life span and gene expression (Grubb *et al.*, 2014). Mice can live up to three to five years. To accelerate aging some genetically modified animals have been generated, many of them mimicking human diseases such as HGPS, dyskeratosis 21yogenesis, CS and many more (Mitchell *et al.*, 2015). In mice, the *klotho* gene has been well described. An overexpression of this gene leads to delayed aging while elimination leads to rapidly aging animals (Dërmaku-Sopjani *et al.*, 2013). Additionally, mouse models addressing the issue of prolonged lifespan have been established and investigated.

Other mammals are being less frequently used for aging research as well, such as dogs (*Canis lupus familiaris*) that are of interest for scientists as they share the same environment as humans and do not require artificial housing (Gilmore and Greer, 2015). Furthermore, they develop a series of aging-related diseases such as heart failure, neoplasia formation and immune-mediated diseases (Fleming *et al.*, 2011).

The naked mole rat *Heterocephalus glaber* is the longest living rodent used for medical research (Lewis *et al*., 2012, 2016). These animals have an extraordinary long lifespan of up to 30 years when compared to other rodents like mice and rats. It has been shown, that naked mole rats have high levels of autophagy (Zhao *et al.*, 2014) and telomerase reverse transcriptase activity that is very likely to contribute to the longevity of the animal (Kim *et al.*, 2011). Hence, naked mole rats are of great interest for longevity and cancer studies.

The bowhead whale *Balaena mysticetus* is by far the largest animal for aging studies (Seim *et al*., 2014; Keane *et al*., 2015). In fact, it is the longest living mammal known to mankind as an animal with an estimated age of over 200 years. While samples from bowhead whales might be difficult to obtain, these animals have provided valuable insights on longevity (Keane *et al.*, 2015). By comparative genome analysis, interesting details such as gene duplication and amino acid changes in genomic regions for cancer and lifespan associated genes were found. The protein proliferating cell nuclear antigen (PCNA), which is involved in DNA repair (Hoege *et al.*, 2002), has shown to be duplicated in the whale, suggesting a protective function that contributes to the old age of the animals.

1.4.2 *Nothobranchius furzeri* is a new model organism for aging research

There has been a demand for suitable models for aging studies that meet the needs for research questions. On the one hand the model organism of choice should resemble the aging process of human aging as close as possible, on the other hand short generation time and hence accelerated aging are preferable, as this latter would allow to perform many different experiments in a short time. With the turquoise killifish *Nothobranchius furzeri* (*N. furzeri*) such a new animal model for aging research has been established most recently, which meets both requirements (Terzibasi *et al*., 2008; Kim *et al*., 2016). The fish have many biological features such as muscular and circulatory system in common with other mammalian model organisms paired with an even shorter lifespan (Hu and Brunet, 2018).

The turquoise killifish belongs to the class of teleost fish (bony fish), which includes most fish species and also other common model organisms such as the zebrafish, tilapia and the Japanese rice fish Medaka. Other species of the *Nothobranchius* family are being investigated in the field of aging research as well. However, compared to *N. furzeri*, they have a longer lifespan. Nevertheless they present aging-associated changes of phenotype (Genade *et al*., 2005; Hsu *et al*., 2008; Lucas-Sánchez *et al*., 2011; Cellerino *et al*., 2016; Dong *et al*., 2017).

The turquoise killifish originates from South-East Africa and occurs mainly in the area of Mozambique and Zimbabwe in a semi-arid plateau region (Fig.1-5A). Small ponds with rather turbid water are the natural habitat in which *N. furzeri* live and reproduce (Fig.1-5B). The weather is bi-seasonal, with a rainy and a dry season each year. During the dry season, the eggs of *Nothobranchius* are in a state of diapause in the ground. When the rainy season starts, these eggs are released from diapause and after a short period of time fish hatch and develop rapidly reaching sexual maturity at three to five weeks of age (Fig.1-5C). With a life span of about 4-9 months under laboratory conditions, the fish

show age-dependent changes of morphology and gene expression, resembling certain hallmarks of aging, such as telomere shortening (Hartmann *et al.*, 2009), spinal curvature (Genade *et al.*, 2005), loss of mitochondrial function (Hartmann *et al.*, 2011), neoplasia formation (Di Cicco *et al.*, 2011), accumulation of lipofuscin in the liver (Ng'oma *et al*., 2014) and many more (Cellerino *et al*., 2016). With its extremely short lifespan, *N. furzeri* represents an interesting model for research, giving the advantages of a vertebrate while living few weeks longer than *Drosophila* (Fig.1-5D) (Wang *et al.*, 2015). The short lifespan can be prolonged by the usage of resveratrol (Valenzano and Cellerino, 2006), a molecule also present in red wine, which is able to prolong the lifespan of several model organisms, but still has to be proven for human extension of lifespan (Timmers *et al.*, 2012). Due to the short time that is needed to reach sexual maturity, it is also possible that more than one generation of killifish hatch during a single rainy season. In the wild, the fish eventually die because of ponds drying out at the end of the rainy season while the eggs survive in the muddy ground. However, even in the laboratories the fish maintain their short lifespan. After a phase of rapid growth declining health and overall frailty can be observed before the fish eventually die (Fig.1-5E).

Different strains with varying maximum lifespan are investigated at the moment. While the highly inbred GRZ strain has the shortest lifespan with just 17 weeks, fish of the MZM strains, that have been brought to the laboratories few years ago, live up to 50 weeks, in some cases even 70 weeks when single-housed (Baumgart *et al*., 2016), and show a more pronounced aging phenotype (Terzibasi *et al.*, 2008). Therefore, the strain MZM-0410 was used for all following studies, if not stated else. Moreover, muscle and heart tissue from *N. furzeri* were investigated to characterize epigenetic changes of aging in this animal model. Three different age periods were selected for the experiments: young, adult and old *N. furzeri*. In a previous study an accumulation of H3K27me3 in old *N. furzeri* brain compared to brain of young *N. furzeri* has been observed (Baumgart *et al.*, 2014a). One aim of this study was to investigate if this phenomenon is occurring also in skeletal muscle and heart tissue derived from this novel animal model. In addition to that, expression of mRNA of enzymes involved in DNA and histone methylation was addressed as well as the gene expression and the overall aging phenotype of *N. furzeri* skeletal muscle tissue.

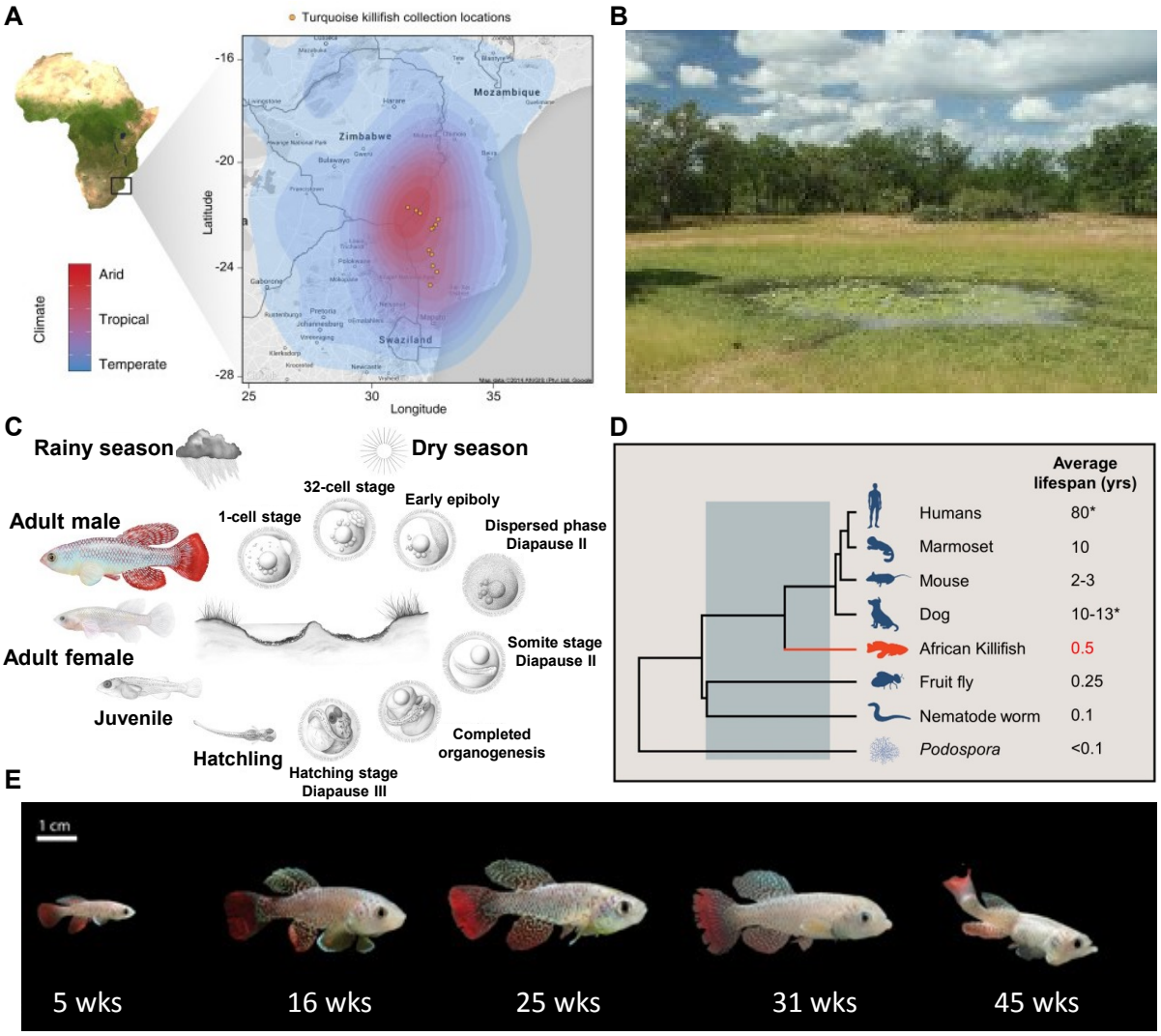

Figure 1-5: The turquoise killifish *Nothobranchius furzeri* (*N. furzeri*) has an exceptional short lifespan . (A) The *N. furzeri* originates from South-East Africa, a semi-arid region between Mozambique and Zimbabwe. Locations of samplings from a field trip are indicated (orange dot; modified after Valenzano *et al.* 2015. (B) Natural habitat of the *N. furzeri*: small ponds as depicted are home of wild *N. furzeri* (modified after Reichard *et al.*, 2015). (C) Life cycle of *N. furzeri*: during the dry season the eggs of *N. furzeri* are arrested in a diapause stage. As the rainy season sets in the embryo develops and hatches soon after. Adult male fish have colorful fins while females are smaller in size and less colorful (modified after Platzer and Englert 2016). (D) Schematic overview on lifespan of various model organisms (modified after A. M. Wang *et al.* 2015). (E) The killifish develops and increases in size rapidly, however a gradual loss of overall fitness can be observed in late adulthood (week 31); wks = weeks after hatching (modified after Hartmann *et al.* 2013).

1.4.2.1 *Skeletal muscle of teleost fish*

Skeletal muscle is the most abundant tissue in vertebrates and has distinct features. It is a heterogeneous tissue built from different types of fibers according to the needs of its surroundings but serves mainly for locomotor activity (Rossi and Messina, 2014). Muscle tissue develops during larval state from muscle progenitor cells, the satellite-cells. These satellite cells, that are also responsible for muscle regeneration, have been identified in several teleost fish species. Myogenesis continues to form skeletal muscle cells in waves of differentiation (Gurevich *et al.*, 2014). Thereby,

myotubes are formed by fusion of myoblasts and are combined into muscle fibers, that grow according to increased body size (Johnston, 2006). Multiple muscle fibers cluster in bundles, the fascicles. Similar to mammalian physiology, muscle tissue of teleost fish can be divided into two subgroups: slow muscle fibers and fast muscle fibers, which come from different progenitor cells and can be found at varying ratios depending on functional demands. The majority of muscle mass is made up by white muscle mass that consists of primarily fast muscle fibers responsible for rapid movement and functions also anaerobically. Red muscle tissue lies directly under the skin, located laterally and is primarily used for endurance movement. It is build up by slow muscle fibers that function aerobically (Fig.1-6) (Jayne and Lauder, 1994). At present, many studies investigating muscular diseases in teleost fish are being performed in zebrafish (Guyon *et al*., 2007; Berger and Currie, 2012; Plantié *et al*., 2015).

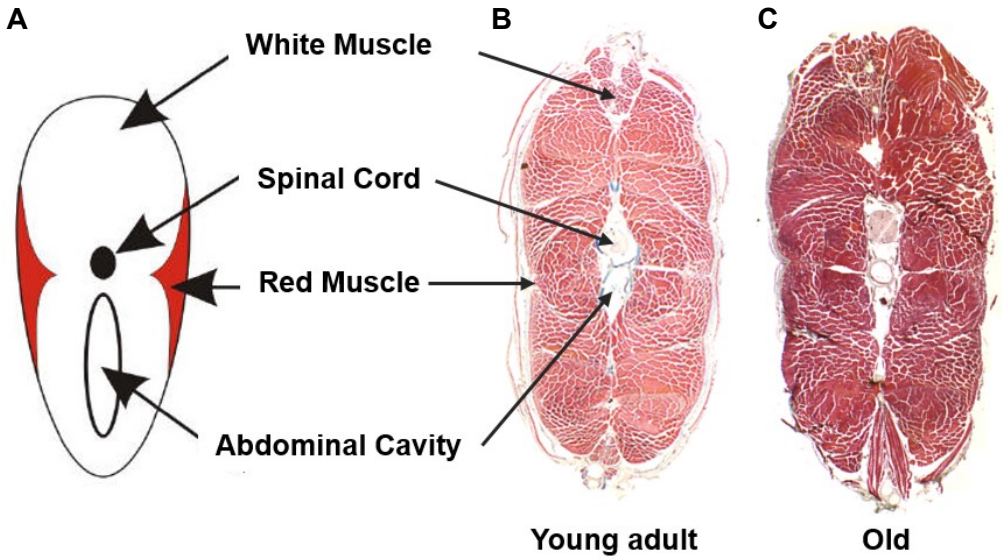

Figure 1-6: Physiology of fish muscle . (A) Scheme of dorsal – ventral cross section of a teleost fish (modified scheme after http://esi.stanford.edu/exercise/exercise6.htm). (B) Representative Masson-Goldner trichrome staining of a section from young adult (12 weeks) *N. furzeri* muscle tissue. (C) Representative Masson-Goldner trichrome staining of a section from old *N. furzeri* muscle tissue. Muscle tissue is stained red, collagenous matrix proteins are stained in blue. No distinction between red and white muscle fibers can be obtained with this staining.

1.4.2.2 *The heart of teleost fish*

Fish hearts consist of two chambers, one atrium and one ventricle. Upstream of the atrium, the *Sinus venosus* can be found where the blood is being gathered before entering the atrium. A structure of the coronary artery, the *Bulbus ateriosus,* regulates the pressure of the blood exiting the ventricle to avoid the gills being damaged. All four compartments are arranged in a serial fashion (Fig.1-7A). With increasing age, the heart of *N. furzeri* grows in size (Fig.1-7B). Teleost fish have a single circulatory system (Farrell, 2011), in which the blood is pumped from the heart to the gills, where it is

oxygenized and then distributed in the organism. Compared to humans that have a double circulatory system and two atria and two ventricles the cardiovascular and circulatory system of fish is somewhat simpler in its physiology (Fig.1-7C). The contractile ventricle of most fish has a loose, sponge-like structure of the muscle tissue that is perfused by blood rather than a compact myocardium like in mammals (Fig.1-7D) (Pieperhoff *et al*., 2009; Parente *et al*., 2013).

The development of the cardiac system has been extensively studied in multiple fish species. While mammals cannot regenerate their heart after injury, the heart of zebrafish is able to regenerate to a certain extent as cardiomyocytes are able to proliferate (Poss *et al*., 2002; Kikuchi, 2014, 2015). Also in Goldfish (*Carassius auratus*) cardiac regeneration of the ventricle has been reported (Grivas *et al.*, 2014). However, not all fish species are able to regenerate the heart after injury, e.g. Medaka show hardly any regenerating capacities (Ito *et al.*, 2014). If the heart of *N. furzeri* is able to regenerate as well remains to be elucidated. Adult zebrafish are largely used as models for cardiac diseases. Cryo-injury, for example, has been shown to mimic myocardial infarction (Chablais *et al.*, 2011) and cardiomyopathy can be induced by stress conditions via doxorubicin treatment or chronic anemia (Shih *et al.*, 2015). These methods, however, have not yet been reported in an aging related context in *N. furzeri*.

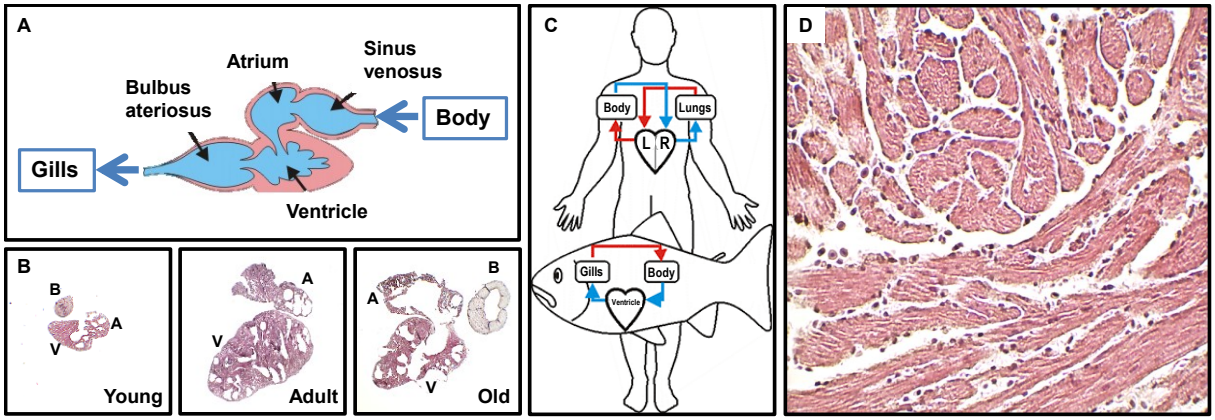

Figure 1-7: Physiology of the heart of teleost fish . (A) Schematic image of the teleost heart. Blood is pumped from the body through the *Sinus venosus* and atrium, the ventricle contracts rhythmically and pumps the blood via the *Bulbus ateriosus* towards the gills; blue arrows indicate direction of blood flow (scheme modified after https://esi.stanford.edu/circulation/circulation5.htm). (B) Representative Masson-Goldner trichrome staining of young (left), adult (middle) and old *N. furzeri* heart; V = ventricle, A = atrium, B = *Bulbus ateriosus*. 4x magnification. (C) Circulatory system of humans (top) and fish (bottom): In humans, blood comes from the body to the right side of the heart (R) and is from there forwarded to the lungs. There, the blood is oxygenized and comes to the left side of the heart (L), from where it is pumped through the body. In fish, the blood comes from the body and is forwarded by the ventricle towards the gills, where it is oxygenized and distributed through the body (scheme modified after https://esi.stanford.edu/circulation/circulation6.htm). (D) Typical sponge-like structure of the *N. furzeri* ventricle; representative Masson-Goldner trichrome staining of an adult ventricle. Erythrocytes are stained dark brown; muscle tissue in red. 40x magnification.

2 Objective

Aging is a universal and physiological phenomenon that progressively impaires proper function of an organism. To investigate changes over age, very diverse model systems have been established over the past decades to elucidate specific mechanisms that lead to aging.

Most recently, the turquoise killifish has gained attention as it is the shortest living vertebrate known today. It has a very short generation time living between 3-9 months. The genome has been published 2015, however, only little is known about epigenetic changes with age and how this influences gene expression. In addition to that, at present only limited information regarding the aging phenotype of heart and muscle of the killifish is available.

Hence, this thesis addresses the following questions:

1) How does the global epigenome change from young to old *Nothobranchius furzeri* in skeletal muscle tissue and heart?
2) What are potential causes for those changes and how may they be influenced?
3) May those changes be responsible for the short lifespan of the killifish?

To investigate the impact of aging on killifish, heart and muscle tissue have been used for experiments, analyses and sequencing. The overall aging phenotype in heart and muscle was very similar. However, for selected experiments the focus was pointed selectively on one type of tissue to study a specific finding and its causes more in detail as specified herein. To investigate global histone methylation mainly *N. furzeri* skeletal muscle tissue was used and some of the findings could be also confirmed in *N. furzeri* heart tissue. Meanwhile, global miRNA expression was analyzed in heart tissue. However, because of its pathophysiological relevance specifically attention was paid to the regulation and function miR-29 in the heart and skeletal muscle. The observations were successfully reproduced human cardiac fibroblasts (HCF) and in cells derived from *N. furzeri* skeletal muscle tissue.

3 Material and Methods

3.1 Material

3.1.1 Consumables

Table 3-1: Consumables used

Item	Manufacturer	Product Number
96well ELISA Microplates	Greiner Bio-One	655101
Amersham Hybond ECL 0.2µm	GE Healthcare Life Sciences	RPN3032D
Blotting paper	Macherey-Nagel	742113
Cellstar 6/12/24 well cell culture plate, with lid	Greiner Bio-One	657 160/665 180/662 160
Cellstar cell culture dishes, 35x10/60x15/100x20mm	Greiner Bio-One	627 160/628 160/664 160
Ceramic sphere (1/4”)	MP Biomedicals	6540-422
Costar cell scraper	Corning Corporate	3010
Cover slides		
Cryo.S freezing tubes	Greiner Bio-One	122263
Feather disposable scalpel	Feather Safety Razor Co. LTD	200130021
Filter system NY250/500 0.22	Corning	430771/430773
Glass beads	Sigma-Aldrich	G4649
Glasstic slide 10 with grids	Kova International Inc.	87144
Injekt 20 mL/Luer Solo	Braun	4606205V
Menzel-Gläser polysine slides	ThermoScientific	J2800AMNZ
Micro tube	Sarstedt	83.34.06
MicroAmp Fast optical 96well Reaction Plate (0.1ml)	Applied Biosystems Life Technologies	4346907 / SAP 360235
MicroAmpOptical adhesive film	Applied Biosystems	4311971 / SAP 329063
Millex-GS 0.22µm sterile filter	Merck Millipore Ltd	SLGS033SS
Millex-HV 0.45µm sterile filter	Merck Millipore Ltd	SLHV033RS
Original perfusor syringe 50ml/Luer Lock	Braun	8728819F
Parafilm	WS Laborservice GmbH	300573
Pipette filter tips (10/100/1000µl)	Greiner Bio-One GmbH	771 257/771 261/ 686 280
Pipettes COSTAR 2/10/25 ml	Costar	327106/327103/302161
Reaction tubes (0.2/0.5/1.5/2/5ml)	Eppendorf	003012-4332/1023/0086/0094/19401
Tubes GREINER 15/50ml	WS Laborservice GmbH	304196/#304198

3.1.2 Equipment

Table 3-2: Equipment used

Instrument	Type	Manufacturer
8-channel multipipette		Eppendorf
Adjustable volume pipettes		Eppendorf
Autoclave	DX-23	Systec
Centrifuge (falcons, plates)	Multifuge 3S-R	Heraeus
Centrifuge (tubes)	5404	Eppendorf
CO2 Incubator Eppendorf	Galaxy 170S	Eppendorf
Confocal Microscope Zeiss	LSM 780 Axio Observer	Zeiss
Heat block	Thermomixer Compact	Eppendorf
Homogenizer	FastPrep24	MP Biomedicals
Hypoxia workstation	SCI-tive	Baker-Ruskinn
Microplate reader	EnSpire Multimode	PerkinElmer
Microscope with camera		Motic Electric Group
Microtome	HM355	Thermo Scientific
Mini gel electrophoresis setup	Protran Tetra	Bio-Rad
Mini Trans Blot Cell setup	Protran	Bio-Rad
Paraffin Embedding Station	TES Valida	Medite
Pipetting aid	Pipetboy	Integra
Plate reader	EnSpire Multimode	PerkinElmer
Power supply	Powerpac HC	Bio-Rad
Real time PCR system	StepOnePlus	Applied Biosystems
Safety cabinet	HeraSafe HS18	Heraeus
Spectrophotometer	NanoDrop 2000	ThermoScientific
Thermocycler	Tprofessional Basic 96	Biometra
Vortexer	Vortex Genie 2	Scientific Industries
Water bath	WTB6	medingLab
Western Blot Imager	Odyssey CLx	LI-COR

3.1.3 Chemicals and reagents

Table 3-3: List of chemicals and reagents used

Name	Product number	Manufacturer
2-Mercaptoethanol	4227.03.00	Carl Roth
2-Propanol	A3928,0500PE	AppliChem
4’,6-Diamino-2-phenylindoledihydrochloride (DAPI)	D8417-5MG	Sigma-Aldrich
Acrylamide 4K Solution (30%)	A0951, 0500	AppliChem
Albumin, from bovine serum (BSA)	A7906-50G	Sigma-Aldrich
Ammoniumperoxodisulfate 98 %	9592.03.00	Carl Roth
Antibody diluent	S3022	DAKO Agilent Technologies
Antigen Retrieval Buffer,	ab94674	Abcam

100X Citrate Buffer pH 6.0		
Chloroform	BP1145-1	FisherScientific
Collagenase A	10 103 578 001	Roche
Dako Antibody Diluent	S3022	Dako
Dako Fluorescent Mounting Medium	S3023	Dako
DMSO	Carl Roth	4720
EDTA	E6758-100G	Sigma-Aldrich
Ethanol (absolute)	BP2818-4	FisherScientific
Ethanol absolute for molecular biology	32205-1L	Sigma-Aldrich
Formaldehyde solution 37% in H_2O	252549-100ML	Sigma-Aldrich
Glycine (99 %)	G7126-1KG	Sigma-Aldrich
HEPES Buffer 1 M	H0887	Sigma-Aldrich
Hydrochloric acid 37 %	30721-1L	Sigma-Aldrich
Hydrogen peroxide	216763-100ML	Sigma-Aldrich
IGEPAL CA-630	I8896-100ML	Sigma-Aldrich
Lipofectamine RNAiMAX transfection reagent	13778075	Life Technologies
Loading buffer	NP0007	ThermoFisher
Methanol	32213-2.5L	Sigma-Aldrich
N-Acetylcysteine (NAC)	A7250-5G	Sigma-Aldrich
Newblot Nitro stripping buffer (5x)	928-40030	LI-COR
Odyssey Protein Molecular Weight Marker	928-40000	LI-COR
ORA qPCR Green ROX H Mix	QPD0205	HighQu
PBS (10x)	P5493	Sigma-Aldrich
Phenylmethanesulfonyl fluoride	P7626-250MG	Sigma-Aldrich
Ponceau S- Solution	A2935,0500	AppliChem
Protease inhibitor cocktail (100x)	M221-1ML	VWR
RG108	CAY13302-10	Cayman
Roti-Histokitt	6638.1	Carl Roth
Roti-Histol	6640.01.00	Carl Roth
SDS – Solution 20 %	A0675,0500	AppliChem
Skim milk powder	70166-500G	Fluka/Sigma-Aldrich
Sodium azide	S2002-25G	Sigma-Aldrich
Sodium chloride	12536	Roche
Sodium chloride	S7653-250G	Sigma-Aldrich
Sodium pyruvate	S8636	Sigma-Aldrich
TEMED	2367.01.00	Carl Roth
TRI reagent	T9424-100ML	Sigma-Aldrich
Tris pufferan	4855.01.00	Carl Roth
Tris-glycine buffer	161-0771	Bio-Rad
Triton X-100	A1388,0500	AppliChem
Tween 20	P1379-500ML	Sigma-Aldrich

3.1.4 Antibodies

3.1.4.1 *Primary antibodies*

Table 3-4: Primary antibodies used for immunofluorescence microscopy (IF) and Western blot (WB)

Antibody against	Dilution used	Product number	Supplier
H3 rabbit pAB	1:100	9715	Cell signaling
H3K27me3	1:100 (IF), 1:1000 (WB)	ab6002	Abcam
H3k9Ac	1:100 (IF), 1:1000 (WB)	ab4441	Abcam
H3K9me3	1:1000	ab8898	Abcam
H4	1:500	sc-10810	Santa Cruz
H4K20me3	1:1000	ab187521	Abcam
HP1α	1:100	bs-3825R	bioss
Nitrotyrosine	1:100	A-21285	ThermoFisher
PCNA	1:500	GTX124496	Genetex
αTubulin	1:4000	3873	Cell signaling
γH2AX	1:500	GTX127343	Genetex

3.1.4.2 *Secondary antibodies*

Table 3-5: Secondary antibodies used for immunofluorescence and Western blot

Antibody	Dilution used	Product number	Supplier
Alexa fluor 488 goat anti-mouse IgG	1:1000	A11029	Molecular Probes
IRDye 800CW goat anti-mouse IgG	1:10000	926-32210	LI-COR
IRDye 800CW goat-anti-rabbit antibody	1:10000	926-32211	LI-COR

3.1.5 Cell culture media and supplements

Table 3-6: Cell culture media and supplements

Item	Product Number	Manufacturer
Amphotericin	15290	Life technologies
DMEM	D6546	Sigma-Aldrich
Fetal Bovine Serum	SH30406.02	Hyclone
Fetal Bovine Serum Superior	S0615	Merck-Millipore
Gentamycin solution	G1397-10ML	Sigma-Aldrich
IMDM	BE12-726F	Lonza
L-Glutamine solution	G7513-100ML	Sigma-Aldrich
OPTI-MEM I	31985-070	Life Technologies
PBS pH 7.4 (1x)	10010-015	Life Technologies
Penicillin/ Streptomycin	P0781-100ML	Sigma-Aldrich
Recombinant human bFGF	233-FB	R&D Systems
TrypLE express trypsin	12605-036	Life Technologies
Water for molecular biology	W4502	Sigma-Aldrich

3.1.6 Kits

Table 3-7: Kits used

Name	Product number	Manufacturer
BCA Protein Assay Kit	23225	Pierce by Thermo Fisher
DNMT activity assay	P-3001	Epigentek
E.Z.N.A. Tissue DNA Kit	D3396-02	VWR OMEGA bio tek
Masson-Goldner's trichrome staining kit	3459.1	Carl Roth
MethylFlash hydroxymethylated DNA quantification KIT (Colorimetric)	P-1036	Epigentek
MethylFlash methylated DNA quantification KIT (Colorimetric)	P-1034	Epigentek
Senescence ß-galactosidase staining kit	9860	Cell signaling technology
Sirius red fast green collagen staining kit	9046	Chondrex
SuperScript III First-Strand Synthesis SuperMix for qPCR	11752050	Invitrogen
Taqman microRNA Assays	4427975	Applied Biosystems

3.1.7 Primers

3.1.7.1 *Primers for mRNA expression analysis*

All primers were designed using the primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Untergasser *et al*., 2012) and ordered from Sigma-Aldrich.

Table 3-8: All primers used for *Nothobranchius furzeri* (*Nfu*), *Danio rerio* (*Dre*) and *Homo sapiens* (*Hsa*)

Species	Target gene	Forward primer	Reverse primer
Nfu	*cbx2*	GTTGGCCTCACCTGACATTT	ATCTGGTTTTCGGTCTGTGG
	cbx7.1	GAGCAAGTGTTTGCTGTGGA	CTTTGGCACCTTTCTTCCTG
	cbx7.2	GGAGACAGGCTGGATTTTGA	GCCATGGTAACCGACTGATT
	cbx8a	CAGTCAATCGGGGTGAAAGT	TTAGACTCCTCCGGGAACCT
	cbx8b	AGGTGGCGAGTATCTGCTGT	CGGTTCCCAAGTGCTGTATT
	cd40	GCTTTGGCTTTGTTTTGCTC	TTTTTCCAGATCCTCCATGC
	cebpb	AACATCTCCACTGCGTCCTC	TGTCCCTGCTCTTCCTCACT
	col11a1	ATGGAGATGCAGAGGTGGTC	AATCCCGATGTTCTCGTCAC
	col15a1 1of2	TACCATGCCTTTTCCAGACC	TTCTCCTCGTTCCCCTTTTT
	col1a1	GACCCACAGGATCTCAAGGA	ACCACTATCACCAGCGGTTC
	col1a2	CAAGAACAGCGTTGCCTACA	CAATGTCCAAAGGTGCAATG
	cyclin b	TCTGATGGAGCTGACCCTCT	TTTTCCCTTCGTTCACCATC
	cyclin d	TCCTCTGGCCATGAACTACC	TGTCCGTGTAGATGCAGAGC
	dnmt1	GTCTCTGCCAGACGATGTCA	GCAAGGCTTTCACCTTTGAG
	dnmt3a	ATTTCTTCGCCAACAACCAC	GCCCACAGTGATGGAATCTT
	dnmt3b	CATGAGAAGGGTGGAGTGGT	AGCTGCCAACTCGATGATCT
	eed	AGTCCTGTGAAAACGCCATC	AACGTAAAGCTTCCCCACCT
	ezh1	CAAGAGGATTCCCAGCGATA	GGGTTGGAGGAAACAGTCGTA
	ezh2	CCCGGCATTCTAGATGATGT	GTCGGTTTCTCATCGGGTTA
	gadd45γ	ATTGCGCTTCAGATCCACTT	CGCAGAACAGACTCAGCTTG
	ir	TGCCTCTTCAAACCCTGAGT	AGGATGGCGATCTTATCACG
	jak3	TGCTGGACTTGTGGAGAATG	CTCGATGCCTGACAGTTCAA
	kdm6a	GTCAAACCCTACCCCCTCAT	TGTGGAGAGAGGAGCCAACT
	kdm6b	CAAAGCCAGCTTTCTGGAAC	TCTGGATGTGAGGAGCACAG
	myog	GTTCGACCAAGCTGGCTATC	CATGGTCACCGTCTTCCTTT
	p15	TGATGATGATGGGGAGCTG	AGTCTCCAGGAAAGCAACGA
	p18	AAAGAGGGTGTTGCCATCAA	GAGGTCGTCATCCACAAGGT
	p19	GCAGCAGCTAAAGGGAACAC	AGCTCCTTTTTCCAACAGCA
	p21	CTTCTCCGACAAACCTCTGG	ACCACCACCCTTCCTCTTT
	p53	TATTCCGCTTTGGAGTCACC	CGAAGCTCCAGCTGGTAATC
	pcna	ACCCTCAGAGCAGAGGACAA	CATGGGAAAGGATCTGGAAA
	polγ	TCCCCGTTAATCAGAACTGG	TCTGCTGCTTTTTGGGAGTT
	ptx3a	GTAGGGAAGAGGCAGACA	GTCAGACAGGAGGGGTTTCA
	ssbp	CTGGAGAGACGGAAACAAGC	CTGACGTTGTCGCTCAGAAA
	stat1a	GCAGGATGAATACGATTTCCA	CTCGGAGATCAGGTCGGTTAT
	suz12	AAAGGAGCAAAGGTGGAGGT	GACGGTTGTGACCACTGATG
	tdg	CACCTGGAAGCAAAGACCTC	GCTGGATGAAGGCATCAAAT
	tet1	TACAAGTTGGGAGGGTCAGG	AAGGACCCTCCTCCTTTTCA

	tet2	CGGAGCAAAGTTCCAAGAAG	CGCAGAAGTCCATACAAGCA
	tet3	CCTCCTGTGAAACCTGTGGT	AAGCAGGGTGTGAAATGTCC
	tfam	GTCCTCGCACTGCCTTTAAC	CATGTGGTCTTCCCAGGACT
	tnfaip2	GACCCAGTCCGATTCAGAAA	CTTCTTCAACAGGCGAGTCC
Dre	*dnmt1*	GGGCTACCAGTGCACCTTTG	GATGATAGCTCTGCGTCGAGTC
	dnmt3a2	TAGGAAAGGCTTGTTTGAGGG	GCGTGAGATGTCTTTCTTGTC
	dnmt3b2	CGCTACATTGCCTCTGAGA	GCCAGATGTTTCCTAGTGATG
	col1a1	CAGACTCCACCTGCTTATTCTACAC	TTGACATCGCCCCTATGGACGTTG
	col1a2	CATGAGGTAGTTTAAACCTTACGG	GGACATTGGCCCAGTCTGTTTCAA
	col15a1b	GATTTCGAGGGTTCTGGTGA	ATACACCTGGCTCACCCTTG
	gapdh	GTGGAGTCTACTGGTGTCTTC	GTGCAGGAGGCATTGCTTACA
Hsa	*dnmt1*	ATCCGAGGAGGGCTACCTG3	CACTTCCCGGTTGTAAGCAT
	dnmt3a	AGCCCAAGGTCAAGGAGATT3	GTTCTTGCAGTTTTGGCACA
	dnmt3b	TCAGGATGGGAAGGAGTTTG	CTGCAGAGACCTCGGAGAAC
	col1a1	GAACGCGTGTCATCCCTTGT	GAACGAGGTAGTCTTTCAGCAACA3
	col3a1	AACACGCAAGGCTGTGAGACT3	GAACGAGGTAGTCTTTCAGCAACA
	col11	GGTCTGCAGTCGCAATTTCG	CCACCTAGAGGACCACGGCT
	col15	AGGCCCTGGATGCACAATG	GACCAATTGCCGTTGG
	p0	TCGACAATGGCAGCATCTAC	ATCCGTCTCCACAGACAAGG

3.1.7.2 ***Primers for microRNA expression analysis***

All primers were obtained from the Taqman miRNA Assay series from ThermoFisher (catalogue number 4427975).

Table 3-9: Assays used for miRNA expression analysis

Target microRNA	Assay ID
hsa-miR-141	000463
hsa-miR-16	000391
hsa-miR-181c	000482
hsa-miR-200a	000502
hsa-miR-200b	002251
hsa-miR-200c	002300
hsa-miR-29a	002112
hsa-miR-29b	000413
hsa-miR-29c	000587
hsa-miR-429	001024

3.1.8 Software Programs

Table 3-10: Software programs used

Software	Indented use	Producer
DAVID	Gene ontology and KEGG pathway analysis	Huang *et al*., 2009
GraphPad 7	Statistical analysis	GraphPad
LI-COR	Signal Intensity analysis	LI-COR
Mendeley	Citation	Elsevier
Microsoft Office	Data analysis, text generation editing	Microsoft
miRBASE	miR analysis	Kozomara and Griffiths-Jones 2014
Oligo Analyzer3.1	Primer design	Integrated DNA Technologies
PAST	Clustering	Hammer *et al*., 2001
Primer3	Primer design	Untergasser *et al,*. 2012
StepOne 2.3	qRT-PCR Analysis	Applied Biosystems
Targetscan	miR target analysis	Ulitsky *et al.* 2012; Agarwal *et al.* 2015
Treeview	Clustering	Swift *et al.* 2004
Venny	Data visualization	Oliveros 2016

3.1.9 Animals

For all experiments *N. furzeri* strain MZM-0410 was used. Tissue from *N. furzeri* was a kind gift from Prof. Dr. Alessandro Cellerino, SNS Pisa and Dr. Mario Baumgart, FLI Jena. Animals were kept according to the legal requirements of the state Thuringia and the Italian Ministry of Health. To avoid detrimental effects from circadian rhythm and feeding, animals were sacrificed at 10 am in a fasted state by authorized personnel. Prior sacrifice and tissue collection animals were anesthetized with Tricane (MS-222) and wet ice. Tissues were immediately snap-frozen with liquid-nitrogen or fixed in PFA. To isolate cells, tissues were placed in growth medium until further processing. Johanna Heid was at least three times in person present for tissue harvest. Dr. Mario Baumgart and Sabine Matz collected and froze skeletal muscle and tissue from *N. furzeri* on multiple occasions when other organs were extracted, too. The material was stored at -80 °C and shipped on dry-ice to the laboratory of Prof. Dr. Carlo Gaetano.

cDNA from *N. furzeri* skeletal muscle and heart was in part provided by Dr. Mario Baumgart.

Paraffin-embedded muscle and liver tissue from *N. furzeri* was a kind gift of Prof. Dr. Alessandro Cellerino.

For all experiments with *N. furzeri* skeletal muscle, whole muscle was isolated and used for subsequent analysis. No separation between red and white muscle mass was operated. For all experiments with *N. furzeri* heart, whole hearts of *N. furzeri* were used. No dissection between ventricle and atrium was performed.

Dr. Roberto Ripa (SNS, Pisa) generated a zebrafish mutant with a miR-29 Sponge construct. A detailed description of construct and cloning strategy can be found described in Heid *et al.* (2017) and Ripa *et al.* (2017).

Echocardiography of zebrafish and killifish was performed by Dr. Giuseppina Milano, Prof. Dr. Guilio Pompilio and Dr. Alessandro Scopece in Milan. All animals were kept and treated according to the legal requirements of the Italian Ministry of Health.

In Tab.10-1, all collaborators and their contributions are listed.

3.2 Methods

All consumables are listed in table 3-1. Equipment and instruments used for this thesis can be found in table 3-2. Chemicals and reagents are mentioned in table 3-3. Commercially available kits that have been used are listed in table 3-7.

3.2.1 Molecular Biology

3.2.1.1 *RNA extraction with phenol chloroform*

Tissue samples were dissected and snap-frozen by Sabine Matz and Dr. Mario Baumgart from the FLI, Jena and shipped on dry ice to the laboratory in Frankfurt. All subsequent specimen preparation of *N. furzeri* tissue was performed in Frankfurt. Tissue samples were homogenized using 500-1000 µl TRI reagent, ceramic beads (MP Biomedicals) and a tissue homogenizer. Cells were washed with PBS and then re-suspended in 500 µl TRI reagent and transferred into reaction tubes. Samples were stored at -80 °C for 1h or until further processing. 100 µl chloroform was added to each tube, mixed well and incubated for 3 min at RT. Centrifugation was performed at 13000 rpm and 4 °C (Eppendorf centrifuge). The upper phase was transferred to a new tube and 250 µl isopropanol were added. After brief mixing, incubation for 10 min at RT was allowed. A second centrifugation step at 11000 rpm and 4 °C was performed. The supernatant was removed carefully and the pellet was washed with 75 % ethanol. After a new round of centrifugation at 10,000 rpm and 4 °C the pellet was re-suspend in 10-20 µl RNA-free water and incubated for 10 min at 55 °C. RNA content was determined by using a NanoDrop 2000 (ThermoFisher). Samples were stored at -80 °C until further use.

3.2.1.2 *DNA extraction*

Tissue samples were dissected and snap frozen by Sabine Matz and Dr. Mario Baumgart from the FLI, Jena and transported on dry ice to the laboratory in Frankfurt. All subsequent specimen preparation of *N. furzeri* tissue was performed in Frankfurt using the E.Z.N.A. tissue DNA kit to extract DNA from samples. Tissue samples were minced and lysed at 55 °C using lysis buffer and proteinase K (both provided by the kit). After a centrifugation step, the supernatant was transferred into a 1.5 ml tube and mixed with binding buffer and incubated for 10 min at 70 °C. Then, ethanol was added and the solution was transferred to a column. After several wash steps the column was dried by centrifugation. Elution of DNA was performed with water or elution buffer. Concentration of nucleic acids was determined using a NanoDrop 2000 apparatus (ThermoFisher). DNA extraction from cells was performed in a similar fashion: Cells were washed twice in PBS (Life Technologies) and then re-suspended in 200 µl PBS before mixing with binding buffer and incubation at 70 °C.

3.2.1.3 *Quantification of nucleic acids*

RNA or DNA concentration was determined using a spectrophotometer (NanoDrop). 1.5 µl solution was applied onto the pedestal. A blank measurement with pure RNAse/DNAse free water (Life Technologies) was performed prior to measurement of samples.

3.2.1.4 *cDNA synthesis*

For reverse transcription the superscript III kit from Invitrogen was used. To transcribe the RNA into cDNA 1 mg RNA and water to adjust to 8 µl, 10 µl buffer and 2 µl enzyme per reaction were placed in a thermocycler running at the conditions below (Tab.3-11):

Table 3-11: Protocol for cDNA synthesis

Reaction mix	cycling program
1 mg RNA	10 min 25 °C
10 µl buffer	30 min 50 °C
2 µl enzyme	5 min 85 °C
x µl water	
20 µl total volume	until use 4 °C

3.2.1.5 *qRT-PCR*

mRNA expression was analyzed via qRT-PCR with a StepOne (Applied Biosystems). All primer sequences were designed with the help of Primer3 (Untergasser *et al.*, 2012) and ordered from Sigma-Aldrich. A list of all primers can be found in table 3-8. A master mix of primer and SYBR green and a 38yogenes master mix with cDNA and water were prepared and ran at the conditions that can be found below (Tab.3-12). Analysis was performed in duplicates and relative gene expression was calculated using the ΔΔCT method (Pfaffl, 2004). *ir* (*Nfu*), *p0* (*Hsa*) and *gapdh* (*Dre*) were used as reference control.

Table 3-12: Protocol for qRT-PCR

qRT-PCR reaction mix	qRT-PCR program
6.5 µl SYBR green reagent 0.5 µl forward primer (10mM) 0.5 µl reverse primer (10mM) 1 µl cDNA 4.5 µl H_2O 13 µl total volume	20 s 95 °C 3 s 95 °C 30 s 60 °C } 30 cycles 15 s 95 °C 60 s 60 °C 15 s 95 °C } Melt curve

3.2.1.6 *qRT-PCR for microRNA analysis*

microRNA expression was analyzed via qRT-PCR with a StepOne machine (Applied Biosystems). All primers were ordered from the Taqman Assay series from ThermoFisher. Reference numbers of all miRNAs assays are listed in table 3-9. Analysis was performed according to assay descriptions and carried out in duplicates; relative gene expression was calculated using the ΔΔCT method (Pfaffl, 2004). miR-181c (*Nfu*) and miR-16 (*Hsa*) were used as reference control.

3.2.1.7 *Mitochondrial copy number*

Mitochondrial copy number was assessed as described previously (Hartmann *et al.*, 2011): primers for mitochondrial DNA (Tab.3-13) were used in qRT-PCR with isolated DNA from *N. furzeri* skeletal muscle and heart and calculated accordingly. Cycle threshold (CT) values of mitochondrial PCR products were normalized to CT-values of a nuclear locus according to the following equation: Relative mtDNA copy number per diploid cell = $2 \times 2^{\Delta Ct}$, where ΔCt is $Ct_{Cdkn2a/b\ \text{locus}} - Ct_{\text{mitochondrial locus}}$.

Table 3-13: Primers for determination of mitochondrial copy number

Target gene	Forward primer	Reverse primer
Cdkn2a/b gene locus	ATCTCGAAGGCAAGCATCC	GCAGAAGCGACTCCACATC
16S rRNA	GCGACCGTGGAGAAAAATAA	GGATTGCGCTGTTATCCCTA

3.2.1.8 *Sequencing of miRNA and mRNA*

mRNA sequencing of *N. furzeri* skeletal muscle tissue and *D. rerio* hearts was performed by the core facility of the SFB834 under Dr. Stefan Günther, Dr. Carsten Künne and Prof. Dr. Thomas Braun. microRNA sequencing of *N. furzeri* hearts was performed by Dr. Mario Baumgart. Detailed description of sequencing procedures are published (Heid *et al.*, 2017).

3.2.2 Immunological methods

3.2.2.1 *Protein extraction*

Tissue samples were dissected and snap-frozen by Sabine Matz and Dr. Mario Baumgart from the FLI, Jena and transported on dry ice to the laboratory in Frankfurt. All subsequent specimen preparation was performed in Frankfurt where samples were weighted and Laemmli lysis buffer (Tab.3-14) was added accordingly, homogenization was performed by using ceramic beads (MP Biomedical) or, for smaller specimens like *N. furzeri* hearts, a hand-held pestle. Cultured cells were washed with PBS (Life Technologies) and were either treated with trypsin (Life Technologies) and collected via centrifugation or removed directly from the plate applying 20-100 µl Laemmli lysis buffer (Tab.3-14) and using a cell scraper. The suspension was collected in a tube and heated for 5 min at 95 °C. Determination of protein concentration was performed using the BCA protein quantification kit (ThermoFisher) according to the instruction of the manufacturer. Suspensions were either used directly for Western blot or stored at -20 °C until further use.

Table 3-14: Laemmli buffer

Laemmli buffer (2x)
100 mM TRIS buffer (pH 6.8)
20 % glycerol
4 % SDS
In ion-free water

3.2.2.2 *SDS-PAGE and Western blot*

Acrylamide gels were prepared according to the size of the protein that was to be detected: proteins larger than 100 kDa required 8% acrylamide gels while proteins < 100 kDa were detected using 15 % acrylamide gels (Tab.3-15). After the separating gel was casted and polymerized, the stacking gel was poured on top.

Samples containing 5-20 µg total amount of protein were supplied with 4x loading buffer and boiled for 5 min at 95 °C. After a short centrifugation step, samples were loaded onto the gel, which ran at 3 mA for about 75 min in a Western blot Mini apparatus filled with running buffer (Tab. 3-16).

Table 3-15: Constitution of stacking and separating gel solution

	Stacking gel	**Separating gel 8 %**	**Separating gel 15 %**
Ion-free water	2.1 ml	4.6 ml	2.3 ml
Acrylamide (30 %)	500 µl	2.7 ml	5 ml
TRIS Buffer	390 µl (0.5 M; pH 6.8)	2.5 ml (1.5 M; pH 8.8)	2.5 ml (1.5 M; pH 8.8)
SDS (10 %)	30 µl	100 µl	100 µl
APS	30 µl	100 µl	100 µl
TEMED	3 µl	6 µl	4 µl

Table 3-16: Running buffer

Running buffer:	**Amount**
TRIS glycine buffer (10x; Bio-Rad)	100 ml
SDS (20 %)	50 ml (0.5 %)
Ion-free water	850 ml

Depending on protein size, transfer of gel was either for 2.5 h at 25 mA for small proteins or overnight (~16 h) at 30 mV for large proteins onto a membrane using a Protran Western blot chamber (Bio-Rad) filled with transfer buffer (Tab.3-17).

Table 3-17: Transfer buffer

Transfer buffer:	**Amount**
TRIS glycine buffer (10x;Bio-Rad)	100 ml
Methanol	100 ml (10 %)
Ion-free water	800 ml

To block for unspecific binding of proteins the membrane was incubated in a solution of 5 % skim milk in phosphate buffered saline with 0.1 % Tween-20 (PBS-T; PBS from Sigma-Aldrich) for 1 h at RT on a rotation platform. Primary antibodies were diluted in 5 % skim milk in PBS-T and applied over

night at 4 °C. After three washes with PBS-T the membrane was incubated with secondary antibodies diluted in 5 % skim milk in PBS-T for 1 h at RT. After three washes with PBS-T, signal was detected using an Odyssey CLx (LI-COR). Signal intensity was calculated using the programs software. Intensity signals from a minimum of three independent experiments with tissue from different animals/ set of cells were used for calculations of bar graphs.

All primary antibodies and dilutions thereof are listed in table 3-4; secondary antibodies can be found in table 3-5.

3.2.2.3 *ELISA assays*

All ELISA assays were performed according to the manufacturer's instruction (Epigentek), all reagents and buffers were included in the kit. In brief, 50-200 ng DNA was added to assay wells and incubated together with binding solution for 90 min at 37 °C. After all steps subsequent multiple washes with wash buffer were performed. Incubation with a capture antibody was performed for 1 h at RT. A secondary antibody solution was incubated for 30 min at RT. An enhancer solution was added and incubated for 30 min at RT. For detection a color changing solution was applied; color development was observed and measured using a plate reader (Enspire). The percentage of methylated/hydroxyl-methylated DNA in a sample was calculated according to the manufacturer's instructions.

3.2.2.4 *Paraffin embedding of tissue*

Tissue was isolated from the animals by Sabine Matz and Dr. Mario Baumgart, fixed with 4 % PFA solution overnight and stored at 4 °C until further use. To embed the specimens, automated fixation program (Medite) was used with xylol as extraction reagent. Tissue specimens were then cut in 5 µm thin slices using a microtome (ThermoScientific) and 3-10 slices were mounted on a single glass slide (Menzel by ThermoFisher).

3.2.2.5 *Immunofluorescence staining*

Specimens were de-paraffinized using Roti-Histol twice for 10 min followed by a descending alcohol series (100-50 % EtOH) and a final 10 min re-hydration step in water. For antigen retrieval, slides were submerged in 1 % citrate buffer solution (Abcam) for 20 min in a 95 °C water bath. When the cuvette was cooled down to RT, slides were carefully dried and specimen were covered in antibody diluent (DAKO) for incubation > 1 h. All primary antibodies were applied overnight at 4 °C. After three washes with 1x PBS, tissue sections were incubated with secondary antibody for 1 h at RT. Slides

were washed anew three times with 1x PBS (Sigma-Aldrich) and nuclear staining either with TO-PRO3 or DAPI was performed, incubating for 20 min at RT. Three final washes were performed before specimens were covered with mounting medium (DAKO) and a cover slide. Until microscopic analysis was performed, slides were kept covered from light at 4 °C.

All primary antibodies and dilutions thereof are listed in table 3-4; secondary antibodies can be found in table 3-5.

3.2.2.6 *Histological staining*

Specimens were de-paraffinized using Roti-Histol twice for 10 min followed by a descending alcohol series (100-50 % EtOH) and a final 10 min re-hydration step in water. Masson-Goldner and Fast Green Sirius Red staining were performed according to the manufacturers' protocol. To enhance staining for connective tissue, Aniline Blue instead of Light Green (Goldner's stain III) was used. Specimens were covered with Roti-Histokitt and a cover slide. After drying overnight, the staining could be analyzed using a light microscope. Sudan Black B staining was performed according to a published protocol (Evangelou and Gorgoulis, 2017); however, no counterstaining with nuclear fast red was performed afterwards. The staining could be analyzed immediately after mounting.

3.2.3 Cell culture

All ingredients for cell culture media and supplements can be found in table 3-6.

3.2.3.1 *Cell isolation from N. furzeri skeletal muscle*

Skeletal muscle tissue was freshly isolated and placed in tissue culture medium appropriate for *N. furzeri* cell culture (Tab.3-18) before transportation on wet-ice. Before processing, specimens were weighted and minced on ice with a scalpel in PBS. Tissues from up to three individuals were pooled. Collagenase solution (1 mg/ml) was added to samples and incubated for 30-40 min on a rotation platform at 37 °C until solution turned opaque. Digestion was stopped by adding 10 ml of culture medium. Cell suspension was filtered through 100 µm, 70 µm and 40 µm nylon mesh filters. A centrifugation step at 350 g for 10 min at RT concluded this part of the procedure. The supernatant was then aspirated and pellet re-suspended in culture medium. To reduce contamination from fibroblasts during the final plating of cells, two pre-plating steps in 100 mm dishes were performed to let cells adhere to the plastic. After 15 min of incubation, the supernatant was transferred to a new dish, while adherent cells were covered with medium and incubated at 28 °C for subsequent

observation for several days. In a final step, cells were plated in collagen-coated 6well plates and incubated at 28 °C. Cell culture medium was renewed every two days. Before trypsinization divided, cells were washed with PBS and incubated with trypsin for about 3 min. To stop the reaction, fresh complete medium was added and detached cells transferred into a new dish.

3.2.3.2 *Cell culture of N. furzeri muscle cells*

To maintain cell viability, medium (Tab.3-18) was changed every two days. Cells were passaged at about 70-90 % confluency; after a washing with sterile PBS trypsin was applied for 3-5 min to detach cells from the cell culture dish. To neutralize trypsin, growth medium was added and cells were counted or transferred into larger cell culture dishes. To grow cells, these were kept in a dedicated incubator at 28 °C and 5 % CO_2.

Cells were seeded into 12-well plates for a treatment with EZH inhibitors. The day after, medium was changed to fresh medium containing either 1 µM reagent or equivalent amount of DMSO.

For a growth curve analysis, cells were seeded in two different concentrations (10,000 and 20,000 cells/well) in a 12-well plate. Every other day, cells from one well were detached and counted with a KOVA slide.

B-galactosidase staining was performed according to the manufacturer's instruction (Cell Signaling). After incubation and fixation, pictures were taken using a light microscope (Motic). 25 pictures of each condition were taken randomly and cells positive for β-galactosidase were counted. Percentage of positively stained cells was assessed by counting all cells per field.

For cryo-conservation, cells were washed with PBS twice and incubated with trypsin at 28 °C for 3 min. Cells were collected with 4 ml medium in a Falcon tube and centrifuged for 5 min at 1500 rpm. Cells were re-suspended in medium containing 10 % DMSO and transferred into cryo-vials. After two hours at -20 °C cells were stored at -80 °C overnight before transfer to liquid nitrogen for long-term storage.

Table 3-18: Constitution of medium used for *N. furzeri* muscle cell culture

Ingredient	Amount
Water	10 ml (10%)
FBS	20 ml (20%)
Penicillin/Streptomycin	2.5 ml
L-Glutamine	2.5 ml
DMEM (high glucose)	65 ml
Amphotericin	250 µl
Gentamycin	500 µl

3.2.3.3 *Cell culture of HFCs*

Cells from human cardiac fibroblasts (HCFs) from human donors were obtained as previously described (Vecellio *et al.*, 2014), fetal and adult HCFs were obtained from Innoprot. Cells were split when 70-90 % confluency was reached. Medium (Tab.3-19) was changed every second day. Cells were used until passage 10 was reached.

To induce oxidative stress 200 µM H_2O_2 or 10 µM CCCP was added to the medium and incubated for 24 h before harvesting the cells. 10 mM NAC was added to the medium 16 h before treatment start.

Table 3-19: Constitution of medium for HCF cell culture

Ingredient	Amount
FBS Hyclone serum	20 ml (20%)
Penicillin/Streptomycin	2.5 ml
L-Glutamine	2.5 ml
IMEM	75 ml
β-FGF	250 µl

3.2.4 Statistical analysis

Data in the graphs are expressed as mean values with standard error of means (SEM). GraphPad Prism was used to test statistical significance. To compare two groups, a student t-test was performed. For multiple comparisons, analysis of variance (ANOVA) was used with the Kruskal-Wallis test in combination with a Dunns post-test. Statistical significance was defined when $p < 0.05$ and indicated with the "*****" symbol.

4 Results

Global histone modifications were evaluated using two different sources to assess potential epigenetic changes associated with age in the muscle tissue of *N. furzeri*. Frozen material from different ages was lysed and used for Western blot analyses while paraffin embedded tissues were used for immunofluorescence and histological analyses. Total RNA and DNA were extracted from a separate set of frozen material to investigate changes of mRNA expression of specific genes and investigate alterations of global DNA methylation and hydroxyl methylation occurring with age. Experiments were carried out not only in skeletal muscle tissue but also in whole hearts from *N. furzeri* to evaluate similarities and/or differences between these two organs.

Isolation of cells from skeletal muscle was performed in addition to the analysis of specific tissues. The cells could be cultured for many passages and were utilized for selected experiments to confirm observations obtained from skeletal muscle tissue and to evaluate the effect of drug treatment in an *in vitro* setting.

Finally, changes in microRNA expression upon aging were investigated. In collaboration with Dr. Roberto Ripa and Prof. Dr. Alessandro Cellerino from SNS in Pisa, Italy, a zebrafish mutant was generated to further explore the role of one specific microRNA, miR-29.

4.1 Histone methylation

4.1.1 Markers for closed chromatin increase with age

Skeletal muscle tissue from *N. furzeri* taken at young, adult and old age was used for immunofluorescence and Western blot to evaluate changes in histone methylation. Immunofluorescence microscopy analysis revealed an accumulation of histone 3, lysine 27, tri-methylation (H3K27me3) in old in *N. furzeri* skeletal muscle compared to young animals (Fig.4-1.A). Moreover, immunofluorescence determination of heterochromatin protein 1 alpha (HP1α) distribution, a marker for closed chromatin, and for H3K27me3 showed a significant co-localization of the two antigens in skeletal muscle tissue of old *N. furzeri* (Fig.4-1.B), suggesting for a predominant closed chromatin state in old animals.

The evidence of an increased H3K27me3 in old *N. furzeri* skeletal muscle was confirmed by Western blot analysis (Fig.4-1C). Interestingly, other histone marks, associated with closed chromatin conformation, accumulated with age in skeletal muscle tissue of *N. furzeri*. Specifically, histone 3, lysine 9, tri-methylation (H3K9me3) increased with age as well as histone 4, lysine 20, tri-methylation (H4K20me3). Densitometry analysis of Western blotting signals showed that histone marks

associated with closed chromatin were up to five-fold higher in old *versus* young animals; in these experiments, total histone 3 (H3) was used as loading control (Fig.4-1C). These results suggested that, in skeletal muscle, the genome of the *N. furzeri* became more compacted with age.

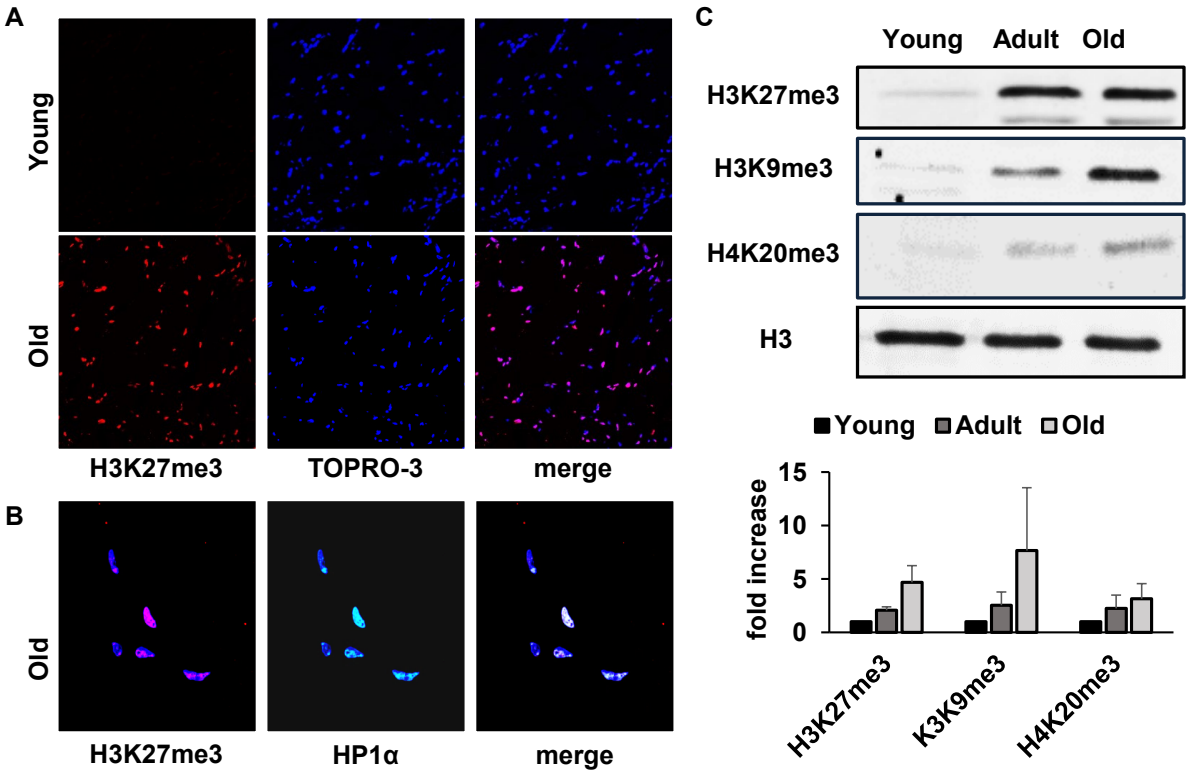

Figure 4-1: Repressive histone marks accumulate in *N. furzeri* muscle tissue with age . (A) Representative confocal microscopy images of H3K27me3 (red) in young (upper panel) and old (lower panel) *N. furzeri* muscle tissue. Nuclei were counterstained with TOPRO-3 (blue). (B) Representative confocal microscopy images of H3K27me3 (red, left panel) and HP1α (green, middle panel) of old *N. furzeri* muscle tissue. Overlapping signal appears in white (right panel), nuclei were counterstained with TOPRO-3 (blue). (C) Representative Western blot analysis H3K27me3, H3K9me3 and H4K20me3 expression in muscle tissue from young, adult and old *N. furzeri*. Protein levels of total histone 3 (H3) were used as loading control. Densitometry analysis of H3K27me3, H3K9me3 and H4K20me3 expression in muscle tissue.

4.1.2 Markers for open chromatin conformation decrease with age

Since a trend towards a more closed chromatin conformation was detected in the skeletal muscle tissue of old *N. furzeri*, changes of histone marks associated with open chromatin were analyzed in order to investigate whether these histone marks were changed according to age in *N. furzeri* skeletal muscle. Confocal immunofluorescence showed that histone 3, lysine 9, acetylation (H3K9ac) is reduced in old *N. furzeri* skeletal muscle (Fig.4-2A). In parallel, Western blotting further indicated that histone 3, lysine 4, tri-methylation (H3K4me3) and also histone 3, lysine 9, acetylation (H3K9ac) and histone 4, lysine 16, acetylation (H4K16ac) as well decreased with age in *N. furzeri* skeletal muscle tissue (Fig.4-2B).

Taken together, these observations suggest that, upon age, the chromatin in the muscle tissue of *N. furzeri* became less accessible and more condensed.

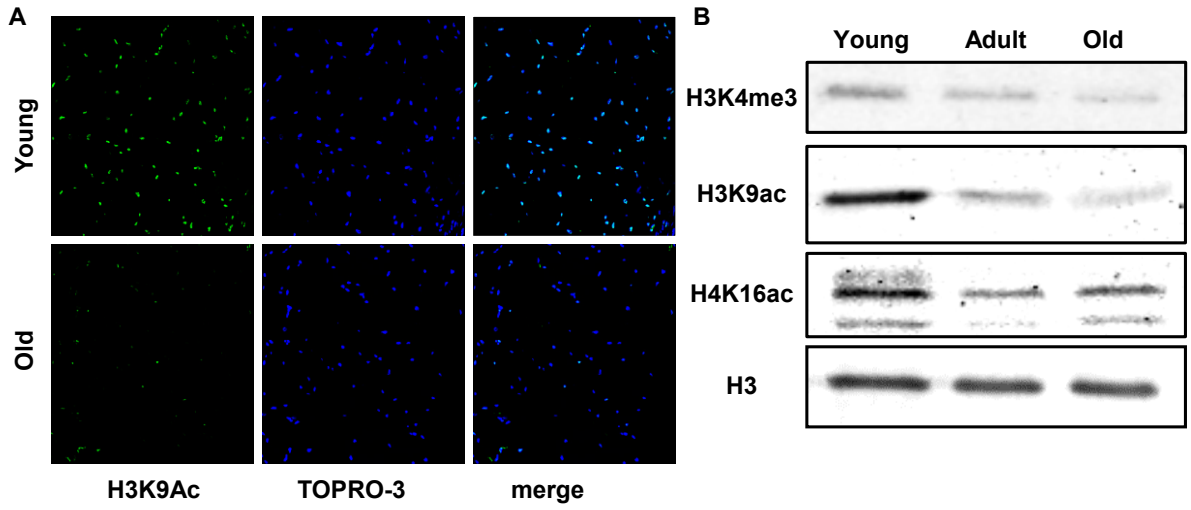

Figure 4-2: Activating histone marks decrease in *N. furzeri* muscle tissue with age . (A) Representative confocal microscopy images of H3K9Ac (green) in young (upper panel) and old (lower panel) *N. furzeri* muscle tissue. Nuclei were counterstained with TOPRO-3 (blue). (B) Representative Western blot analysis of H3K4me3, H3K9ac and H4K16ac expression in muscle tissue from young, adult and old *N. furzeri*. In each condition, protein level of total histone 3 (H3) was used as loading control.

4.1.3 Expression of methylating enzymes increases in *N. furzeri* muscle tissue with age

Histone methylation is accomplished by specific enzymes, the so-called epigenetic writers. In the light of evidence that the chromatin of *N. furzeri* became enriched in methylation compatible with more condensed structure, evaluation of mRNA expression of these enzymes was of particular interest. The proteins of the polycomb repressive complex 2 (PRC2) are responsible for establishing the histone mark for H3K27me3. By quantitative PCR (qRT-PCR) analysis, mRNA levels of genes of interest might be assessed. With this method, high expression levels of PRC2 mRNAs were detected in skeletal muscle tissue of old *N. furzeri* (Fig.4-3A). mRNAs of *enhancer of zeste 1* and *2* (*ezh1* and *ezh2*) and *embryonic ectoderm development* (*eed*) increased with age, paralleled by other members of the PRC1 and 2. In fact, a steep increase of expression in mRNA encoding for levels of *chromobox 7* (*cbx7*) and *chromobox 8* (*cbx8*) could be observed in old animals (Fig.4-3B). In addition, expression of mRNAs encoding for enzymes responsible for removal of methyl residues could be determined: specifically, the mRNA level of *lysine demethylases 6a* (*kdm6a*) and *6b* (*kdm6b*) was significantly altered in the skeletal muscle tissue of adult *N. furzeri* (Fig.4-3C). This finding suggested that higher mRNA levels of methylated histones could result from the coordinated increase in mRNA of PRC members and declined expression of *kdms*.

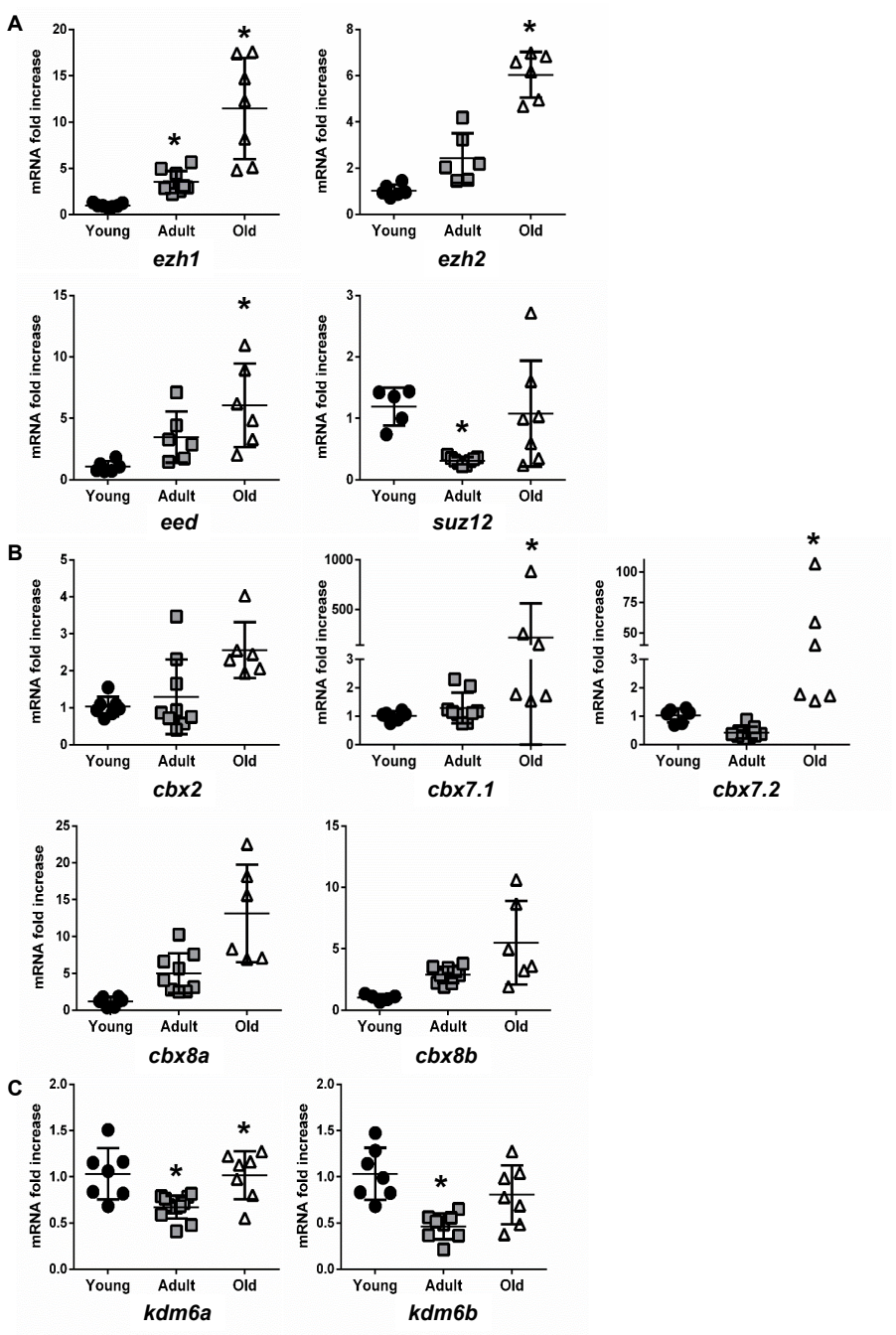

Figure 4-3: Methylating enzymes have increased gene expression levels with age in *N. furzeri* skeletal muscle . (A) qRT-PCR analysis of polycomb repressive complex 2 mRNAs in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* muscle tissue expressed as fold increase versus young samples. (B) qRT-PCR analysis of selected polycomb repressive complex 1 mRNAs in young, adult and old *N. furzeri* muscle tissue expressed as fold increase versus young samples. (C) qRT-PCR analysis of *kdm6a* and *kdm6b* mRNAs in young, adult and old *N. furzeri* muscle tissue expressed as fold increase versus young samples. *p<0.05 vs. young.

4.1.4 Histone methylation in other tissue

In the light of the finding that upon age H3K27me3 accumulated in the brain of *N. furzeri* (Baumgart *et al.*, 2014b), it was considered of particular interest to investigate whether H3K27me3 accumulated also in other tissues of the killifish. To this aim, a culture of primary *N. furzeri* skeletal muscle cells was established. A mix of cells mainly consisting of fibroblasts and myoblasts was obtained which could be passaged multiple times. Western blot analysis was performed to investigate whether histone modifications were maintained in cells from animals at different ages. It could be shown that H3K27me3 was elevated in cells from old *N. furzeri* compared to those isolated from young animals. Conversely, H3K9ac was decreased in cells from old muscle tissues confirming previous findings from skeletal muscle tissue (Fig.4-4A).

Further, an accumulation of H3K27me3 could be detected also in the heart of aged *N. furzeri*, (Fig.4-4B). Opposite to skeletal muscle tissue H3K9ac also increased in the heart suggesting for differential regulation of this modification occurring in the two types of tissue.

As a control tissue the epigenetic landscape of liver from young and old *N. furzeri* was investigated. Remarkably, no increase of H3K27me3 was detected (data not shown). This is of particular interest as liver tissue is known to be able to efficiently regenerate and could be associated to the particular features of this organ. Further experiments are necessary to elucidate this interesting aspect.

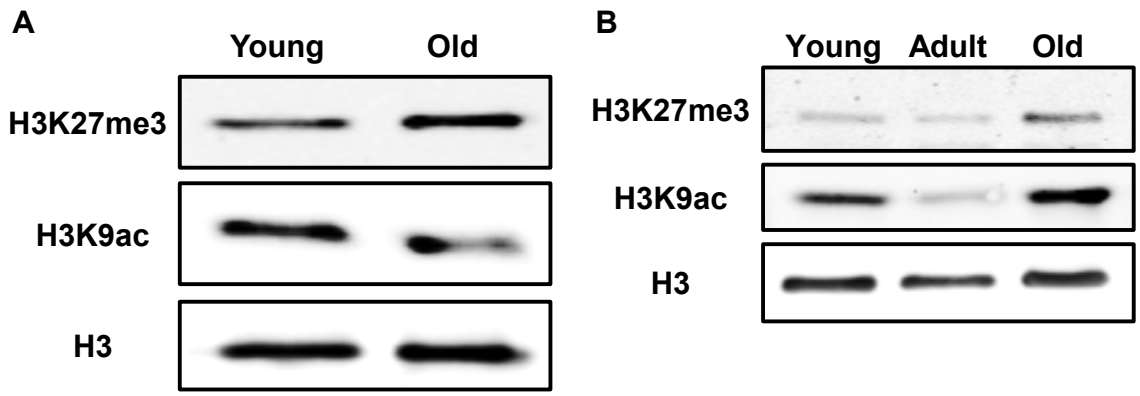

Figure 4-4: Histone modifications in cells and heart from *N. furzeri* . (A) Representative Western blot analysis of H3K27me3 and H3K9ac expression in cultured cells derived from young and old *N. furzeri* muscle tissue. Total histone 3 (H3) was used as loading control. (B) Representative Western blot analysis of H3K27me3 and H3K9ac expression in heart tissue from young, adult and old *N. furzeri*. Total histone 3 (H3) was used as loading control.

4.1.5 mRNA sequencing of *N. furzeri* muscle tissue

To gain insight into changes in gene expression associated with aging, a sequencing analysis of the mRNA of *N. furzeri* skeletal muscle was technically performed by the core facility of the Max-Planck-Institute in Bad Nauheim, under supervision of Dr. Carsten Künne, Dr. Stefan Günther and Prof. Dr. Thomas Braun. Total mRNA from young, adult and old *N. furzeri* was isolated and used for sequencing. All subsequent analyses of the sequencing results were performed by the author of this thesis. Comparing the mRNA expression in *N. furzeri* skeletal muscle tissue from young, adult and old animals showed that many genes vary in their expression level upon aging. Analysis and validation of mRNA decreased in expression will be presented in the next subsections (4.1.5.1 and following). Afterwards results from mRNAs, whose expression is increasing with age, will be presented (4.1.5.8).

4.1.5.1 *Reduction of cell cycle and DNA repair-associated gene expression with age*

Venn analysis revealed that several mRNAs are continuously decreased while others are diminished in their expression only between certain age cohorts (Fig.4-5A). In general, expression of 2220 mRNAs of genes was reduced in adult *N. furzeri* muscle compared to young muscle tissue (see sum of the mRNAs in the blue circle). A total of 1620 mRNAs were decreased in expression comparing old versus young muscle tissue (see sum of the mRNAs in yellow circle). 281 mRNA were downregulated in old muscle tissue in comparison to adult (see sum of the mRNAs in the green circle). Furthermore, this analysis demonstrated that many mRNAs were reduced throughout lifespan (see overlapping areas of circles). For subsequent analysis focus was laid on mRNA expression changes occurring between young and old (see yellow circle).

Analysis was performed using online available resources. Namely the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al.*, 2009) was used for enrichment and classification of sequencing data. With the Kyoto Encyclopedia of Genes and Genomes (KEGG) identification of specific molecular patterns and pathways was achieved. The KEGG pathway analysis of the 1620 mRNAs downregulated from young to old *N. furzeri* skeletal muscle tissue indicated that pathways associated with cell cycle, DNA synthesis and DNA repair are less expressed (Fig.4-5B). Therefore, cell cycle and DNA synthesis and repair might be impaired with age in *N. furzeri* skeletal muscle.

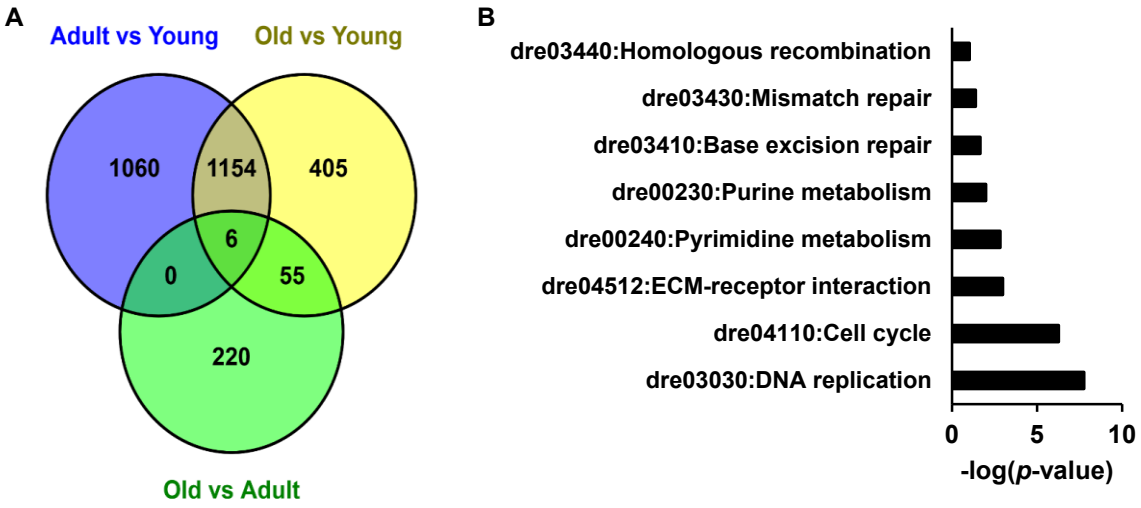

Figure 4-5: Sequencing analysis of genes that are downregulated in *N. furzeri* skeletal muscle while aging . (**A) Venn diagram depicting downregulated mRNA of genes in *N. furzeri* muscle tissue at different age. (B) KEGG pathway analysis of genes that are downregulated in their expression at old versus young age: signal transduction of cell cycle (dre04110) and DNA replication (dre03030) are the most strongly downregulated pathways. In addition to that pathways related to DNA damage repair (dre03430 and dre03410) are also reduced with age.**

4.1.5.2 *Cell cycle and proliferation are reduced upon aging*

KEGG pathway analysis of the sequencing data indicated that mRNA expression of genes associated to cell cycle was reduced with age in *N. furzeri* skeletal muscle. Western blot and qRT-PCR analyses were carried out on selected cell cycle and differentiation markers to validate these findings. Expression of *cyclinB* and *cyclinD* was found reduced in tissue from old animals (Fig.4-6A). Moreover, mRNA expression for the *proliferating antigen* (*pcna*) was significantly lower in *N. furzeri* muscle tissue with aging (Fig.4-6A). Indeed, Western blot analysis confirmed a significant decline in PCNA with age in lysates from *N. furzeri* muscle tissue (Fig.4-6B). Hence it could be demonstrated that cell cycle and proliferation were impaired in skeletal muscle tissue from old *N. furzeri*. In addition, mRNA expression of *myogenin* (*myog*), a marker of differentiation and myogenesis of muscle cells, was also reduced in old skeletal muscle tissue (Fig.4-6C).

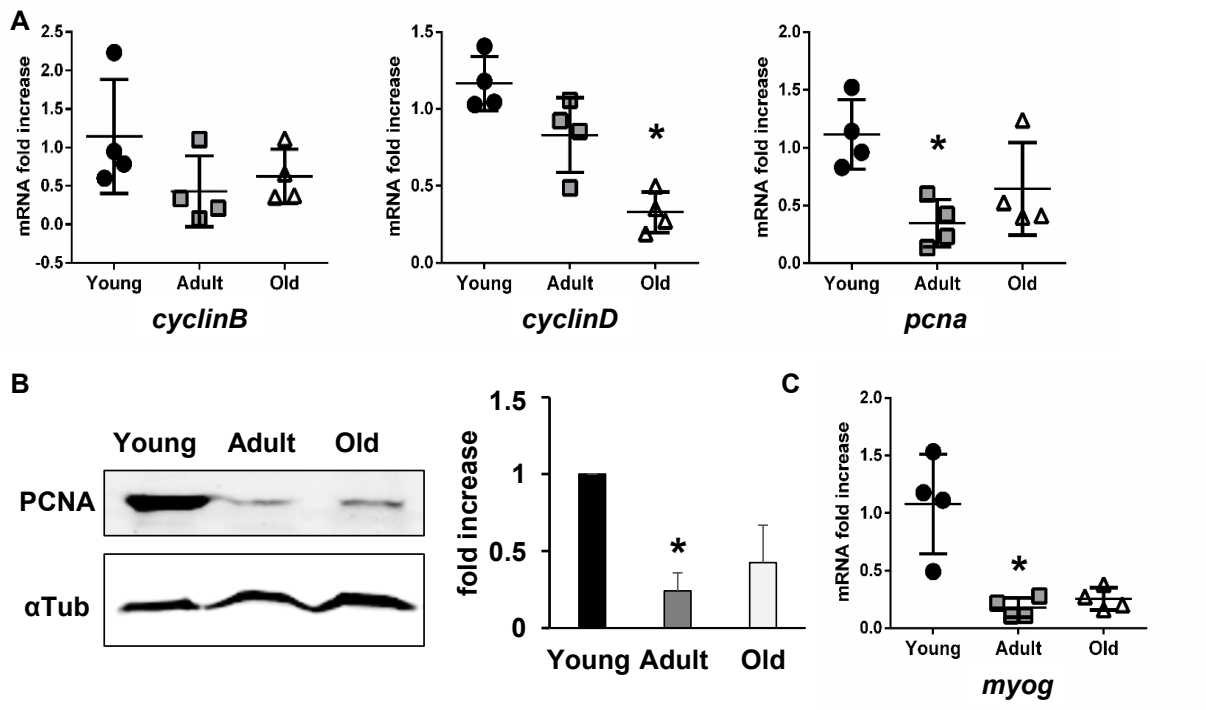

Figure 4-6: Expression of cell cycle genes declines with age in *N. furzeri* skeletal muscle . (A) qRT-PCR analysis of markers of cell cycle progression *cyclinB* and *cyclinD* as well as for *pcna* in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* muscle tissue. (B) Representative Western blot analysis of PCNA protein levels in muscle tissue from young, adult and old *N. furzeri*. Protein levels of alpha tubulin (αTub) were used as loading control. Densitometry of signal intensity was calculated from PCNA protein levels in skeletal muscle of three individual *N. furzeri*. Intensity signal was normalized to young samples (black bars). Adult samples depicted with gray bar, old samples with white bars. (C) qRT-PCR analysis of *myogenenin* (*myog*) in young, adult and old *N. furzeri* muscle tissue expressed as fold increase versus young samples.*p<0.05 vs. young.

To determine whether cell cycle and proliferation are arrested also in the aging heart of *N. furzeri*, Western blot and qRT-PCR analysis were carried out on cell cycle specific markers: mRNA expression of *cyclinB* was found to be diminished in old *N. furzeri* hearts (Fig.4-7A), for *cyclinD* no clear trend could be observed. mRNA expression levels for *pcna* are significantly reduced in old *N. furzeri* hearts compared to expression levels in young *N. furzeri* hearts (Fig.4-7A). Western blot analysis of PCNA confirmed these findings as it shows that protein levels for PCNA decline with age in lysates from *N. furzeri* hearts (Fig.4-7B). Moreover, one could also detect an accumulation of DNA damage in heart tissue lysates from old *N. furzeri* hearts (Fig.4-7C).

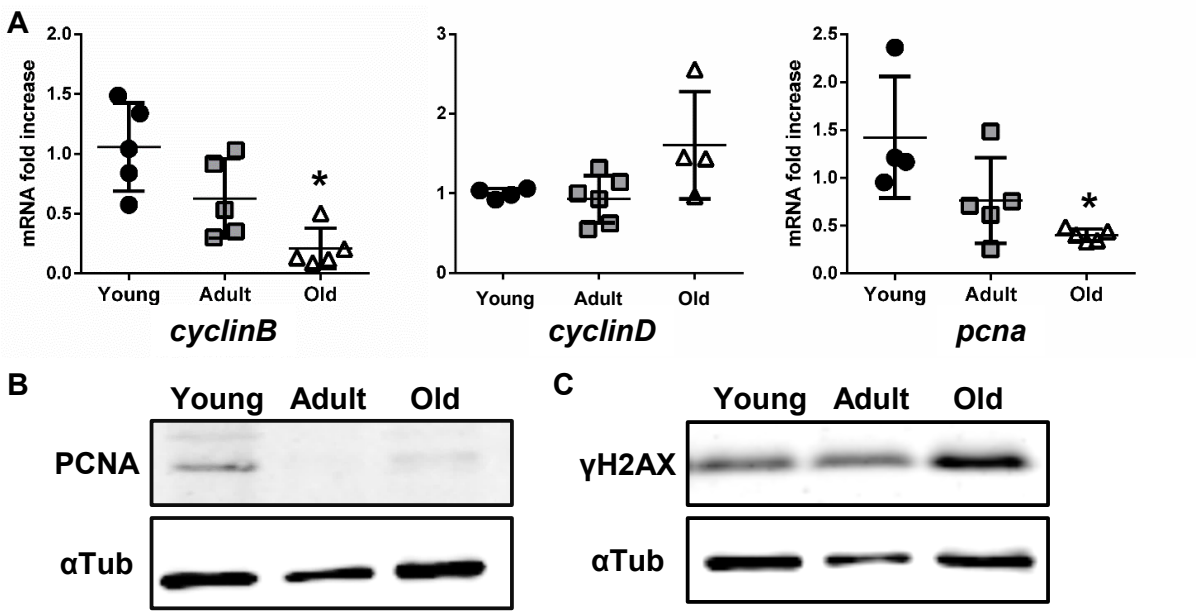

Figure 4-7: Proliferation declines in *N. furzeri* heart while γH2AX, a marker for DNA damage, accumulates . (A) qRT-PCR analysis of *cyclinB*, *cyclinD* and *proliferating antigen* (*pcna*) in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* heart. (B) Representative Western blot for PCNA protein levels in young, adult and old *N. furzeri* heart. Protein levels of αTubulin (αTub) were used as loading control. (C) Representative Western blot for γH2AX in young, adult and old *N. furzeri* heart. Protein levels of αTub were used as loading control. *p<0.05 vs. young

4.1.5.3 *DNA damage accumulates with age in N. furzeri muscle*

Sequencing data showed that genes associated with DNA repair and synthesis are also significantly reduced with age in *N. furzeri* skeletal muscle. KEGG pathways analysis also showed that excision repair and homologous recombination as well as DNA repair and purine and pyrimidine metabolism were downregulated in old *N. furzeri* skeletal muscle tissue compared to young *N. furzeri* muscle (Fig.4-8B). Therefore, it could be speculated that damaged DNA might accumulate over time in aging *N. furzeri* skeletal muscle. To explore this possibility, Western blot analysis of the DNA damage marker γH2AX was performed using lysates from young, adult and old *N. furzeri* skeletal muscle. Indeed, γH2AX accumulated in muscle tissue from old *N. furzeri* (Fig.4-8A). Furthermore, qRT-PCR evaluation of the *growth arrest and DNA damage inducible 45γ* (*gadd45γ*) mRNA was performed in young, adult and old *N. furzeri* muscle samples showing levels progressively increasing with age (Fig.4-8B). These results from markers for DNA damage suggest an accumulation of DNA damage due to reduced expression of DNA repair genes, which were found to be reduced in the sequencing data. The observations from aging heart and skeletal muscle of *N. furzeri* were very similar, pointing towards a common aging phenotype of both tissues.

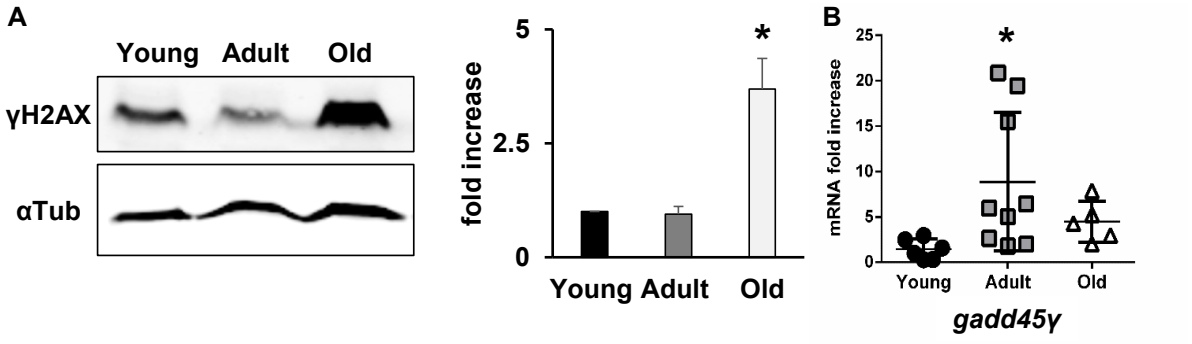

Figure 4-8: DNA repair capacity declines with age in *N. furzeri* skeletal muscle leading to increase of marker for DNA damage . (A) Representative Western blot analysis of γH2AX protein levels in muscle tissue from young, adult and old *N. furzeri*. Protein levels of alpha tubulin (αTub) were used as loading control. Densitometry of signal intensity was calculated from PCNA protein levels in skeletal muscle of three individual *N. furzeri*. (B) qRT-PCR analysis of *growth arrest and DNA damage inducible 45γ* (*gadd45γ*) mRNAs in young (black circles), adult (gray squares) and old (white triangles). *p<0.05 vs. young

4.1.5.4 *Negative regulators of cell cycle progression are reduced with age*

Genes of cell cycle-negative regulators such as members of the INK4 locus are well-known targets for H3K27me3 by EZH2. Their expression is greatly reduced and less INK4 proteins become available for cell cycle control, when tri-methylated. Moreover, disturbed expression of cell cycle regulators is a hallmark of aging-associated diseased and hence their expression levels were analyzed via qRT-PCR. While there was no change of expression level of *p15* with age in *N. furzeri* muscle tissue, the expression of mRNAs for *p18* and *p19* was significantly reduced in *N. furzeri* tissue from adult and old *N. furzeri* compared to expression levels in young muscle tissue (Fig.4-9A). In contrast to mammalians, fish do not have INK4 member *p16* in their genomic sequence. Another cell cycle regulator and tumor suppressor, *p53*, had also significantly reduced mRNA expression levels in adult and old *N. furzeri* tissue compared to young muscle tissue (Fig.4-9B). In summary, these results could indicate that silencing of negative cell cycle regulators might occur in consequence of H3K27 tri-methylation or by overall reduced cell activity. However, this finding needs further investigation on the methylation status of the promotor region of these particular genes.

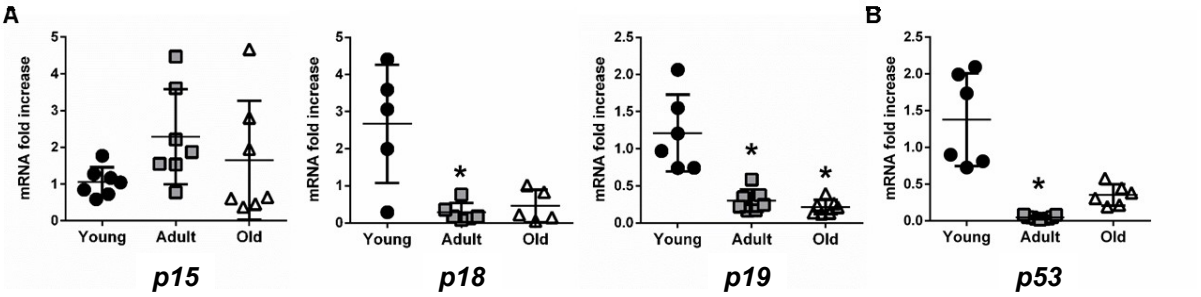

Figure 4-9: Decreased expression of INK4 locus . (A) qRT-PCR analysis of mRNAs of the INK4 locus member *p15*, *p18* and *p19* in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* muscle tissue expressed as fold increase versus young samples. (B) qRT-PCR analysis of tumor suppressor *p53* in young, adult and old *N. furzeri* skeletal muscle tissue expressed as fold increase versus young samples. *p<0.05 vs. young

4.1.5.5 *Markers for cellular senescence increase with age in N. furzeri muscle tissue*

Cell cycle arrest and cellular damage might be associated with oxidative stress often present during cellular or tissue senescence. From the first analyses it was evident that signs of these processes progressively accumulated in *N. furzeri* skeletal muscle upon age. Yet, histological analysis with a Masson-Trichrome staining did not show major changes in muscle morphology (see Fig.1-4). However, aggregation of lipofuscin spots could be observed in paraffin embedded sections of old skeletal muscle with Sudan black b (SBB) (Fig.4-10A). Lipofuscin is a granule pigment that stains depositions of oxidized lipids and proteins, which have been damaged e.g. by ROS. Another marker of cellular senescence is the *cyclin dependent kinase inhibitor 1* (also named *p21*). *P21* expression strongly increased with age in *N. furzeri* skeletal muscle tissue in most individuals (Fig.4-10B). The results from these experiments pointed to an accumulation of markers for cellular senescence in the old *N. furzeri* skeletal muscle.

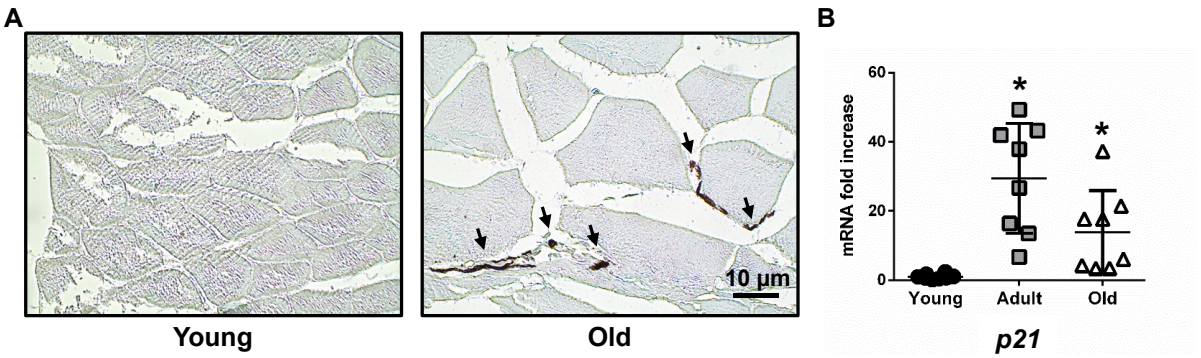

Figure 4-10: Senescence occurs in the aging *N. furzeri* muscle . (A) Representative light microscopic pictures from young (left) and old (right) *N. furzeri* muscle stained with Sudan Black B. Accumulation of lipofuscin staining in the old muscle is indicated with black arrows. Calibration bar = 10 µm. (B) qRT-PCR analysis of *p21* mRNAs in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* muscle tissue. *p<0.05 vs. young

4.1.5.6 *N. furzeri cells recapitulate the aging phenotype of muscle tissue*

Freshly isolated skeletal muscle tissue was digested with collagenase and incubated with medium supplemented with fetal bovine serum (FBS) and water in order to investigate, whether cells from skeletal muscle could be isolated and kept in culture as it has been described for *N. furzeri* fibroblasts derived from skin tissue (Graf *et al.*, 2013) while maintaining epigenetic features that previously had been observed in skeletal muscle tissue. Cells attached to culture dishes and proliferated well. Multiple passages, especially of cells derived from young *N. furzeri* skeletal muscle tissue could be obtained without cells becoming obviously senescent. Several experiments were carried out to determine if cells could maintain the properties and features of their origin.

Cells derived from *N. furzeri* muscle tissue reproduced some features of origin, particularly about gene expression, proliferative capacity and DNA repair. Specifically PCNA protein levels were elevated in cells from young *N. furzeri* muscle, while signals from markers of DNA damage such as γH2AX were higher in cells isolated from muscle tissue of old animals (Fig.4-11A). Interestingly, cells derived from young muscle tissue proliferated more than cells from old muscle tissue as shown by growth curve (Fig.4-11B). In this context, staining for the senescence marker β-galactosidase (β-gal) revealed that in cells from old muscle the number of senescent cells was higher than in cells from young *N. furzeri* muscle (Fig.4-11C). This finding was paralleled by the evidence that mRNA expression level for *p21* was also higher in cells from old *N. furzeri* muscle compared to young muscle (Fig.4-11D). Taken altogether, these results suggest that cells isolated from young and old *N. furzeri* skeletal muscle reveal features similar to those of the tissue origin and that their characteristics remain stable even after a number of passages.

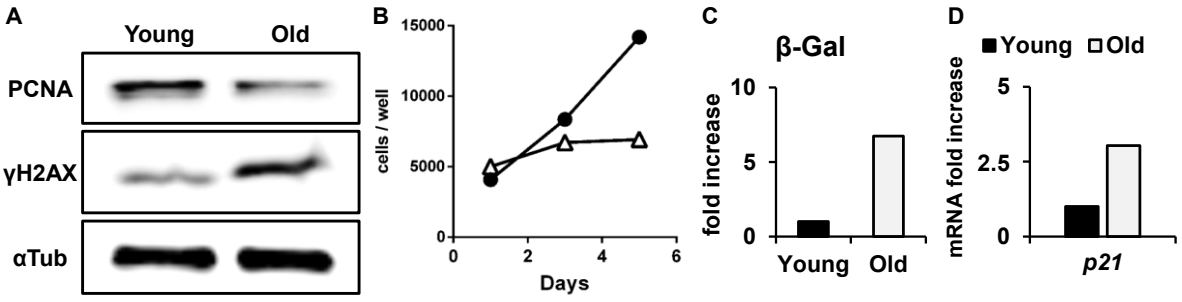

Figure 4-11: Cells obtained from *N. furzeri* muscle tissue resemble the aging phenotype of *N. furzeri* . (A) Representative Western blot for Proliferating Antigen (PCNA) and γH2AX in cells derived from young and old muscle tissue. αTubulin (αTub) was used as loading control. (B) Growth curve of cells derived from young (black dots) and old (white triangles) muscle tissue. (C) Quantification of a β-galactosidase staining; positive cells were counted; percentages were calculated for young (black bar) and old (white bar) cells from muscle tissue, expressed as fold change versus young sample. (D)qRT-PCR analysis of *p21* in cells from young and old muscle tissue.

4.1.5.7 *Cells from N. furzeri can be used for drug treatments*

Cells derived from *N. furzeri* skeletal muscle were used for drug testing of novel histone methylation inhibitors. These drugs described by Ciarapica *et al.* (2014) are specific inhibitors of both EZH proteins, they differ in their residues and are named for subsequent description EZH inhibitors 1 and 2 (EZHi1 and EZHi2) (Fig.4-12A).

Western blotting analysis showed that both inhibitors prevented accumulation of H3K27me3 (Fig.4-12B). However, treatment with EZHi also led to an increase of γH2AX accumulation (Fig.4-12B) – this effect was more evident in cells treated with EZHi2 than in cells exposed to EZHi1. Additionally, qRT-PCR showed that EZHi1 lead to INK4 locus expression more efficiently than EZHi2 treatment (Fig.4-12C). Furthermore, cells under EZHi treatment proliferated about 50 % less than cell under control conditions (Fig.4-12D). Although treatment with EZHi has shown significant reduction in H3K27 tri-methylation and activation of expression of negative cell cycle regulators, more DNA damage and less cell growth was also observed compared to the untreated cells. Due to difficulties in drug administration in the aquatic environments no further testing of these drugs was performed in living animals.

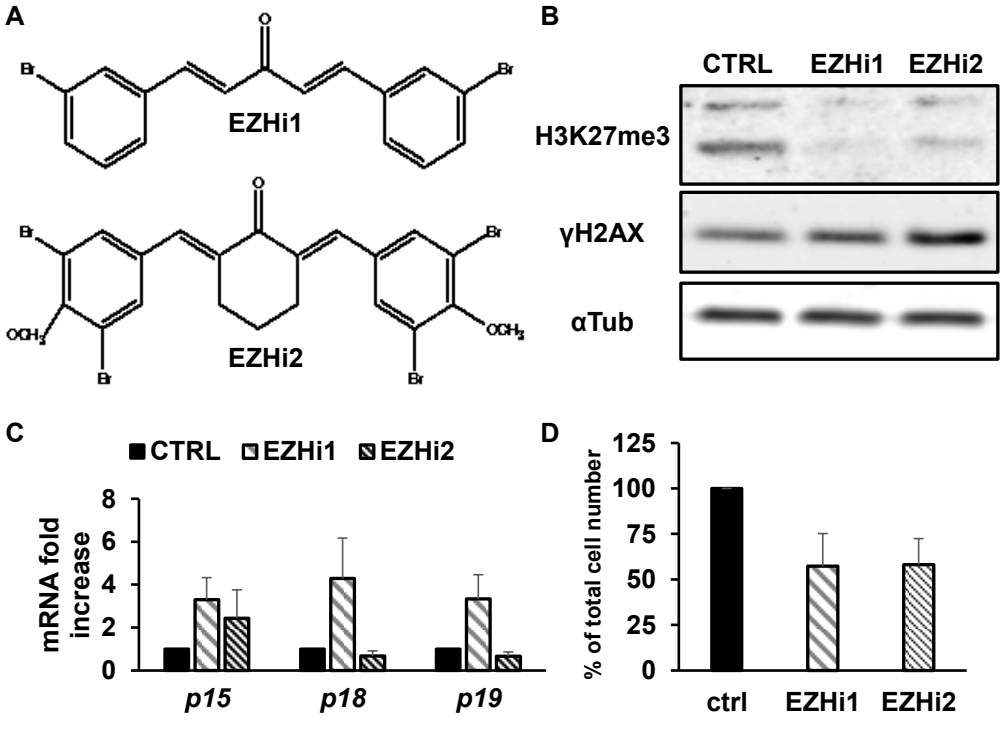

Figure 4-12: Cells from *N. furzeri* muscle tissue can be used for initial drug screens . (A) Chemical structure of inhibitors of enhancer of zeste (EZHi): compound 1 (EZHi1, upper structure) differs in its aromatic residues and mid-part structure from the second compound (EZHi2, lower structure). (B) Representative Western blot for H3K27me3 and γH2AX in cells derived from *N. furzeri* muscle tissue under control (CTRL) and either EZHi1 or EZHi2 treatment. Alpha Tubulin (αTub) was used as loading control. (C) qRT-PCR analysis of mRNA for INK4 locus members *p15*, *p18* and *p19* of cells under control conditions (CTRL, black bars), EZHi1 (lightly striped bars) and EZHi2 (dark gray striped bars). (D) Cells under EZHi treatment proliferate less compared to control cells: % of total cell number of control group after seven days of treatment is plotted.

4.1.5.8 *Inflammation and glycolysis associated genes are upregulated upon aging*

To investigate changes in gene expression, not only the downregulated mRNAs were analyzed but also those mRNAs that are upregulated with age (Fig.4-13): A total of 2031 mRNAs from genes were investigated that are differentially expressed between adult and young *N. furzeri* muscle tissue (see sum mRNAs blue circle). 1438 mRNAs were found upregulated in their expression levels comparing old to young muscle (see sum RNAs yellow circle). Between old and adult animals, 172 genes increased their expression levels (see sum RNAs green circle) (Fig.4-13A).

KEGG pathway analysis of the 1438 genes that are upregulated between young and old age demonstrated that genes associated with inflammation and immune system response were accumulating (Fig.4-13B). Furthermore, genes associated with glycolysis had increased mRNA expression as well, suggesting a possible switch of metabolites and energy supply with age.

In order to validate sequencing, qRT-PCR was performed for selected genes related to inflammatory signaling, confirming evidence of their elevation (Fig.4-13C). Together, these results point to an increased inflammatory signaling in old *N. furzeri* skeletal muscle compared to skeletal muscle of young *N. furzeri*.

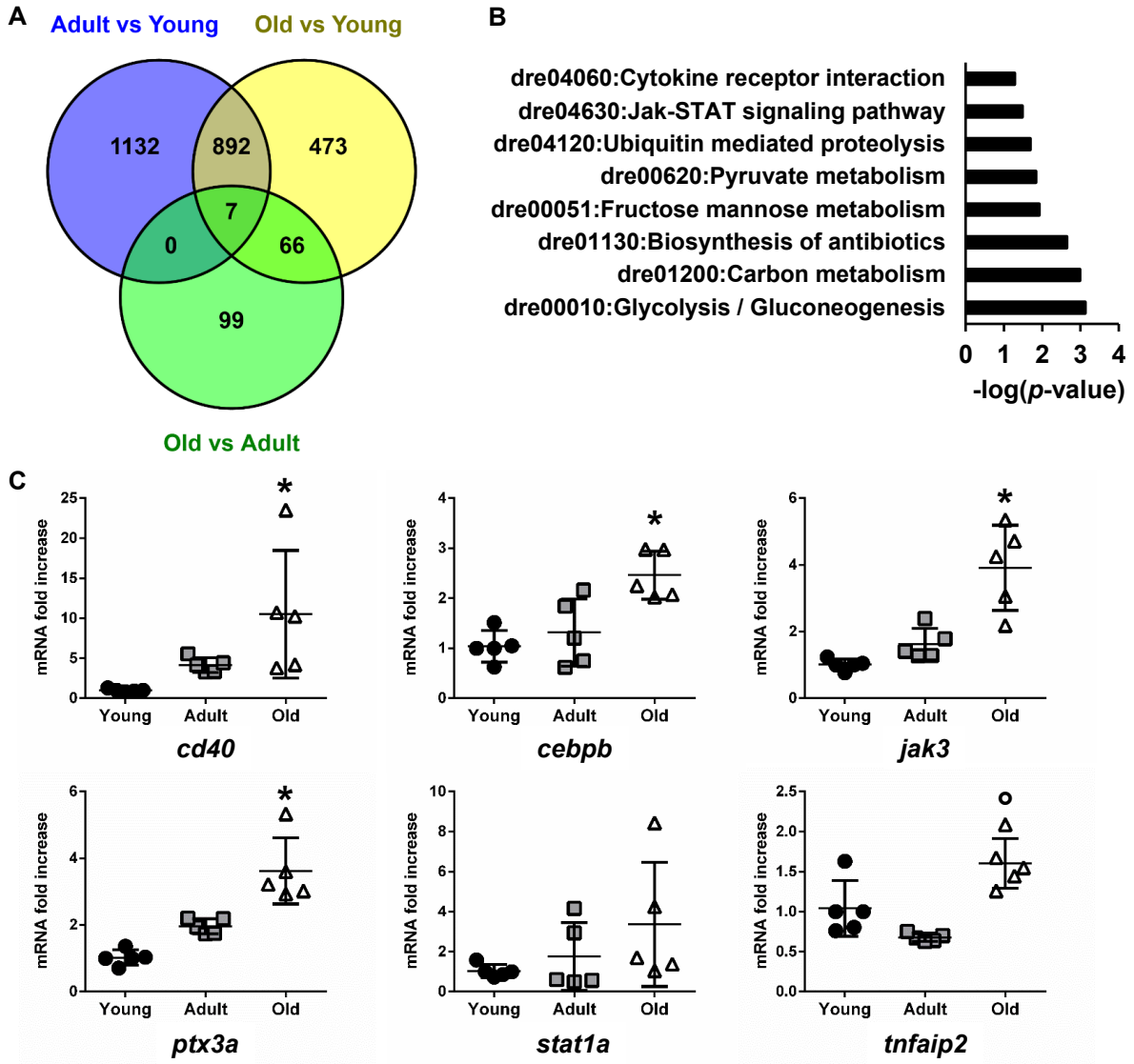

Figure 4-13: Inflammation and glycolysis increase with age . (A) Venn diagram depicting upregulated mRNA of genes in *N. furzeri* muscle tissue at different age. (B) KEGG pathway analysis of genes that are upregulated in their expression at old versus young age. (C) Validation of sequencing results via qRT-PCR analysis of mRNAs related to inflammatory signaling: *cd40 molecule* (*cd40*), *CCAAT/enhancer binding protein beta* (*cebpb*), *janus kinase 3* (*jak3*), *pentraxin 3a* (*ptx3a*), *signal transducer and activator of transcription 1a* (*stat1a*) and *TNF alpha induced protein 2* (*tnfaip2*) in young (black circles), adult (gray squares) and old (white triangles). *p<0.05 vs. young, °p<0.05 vs. adult.

4.1.5.9 *Gene expression of different tissues in old N. furzeri underlying similar findings*

Sequencing results from published databases generated by using other tissues of *N. furzeri* as source were analyzed to compare the results obtained from *N. furzeri* skeletal muscle. In fact, a similar gene expression pattern present in various tissue types suggest a global aging-associated process, while differences might point to tissue specific changes in gene expression occurring upon age. Baumgart *et al.* (2011) published sequencing results on brain, liver and skin of young, adult and old *N. furzeri*. These datasets were crossed with results from sequencing analysis of skeletal muscle tissue and a common age-depended gene expression profile emerged clearly in all four tissues. Six KEGG

pathways from mRNAs that were downregulated with age were found in all four tissues (Fig.4-14A; white circle) and two KEGG pathways from mRNAs were upregulated with age (Fig.4-14B; white circle). The six downregulated KEGG pathways were: DNA replication, cell cycle, ribosome biogenesis in eukaryotes, aminoacyl-tRNA biosynthesis, pyrimidine metabolism and RNA transport. Hence, in muscle, brain, liver and skin, signals associated with cell cycle regulation and DNA synthesis and repair were significantly reduced upon aging. Remarkably, the two upregulated KEGG pathways were JAK/STAT signaling and cytokine/cytokine receptor interaction, suggesting that in all four tissues inflammatory signals might be upregulated with age.

An independent study by Reichwald *et al.* (2015) analyzed gene expression during the diapause stage of the embryo and compared results to gene expression in the aging *N. furzeri* brain: Amongst other pathways they observed a downregulation of DNA replication and cell cycle in diapause and old *N. furzeri* brain. Their dataset was used to compare also mRNA of old *N. furzeri* muscle to mRNAs that were either up or downregulated during diapause and similar results as above were obtained. The mRNAs from 140 genes are downregulated in their expression levels in old *N. furzeri* skeletal muscle compared to young, which were also downregulated during diapause (Fi.4-14C, white circle). KEGG pathway analysis of these 140 genes showed that pathways associated with DNA replication and repair, but also cell cycle were downregulated (Fig.4-14D). The mRNAs of 12 genes were upregulated during diapause, but decreased expression in old muscle tissue was found. Here no pathway analysis was possible due to the low amount of common genes. A further comparison of mRNAs that increase their expression levels during aging and are also increased during diapause, showed an overlap of 38 genes, however, no results from pathway analysis of these genes could be obtained (data not shown). Similarly, eleven genes were found to be upregulated in old *N. furzeri* tissue but downregulated during diapause, again no pathway analysis was technically possible.

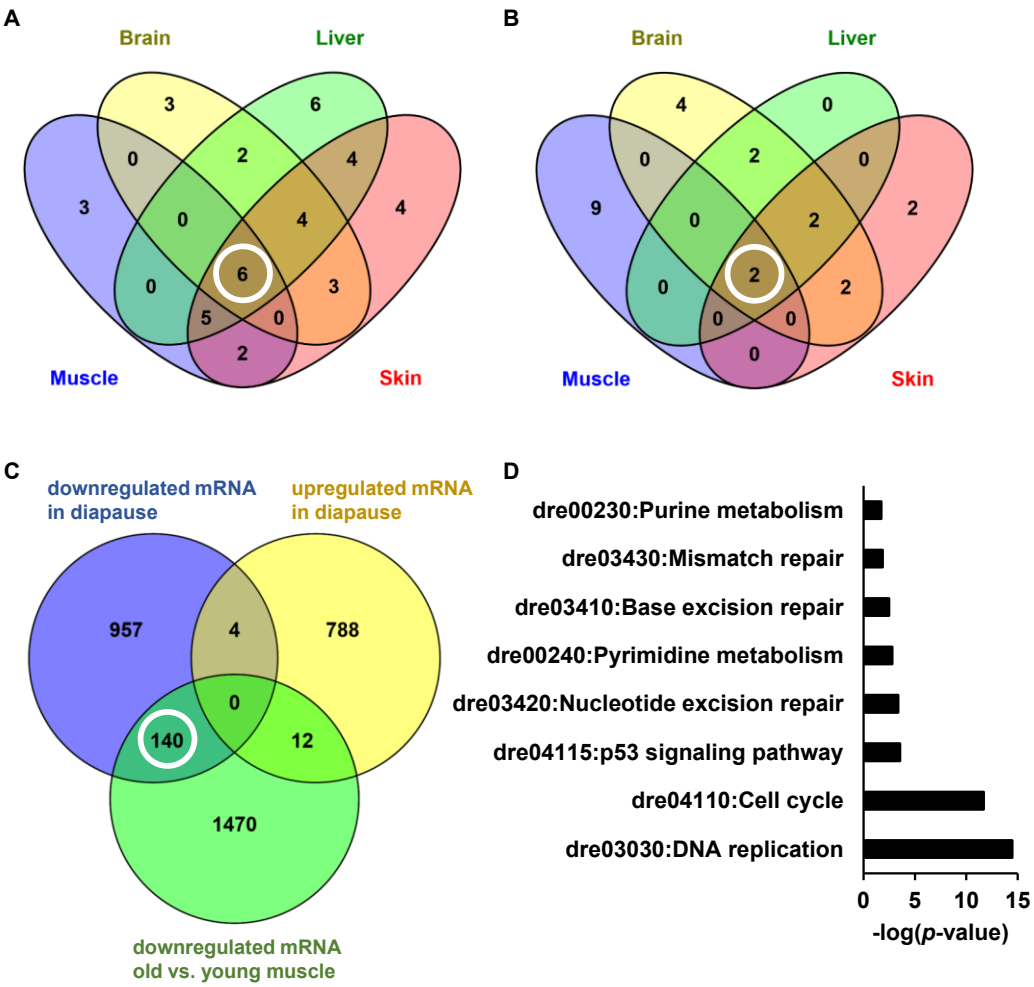

Figure 4-14: Common mRNA gene expression profiles in various tissue types of *N. furzeri* during aging . (A) Venn diagram depicting downregulated KEGG pathways in *N. furzeri* muscle (blue), brain (yellow), liver (green) and skin (red) tissue comparing young versus old samples. Six common KEGG pathways are found (white circle): Ribosome biogenesis in eukaryotes, DNA replication, cell cycle, aminoacyl-tRNA biosynthesis, pyrimidine metabolism and RNA transport. (B) Venn diagram depicting upregulated KEGG pathways in *N. furzeri* muscle (blue), brain (yellow), liver (green) and skin (red) tissue comparing young versus old samples. Two common KEGG pathways are found (white circle): JAK/STAT signaling and cytokine/cytokine receptor interaction. (C) Common mRNA expression profile of downregulated mRNAs during diapause (blue circle), upregulated mRNA during diapause (yellow circle) and downregulated mRNA in old *N. furzeri* muscle tissue (green circle): mRNAs of 140 genes are downregulated during diapause and during aging (white circle). (D) KEEG pathway analysis of 140 genes that are downregulated during diapause and aging. Data from Baumgart *et al.* (2014) (A and B) and Reichwald *et al.* (2015) (C and D) was used to compare with sequencing results from skeletal muscle.

4.2 DNA methylation of skeletal muscle and heart tissue

Histone methylation is often closely associated with DNA methylation (Rose and Klose, 2014). In the experiment performed here, an accumulation of methylated histone marks could be observed in the aging *N. furzeri* skeletal muscle and heart, paralleled by global DNA methylation (5mC) and hydroxyl-methylation (5hmC).

In *N. furzeri* skeletal muscle tissue, global DNA methylation increased with age (Fig.4-15A), despite the reduced expression of DNA methyltransferases. Global hydroxyl-methylation of DNA also increased at adult stage. However, this level declined again with old age (Fig.4-15A). Expression of mRNAs for *DNA methyl transferases 1* and *3a* (*dnmts*) significantly decreased at adult stage and even further at old age, while expression of *dnmt3b* was more variable being in some individuals higher than in others (Fig.4-15B). *Ten-eleven translocases* (*tets*) *1* and *2* are not regulated in an age-dependent manner. However, *tet3* and *thymine DNA glycosylase* (*tdg*) were significantly reduced in their expression levels with age in *N. furzeri* muscle tissue (Fig.4-15C).

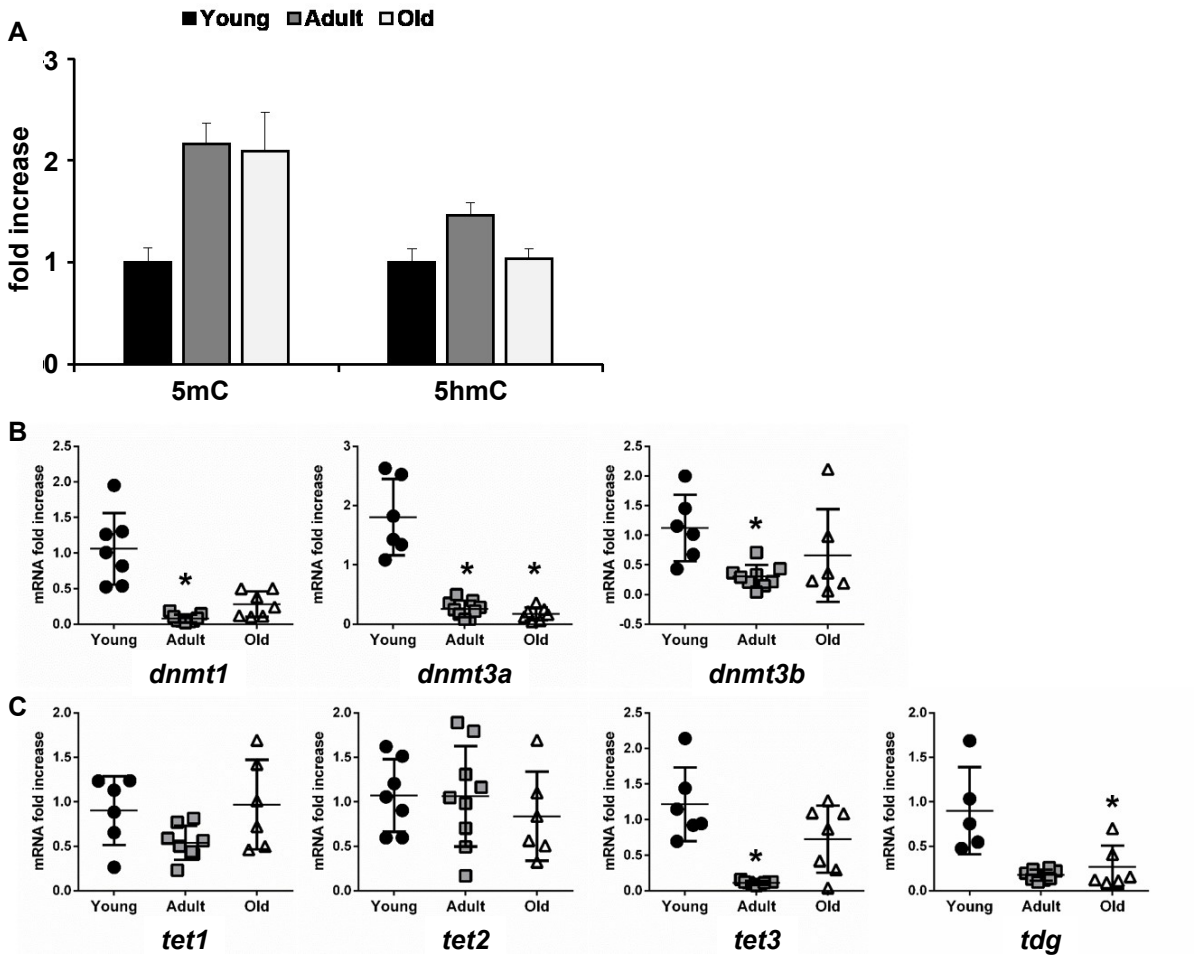

Figure 4-15: DNA methylation pattern changes with age in *N. furzeri* skeletal muscle . (A) ELISA-based analysis of 5-methyl cytosine (5mC) and 5-hydroxyl-methyl cytosine (5hmC) in *N. furzeri* skeletal muscle tissue. (B) qRT-PCR analysis of *DNA-methyl transferases* (*dnmts*) in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* muscle tissue expressed as fold increase versus young samples. (C) qRT-PCR analysis of *ten-eleven translocases* (*tets*) and *thymine DNA glycosylase* (*tdg*) in young, adult and old *N. furzeri* skeletal muscle tissue expressed as fold increase versus young samples. *p<0.05 vs. young

In contrast to skeletal muscle, in the heart global DNA methylation and hydroxyl-methylation decreased with age (Fig.4-16A). While expression of *dnmt1* and *dnmt3a* were significantly reduced with age, *dnmt3b* expression was comparable in muscle tissue only reduced in some hearts of old individuals (Fig.4-16B). Additionally, mRNA expression levels of *tet2*, *tet3* and *tdg* were significantly reduced in old *N. furzeri* hearts when compared to expression levels in the heart of young animals (Fig.4-16C).

While expression of DNA methylating enzymes appeared to be reduced in skeletal muscle as well as in heart tissue of old animals, DNA methylation was reduced only in hearts but not in skeletal muscle tissue from old *N. furzeri*. This might be consequence of functional and biological differences between the two tissue types.

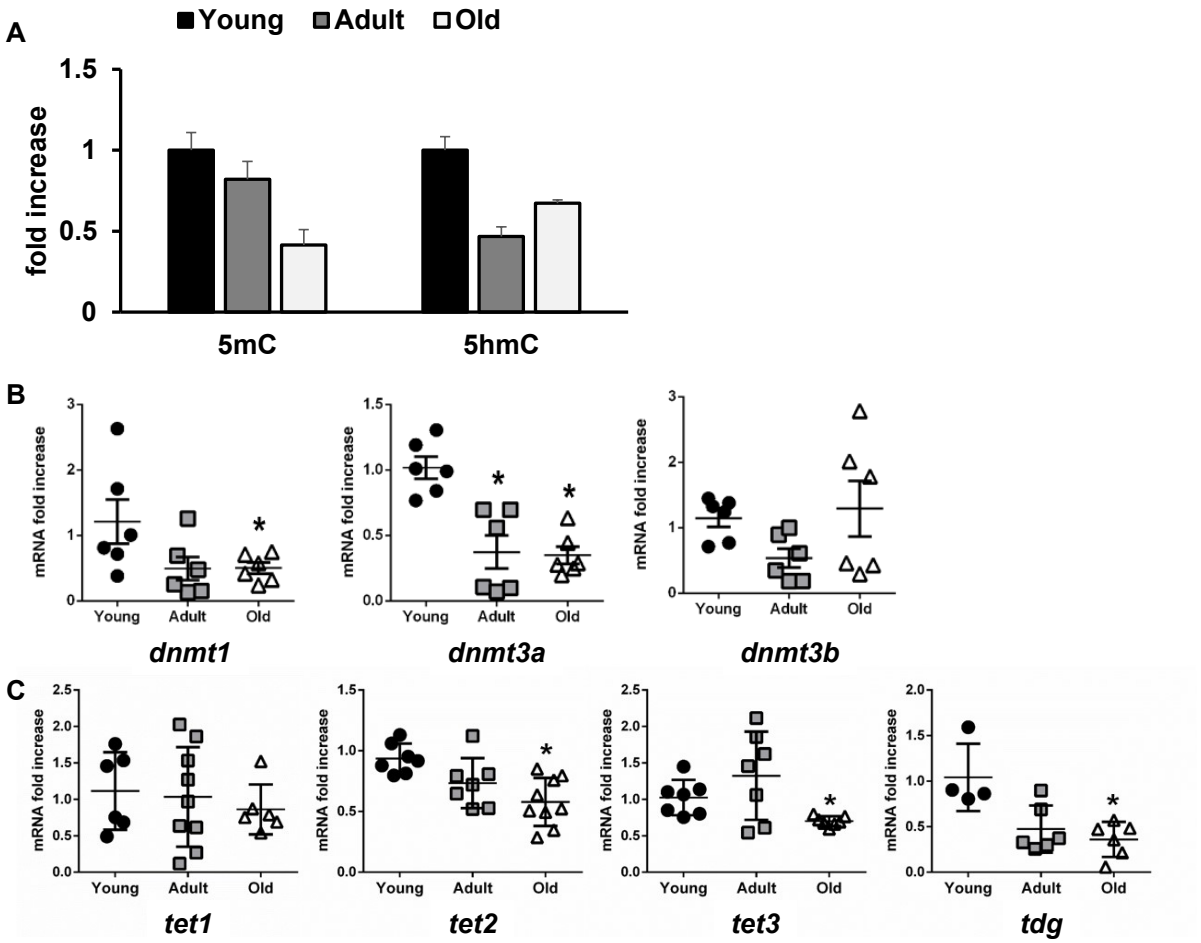

Figure 4-16: DNA methylation pattern changes with age in the heart of *N. furzeri* . (A) ELISA-based analysis of 5-methyl cytosine (5mC) and 5-hydroxyl-methyl cytosine (5hmC) in *N. furzeri* hearts. (B) qRT-PCR analysis of *DNA-methyl transferases* (*dnmts*) in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* hearts expressed as fold increase versus young samples. (C) qRT-PCR analysis of *ten-eleven translocases* (*tets*) and *thymine DNA glycosylase* (*tdg*) in young, adult and old *N. furzeri* hearts expressed as fold increase versus young samples. *p<0.05 vs. young. Data in parts from Heid *et al.* (2017).

4.3 microRNA analysis

Non-coding RNAs (ncRNAs) are important regulators of the epigenetic machinery. They are transcribed from the genome but are not translated into functional proteins but have rather regulatory functions – similar to histone modifications they can repress gene expression, stabilize or change conformation (Winter *et al*., 2009). Among the several different types of ncRNAs the microRNAs (miRNAs) play an important regulatory role. Moreover, in contrast to long-non-coding RNAs, the sequence of miRNAs are highly conserved among species and distributed in a tissue-specific fashion. To gain insight into changes in miRNA expression with age the microRNAome in the heart of *N. furzeri* was sequenced in collaboration with the core facility of the Fritz-Lippmann-Institute under the supervision of Dr. Mario Baumgart.

Expression of miRNAs was strictly age dependent, as shown by the so-called principal component analysis (PCA) (Fig.4-17A). Remarkably, hierarchical clustering showed that miRNAs expressed at low levels at young age were highly expressed at old age and *vice versa* (Fig.4-17B). Although the mechanistic reason for inversion of expression remained unclear, the miRNA expression profile seemed to be age-specific.

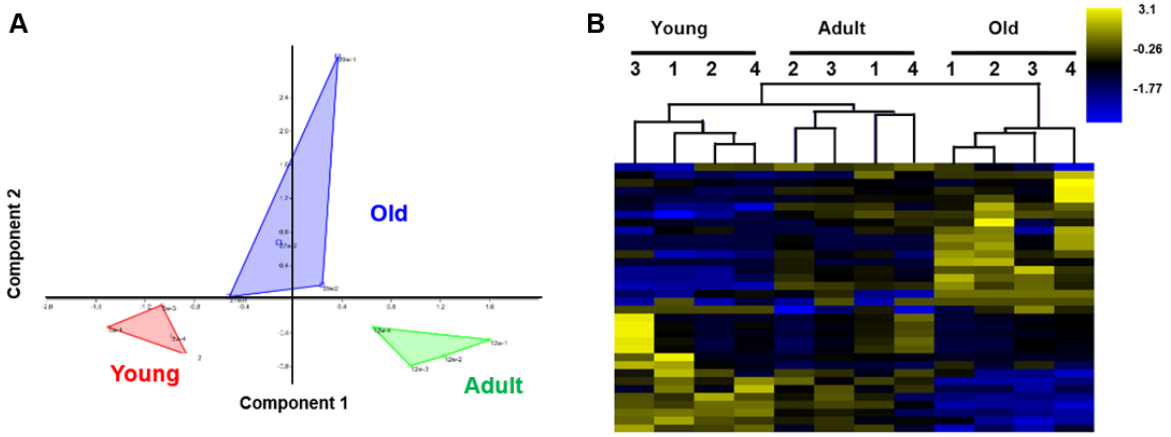

Figure 4-17: In *N. furzeri* heart, miRNAs are expressed in an age-dependent manner . (A) Principal Component Analysis (PCA) of miRNAs in young (red triangle), adult (green triangle) and old (blue triangle) *N. furzeri* hearts. (B) Hierarchical clustering of miRNAs regulated more than ±1.3 log2 fold change. Yellow and blue represent low and high-expressed miRNAs, respectively. Data from Heid *et al.* (2017).

4.3.1 miR-29 increases with age in *N. furzeri* tissue

Analyzing the miRNA sequencing in the heart of *N. furzeri* it became apparent that miR-29 was among the most strongly upregulated miRNAs in old animals. This miRNA is of particular interest as it is reduced upon myocardial infarction (MI) (van Rooij *et al.*, 2008) and seems to protect from fibrosis (He *et al*., 2013; Cushing *et al*., 2015). For this reason, miR-29 has been selected for further analysis. miR-29 levels have been reported to increase with age (Seeger T, 2015). Consistently, in the *Nothobranchius* model, miR-29 clearly increased with age in the heart (Fig.4-18A) and skeletal muscle tissue (Fig.4-18B), as well as in other tissues (Heid *et al.*, 2017; Ripa *et al.*, 2017). To validate these findings in an independent system, it could be shown that also in human cardiac fibroblasts (HCFs), miR-29 increased with the age of the donors (Fig.4-18C). Therefore, miR-29 seemed to increase with age not only in different tissues of the fish but also in mammalian cells suggesting an epigenetic regulation possibly under control of the aging clock (Lowe *et al*., 2016).

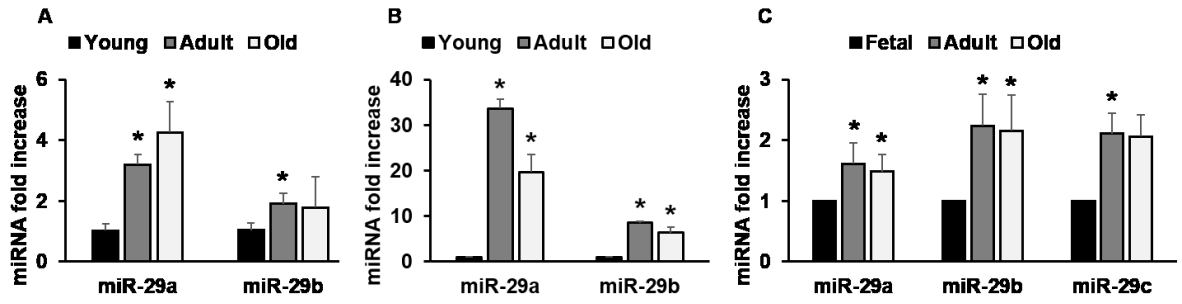

Figure 4-18: miR-29 is upregulated upon aging in *N. furzeri* heart and skeletal muscle, but also in human cardiac fibroblasts . (A) qRT-PCR analysis of miR-29 family members in young (black bars), adult (gray bars) and old (white white) *N. furzeri* hearts expressed as fold increase versus young samples. (B) qRT-PCR analysis of miR-29 family members in young, adult and old *N. furzeri* skeletal muscle expressed as fold increase versus young samples. (C) qRT-PCR analysis of miR-29 family members in human cardiac fibroblasts (HCF) from fetal, adult and old donors expressed as fold increase versus fetal samples. *p<0.05 vs. young/fetal. Data from Heid *et al.* (2017).

4.3.2 Loss of mitochondria and induction of oxidative stress in *N. furzeri* tissue

Loss of mitochondria is a hallmark of aging (López-Otín *et al.*, 2013). Therefore it could be reasoned to assess mitochondrial number also in *N. furzeri* tissues in this study. By using qRT-PCR, mitochondrial and nuclear DNA levels were determined and mitochondrial copy number calculated. As expected, mitochondrial copy numbers decreased with age in *N. furzeri* skeletal muscle tissue (Fig.4-19A) and heart (Fig.4-19B). This may lead to increased reactive oxidative species (ROS) levels, oxidative stress and DNA damage, which accumulated in skeletal muscle (Fig.4-8A) and heart (Fig.4-7C). However, not only mitochondria decreased in number, but also expression levels of mitochondrial proteins such as *polymerase γ* (*polγ*), *mitochondrial transcription factor* (*tfam*) and *mitochondrial single-stranded DNA binding protein* (*ssbp*) were reduced with age in *N. furzeri* muscle tissue (Fig.4-19C).

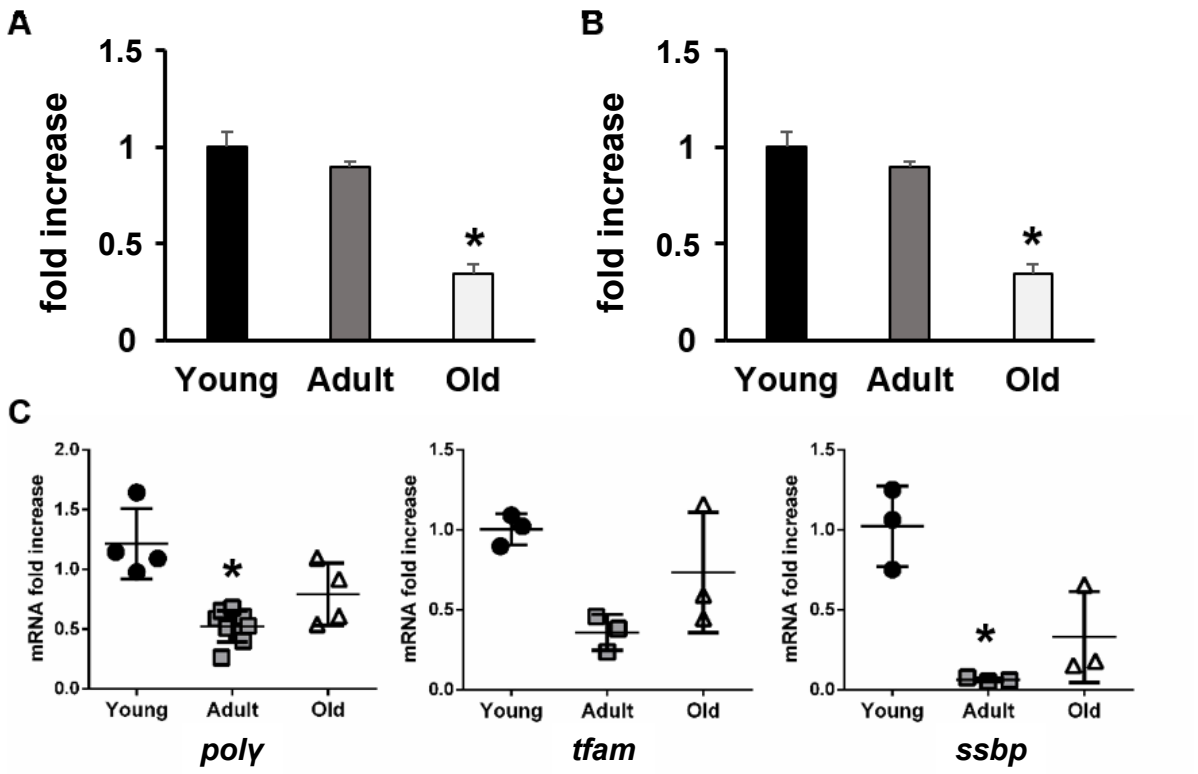

Figure 4-19: Loss of mitochondria and mitochondrial protein expression can be observed in aging *N. furzeri* skeletal muscle tissue . (A) qRT-PCR analysis of mitochondrial copy number in young (black bar), adult (gray bar) and old (white bar) *N. furzeri* skeletal muscle tissue expressed as fold increase versus young samples. (B) qRT-PCR analysis of mitochondrial copy number in young, adult and old *N. furzeri* hearts expressed as fold increase versus young samples. (C) qRT-PCR analysis of *mitochondrial polymerase gamma* (*poly*), *mitochondrial transcription factor* (*tfam*) and *mitochondrial single-stranded DNA binding protein* (*ssbp*) mRNAs in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* muscle tissue expressed as fold increase versus young samples. *p<0.05 vs. young

To evaluate whether loss of mitochondrial copy number contributes to oxidative stress, specific oxidative markers were analyzed in the heart.

Remarkably, an accumulation of Nitrotyrosine – a hallmark compound to identify oxidative stress – with age in *N. furzeri* heart was observed by using confocal microcopy (Fig.4-20A). Further, high expression of miR-200 family members has been associated to oxidative stress (Magenta *et al*., 2011, 2013). Consistently, in the *Nothobranchius* model system, qRT-PCR analysis of the miR-200 family, and in particular that of miR-200c showed an increased expression in the heart (Fig.4-20B). However, in skeletal muscle, miR-200c was increased only in some individuals (Fig.4-20C). Taken together, these results suggested for an accumulation of oxidative stress with age in heart and skeletal muscle. Further, it could be reasoned that the heart could have been more sensitive than skeletal muscle to the molecular influence of ROS production.

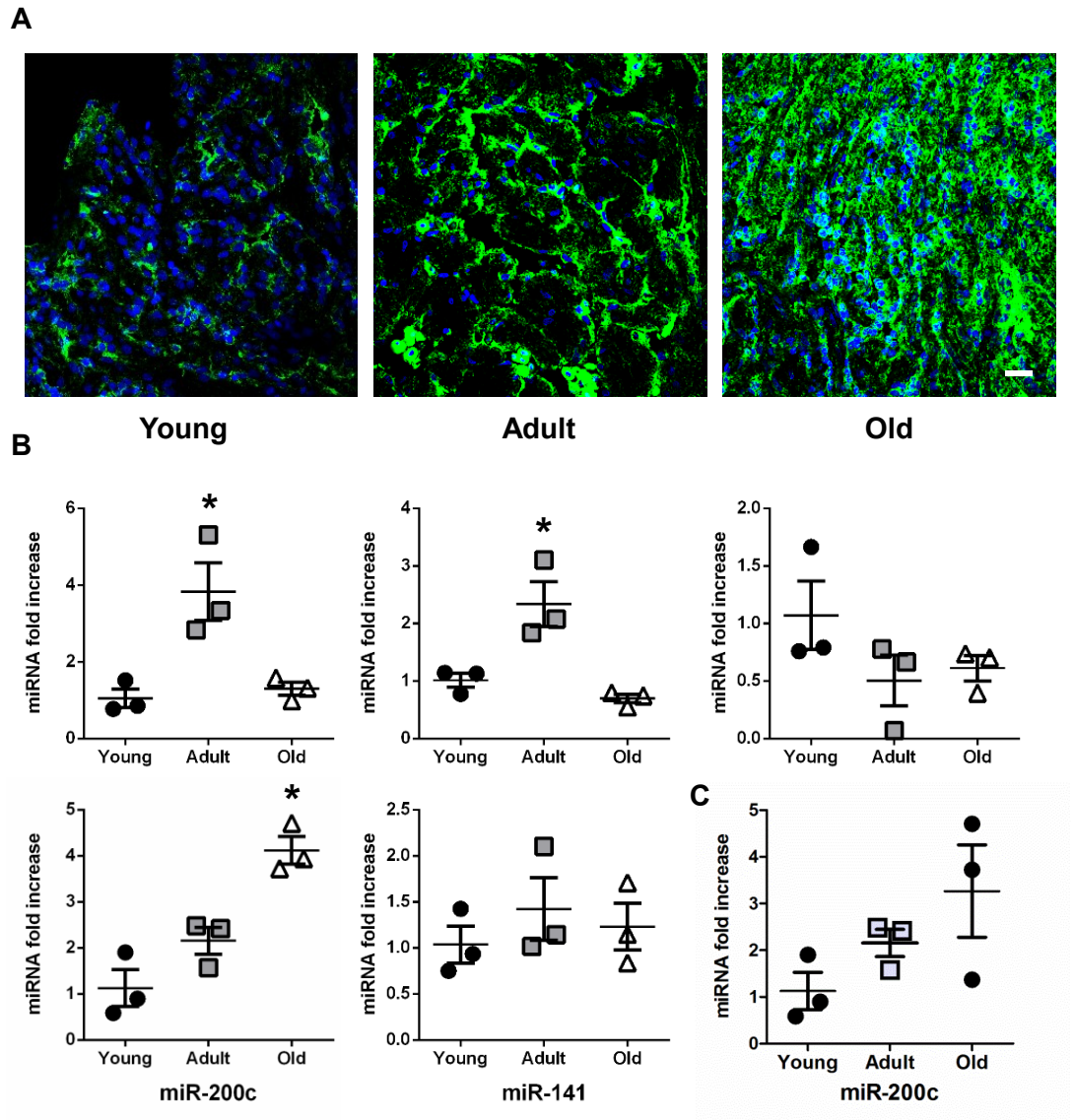

Figure 4-20: Oxidative stress accumulates in the hearts of *N. furzeri* with age . (A) Representative confocal microscopy images of Nitrotyrosine staining (green) in young (left panel), adult (middle panel) and old (right panel) *N. furzeri* hearts. Nuclei were counterstained with DAPI (blue). Calibration bar = 15 µm. (B) qRT-PCR analysis of miR-200 family members in young (black circles), adult (gray squares) and old (white triangle) *N. furzeri* hearts expressed as fold increase versus young samples. (C) qRT-PCR analysis of miR-200c in young, adult and old *N. furzeri* skeletal muscle expressed as fold increase versus young samples. *p<0.05 vs. young. Data from Heid *et al.* (2017).

4.3.3 miR-29 expression is controlled by oxidative stress

To investigate whether oxidative stress could modulate miR-29 expression in cells of cardiac origin, human cardiac fibroblasts (HCFs) were treated with 200 µM hydrogen peroxide (H_2O_2) to induce oxidative stress. In this condition, oxidative stress led to a significant upregulation of miR-29 (Fig.4-21A). A similar result occurred in cardiac myocytes exposed to H_2O_2 (data not shown) (Heid *et al.*, 2017). Additionally, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), known to increase mitochondrial ROS, led to a similar result (data not shown) (Heid *et al.*, 2017).

Dnmts (Fig.4-21B) and *collagens* (Fig.4-21C) are listed among the target genes of miR-29 action and are repressed by miR-29. Accordingly compared to controls, they were reduced in their mRNA expression levels under hydrogen peroxide treatment. The increased miR-29 expression and target gene reduction could be abolished in the presence of a ROS scavenger such as N-acetylcysteine (NAC) (Fig.4-21A, B, C; striped bars). In addition, it could be observed, HCFs under oxidative stress were enriched in H3K27me3 when compared to cells under control conditions (Fig.4-21D). Taken together, these experiments demonstrated that mimicking oxidative stress conditions resemble the observations that could be made in *N. furzeri* skeletal muscle and heart. Levels of miR-29 were increased and H3K27me3 accumulated under oxidative stress, while *dnmts* and *collagens* (*cols*) levels were reduced in their expression.

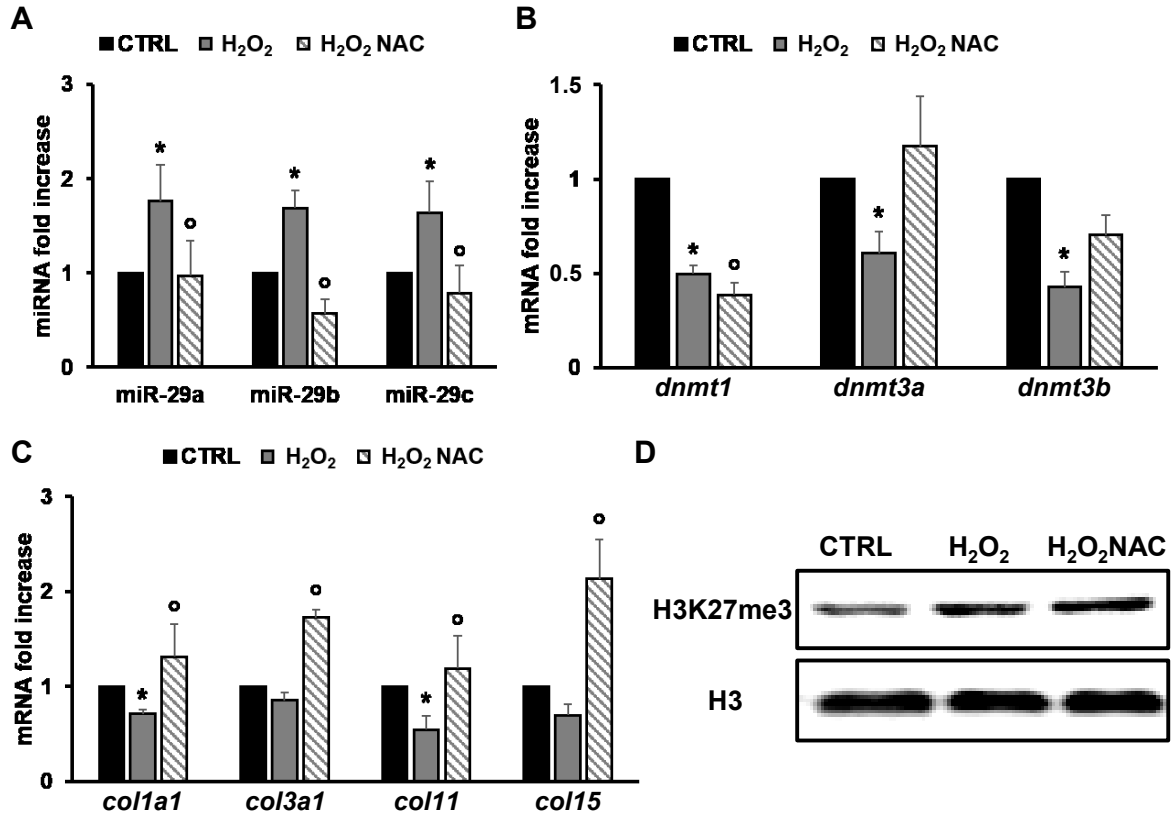

Figure 4-21: Oxidative stress affects miR-29 expression and its targets in human cardiac fibroblasts (HCFs) . (A) qRT-PCR analysis of miR-29 family members in HCFs cultured in control conditions (CTRL; black bars), in the presence of H_2O_2 (gray bars) and of H_2O_2 NAC (striped bars) expressed as fold-change versus control samples. (B) qRT-PCR analysis of *dnmt* mRNAs in HCFs cultured in control conditions, in the presence of H_2O_2 and of H_2O_2 + NAC expressed as fold-change versus control samples. (C) qRT-PCR analysis of *collagen* mRNAs in HCFs cultured in control conditions, in the presence of H_2O_2 and of H_2O_2 + NAC expressed as fold-change versus control samples. (D) Western blot for H3K27me3 of HCFs cultured in control conditions (CTRL), in the presence of H_2O_2 and of H_2O_2 + NAC. Total Histone 3 (H3) was used as loading control. *p<0.05 vs. control; °p<0.05 vs. H_2O_2. Data from Heid *et al.* (2017).

4.3.4 Histology and functional analysis of the heart

The most frequently studied targets of miR-29 are *collagens* (*cols*) as miR-29 represses the expression and translation of *cols*. Collagens are structural proteins of the extracellular matrix (ECM) that are important for e.g. wound healing. However, they also build up during heart disease leading to cardiovascular stiffening. Low levels of miR-29 are postulated to be involved in scar formation through collagen accumulation after myocardial infarction (van Rooij *et al.*, 2008). In the heart of *N. furzeri* miR-29 increased with age and hence the expression of collagens was found significantly reduced (Fig.4-22A). Therefore, the heart of *N. furzeri* did not accumulate collagen depositions with age as could be shown with a Fast Green/Sirius Red staining for collagens (Fig.4-22B, C). Together with Dr. Giuseppina Milano, Dr. Guilio Pompilio and Prof. Dr. Alessandro Scopece from Milan contractility and functionality of the *N. furzeri* heart at different ages were assessed. Naturally the heart grows between young and old, because animals are becoming bigger towards adult stage and the heart size increases accordingly. Hence, the area of the contracted heart (end systolic area; ESA) and the area of the relaxed heart (end diastolic area; EDA) were larger in hearts of old *N. furzeri* compared to hearts of young *N. furzeri* (Fig.4-22D). However, no functional impairment could be measured, as the contractile area was not significantly altered (fractional area change; FAC; Fig.4-22E). Therefore, it could be concluded that collagen content remained stable due to reduced expression of *collagens* and hence the heart of *N. furzeri* should be functionally intact even at old age.

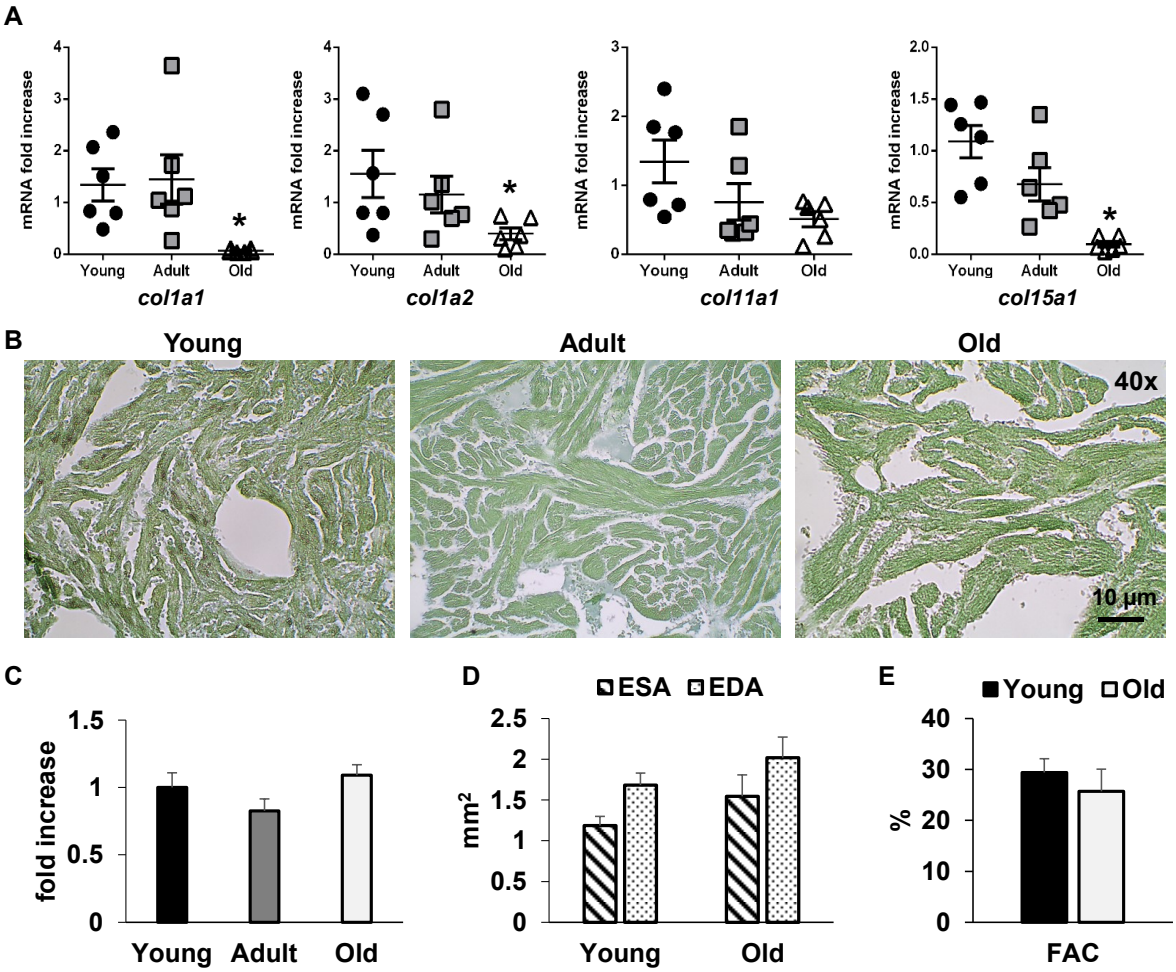

Figure 4-22: Collagen does not accumulate in *N. furzeri* heart with age and hence contractility is not impaired by aging . (A) qRT-PCR analysis of collagen mRNAs in young (black circles), adult (gray squares) and old (white triangles) *N. furzeri* heart expressed as fold increase versus young samples. Data from Heid *et al.* 2017. (B) Representative Fast Green/Sirius Red staining of young (left panel) adult (middle panel) and old (right panels) *N. furzeri* ventricles: Collagenous proteins are depicted in light purple and non-collagenous ones in green. Magnification 40x in all panels. Calibration bar = 10 µm (C) Collagen deposition quantification in sections derived from young (black bar), adult (gray bar) and old (white bar) *N. furzeri* hearts. (D) Graph shows systolic area (black striped bars) and diastolic area (gray dotted bars) in young and old *N. furzeri* hearts. (E) Graph shows Fractional Area Change (FAC) in young (black bar) and old (white bar) *N. furzeri* hearts. *p<0.05 vs. young

4.3.5 miR-29 is important for a functional fish heart

To further elucidate the role of miR-29 in the heart of fish, a transgenic zebrafish with non-functional miR-29 expression was generated in collaboration with Dr. Roberto Ripa and Prof. Dr. Alessandro Cellerino from SNS in Pisa, Italy (miR-29 Sponge) (for mechanism of action see Ebert and Sharp, 2010). When these investigations were started, only little knowledge of techniques for genetic manipulation of the *N. furzeri* was available, hence the zebrafish was used to explore functionally the properties of miR-29 in a fish model. Complete knock-down of miR-29 has been shown to be embryonic lethal, therefore a miR-29 Sponge construct was generated, leading to overexpression of a non-functional version of miR-29 in a tissue-specific manner.

In this system qRT-PCR demonstrated that *collagen* expression was highly increased after miR-29 depletion (Fig.4-23A). Interestingly, the shape of the ventricle changed from a conical structure towards a more roundish shape (Fig.4-23B). In addition to that, the typical Sponge-like structure of the ventricle was lost when no functional miR-29 was present (Fig.4-23C). Moreover, severe cardiac hypertrophy could be observed. With a Fast Green/Sirius Red staining an accumulation of collagenous proteins could be detected in ventricles from miR-29 Sponge zebrafish (Fig.4-23D) showing expression significantly higher than in the ventricles of control animals (Fig.4-23E).

Together with Dr. Giuseppina Milano, Prof. Dr. Giulio Pompilio and Dr. Alessandro Scopece from the Istituto Cardiologico Monzino, Milan, the cardiac function of these miR-29 Sponge zebrafish was assessed and compared to Wild Type zebrafish. Echocardiography revealed that the end-systolic area (ESA) was much larger of miR-29 ventricles compared to the area of control ventricles. Further, the diastolic area was slightly higher in miR-29 Sponge ventricles (Fig.4-23F) and hearts of miR-29 Sponge zebrafish were less contractile, which could be demonstrated with the calculation of the fractional area change that was significantly reduced in hearts from miR-29 Sponge animals (Fig.4-23G). Hence, these results suggested that depletion of functional miR-29 led to collagen accumulation impairing cardiac function in zebrafish.

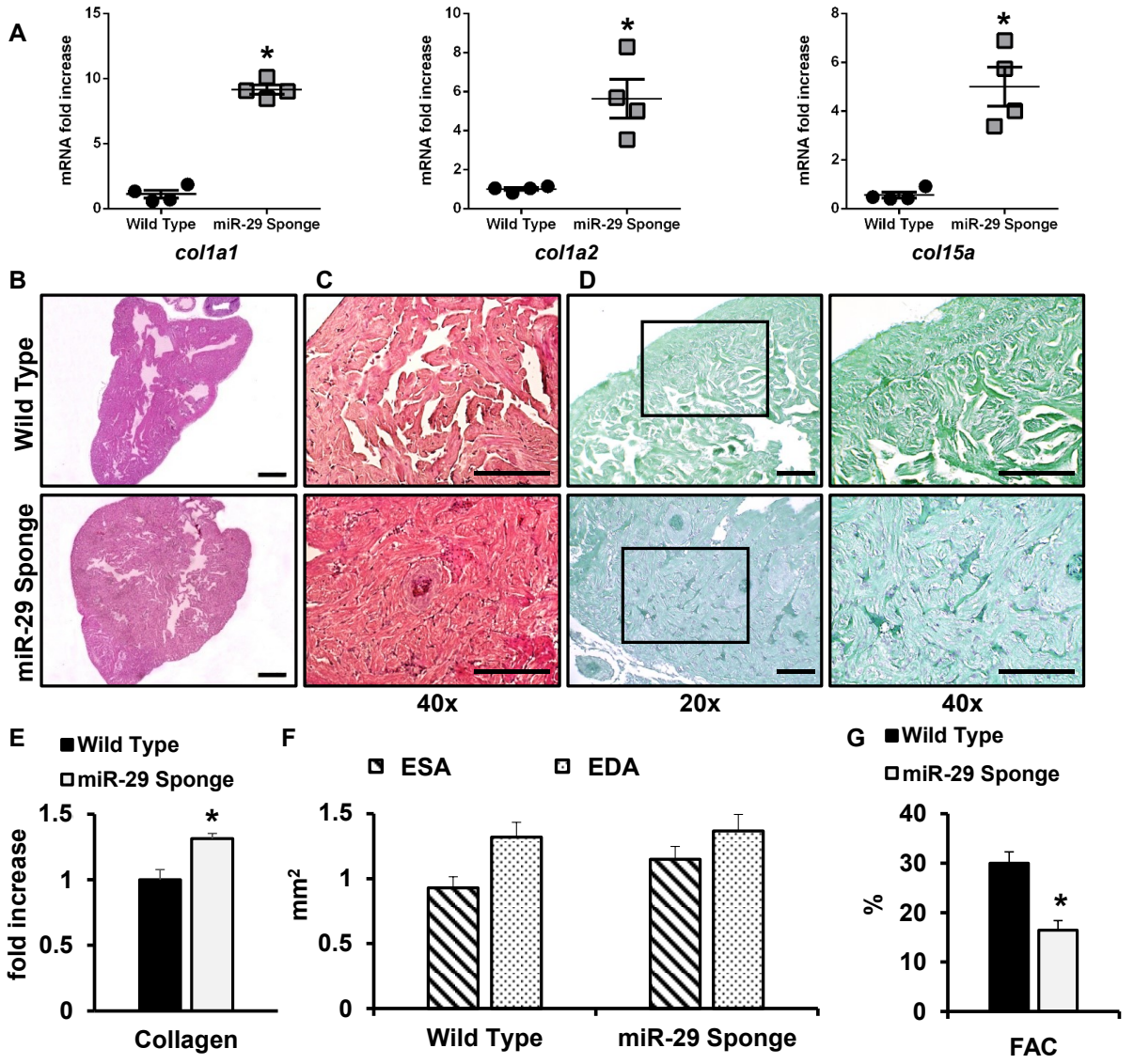

Figure 4-23: miR-29 family knock-down associates with collagen deposition in the zebrafish heart . (A) qRT-PCR analysis of *col* mRNAs in Wild Type (black circles) and miR-29-Sponge (gray squares) hearts expressed as fold increase versus Wild Type samples. (B) Hematoxylin-Eosin staining of whole ventricles from zebrafish. The ventricle of Wild Type (upper panel) has a conical shape, while the ventricle of miR-29 Sponge animals (lower panel) appears more roundish (images kindly provided by the collaborators from Milan, Italy). (C) Representative Masson-Goldner-trichrome staining of ventricles from Wild Type (upper panel) and miR-29 Sponge (lower panel). Calibration bar = 25 µm. (D) Representative Fast Green/Sirius Red staining of Wild Type (left panels) and miR-29-Sponge (right panels) ventricles. Collagenous proteins are depicted in light purple and non-collagenous ones in green. Magnification 20x in first and third panel and 40x in second and fourth panel. Calibration bar = 25 µm (E) Collagen deposition quantification in sections derived from Wild Type (black bar) and miR-29-Sponge (gray bar) hearts. (F) Graph shows systolic area (black striped bars) and diastolic area (gray dotted bars) in zebrafish hearts of Wild Type and miR-29-Sponge. (E) Graph shows Fractional Area Change (FAC) in Wild Type and miR-29-Sponge hearts. *p<0.05 vs. Wild Type. Data from Heid *et al.*(2017).

Cardiac hypertrophy and dysfunction is often associated with hypoxia. To investigate, whether depletion of functional miR-29 led to hypoxic conditions, markers thereof were assessed in hearts of control and miR-29 Sponge fish. Hypoxia-inducible factor 1 alpha (HIF1α) was found to be stabilized in protein lysates (Fig.24-A) and mRNAs of hypoxia-associated genes were significantly upregulated in hearts of miR-29 Sponge zebrafish (Fig.24-B).

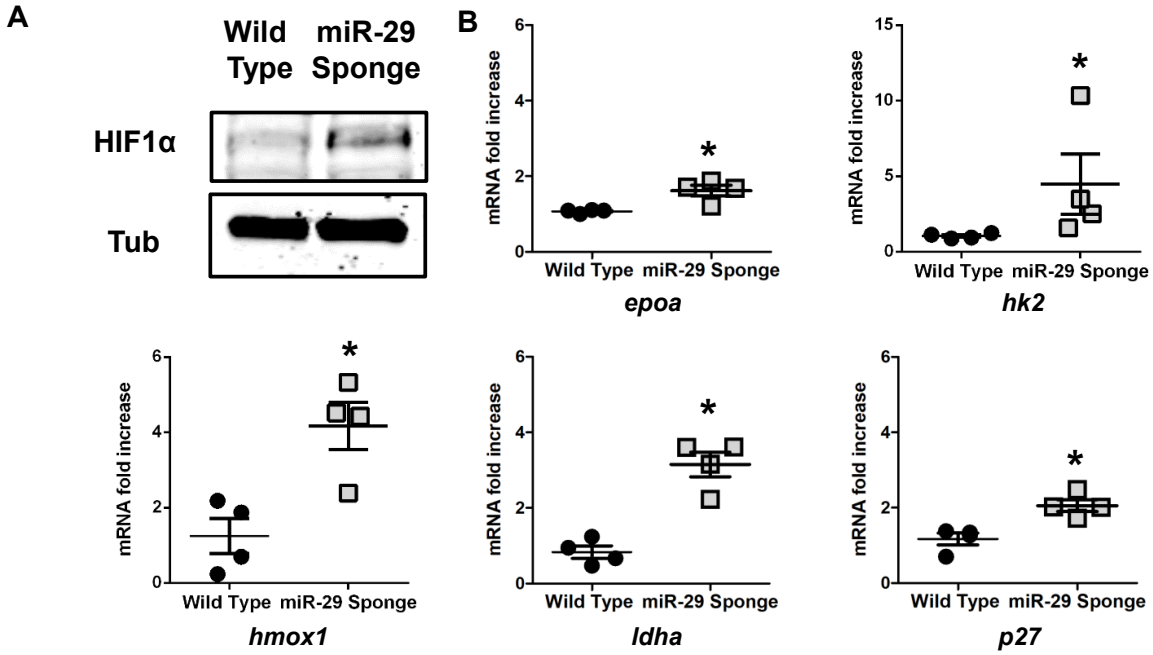

Figure 4-24: Validation of hypoxic conditions in zebrafish hearts : (A) Representative Western blot analysis of hypoxia inducible factor 1 alpha (HIF1α) expression in Wild Type and miR-29-Sponge zebrafish heart. In each condition, α-tubulin was used as loading control. (B) qRT-PCR mRNA analysis of mRNAs of hypoxia associated genes: *erythropoietin alpha* (*epoa*); *Hexokinase2* (*hk2*); *heme oxygenase1a* (*hmox1a*); *lactate dehydrogenase A* (*ldha*); *cyclin-dependent kinase inhibitor 1B* (*p27*) in Wild Type (black circles;) and miR-29-Sponge (gray squares) zebrafish hearts expressed as fold-change versus Wild Type. *p<0.05 Vs Wild Type. Data from Heid *et al.* (2017).

miR-29 does not only target *collagens*, but also *DNA methyltransferases* (*dnmts*) (Morita *et al.*, 2013): with an ELISA assay for global DNA methylation, high levels of 5-mC in the hearts of miR-29 Sponge models could be detected and compared to control animals (Fig.4-25A). This upregulation of global DNA methylation levels might be due to significantly higher expression of *dnmts* in hearts of miR-29 Sponge animals when compared to control zebrafish (Fig.4-25B).

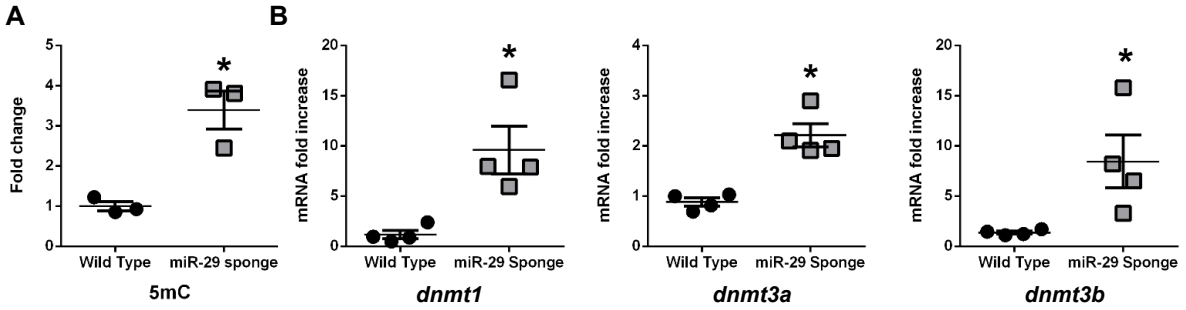

Figure 4-25: DNA methylation increases in the hearts of miR-29 Sponge zebrafish . (A) Global DNA methylation quantification of 5mC in Wild Type (black circles) and miR-29-Sponge (gray squares) hearts expressed as fold-change versus Wild Type samples. (I) qRT-PCR analysis of *DNA methyltransferases* (*dnmts*) mRNAs in Wild Type and miR-29-Sponge hearts expressed as fold increase versus Wild Type samples. *p<0.05 vs. Wild Type. Data from Heid *et al.* (2017).

With the contribution of the sequencing core facility under Dr. Carsten Künne, Dr. Stephan Günther and Prof. Dr. Thomas Braun the mRNA isolated from miR-29 Sponge model and from control hearts was sequenced. The results indicated that miR-29 depletion led to gene expression changes, moreover to an upregulation of genes associated to cardiovascular function and disease, alterations of tissue morphology cell cycle regulation (data not shown). Therefore, it could be postulated that miR-29 played a major role during healthy cardiac aging as recently reported in the publication by Heid *et al.*, (2017).

5 Discussion

5.1 The epigenetic landscape of *Nothobranchius furzeri* changes with age

This study focused on the epigenetic landscape of the turquoise killifish *Nothobranchius furzeri* (*N. furzeri*), in order to obtain insight about changes occurring with age in this interesting but still poorly characterized animal model of aging. Specific changes emerging in aged *N. furzeri* muscle and heart have been detected. Their impact on gene expression has been investigated putting in correlation DNA methylation and microRNA expression. In addition to this, sequencing data from other studies were used for comparison. In fact, this study has been one of the first attempting to assess epigenetic changes occurring with age in skeletal muscle and heart of *N. furzeri*. Therefore, certain limitations in the experimental design and data interpretation must be taken into consideration. Moreover, it has to be emphasized that in the present study different model systems have been used for distinct applications. The turquoise killifish *N. furzeri* was used as the main model for epigenetic studies during aging and the well characterized zebrafish *Danio rerio* has been adopted in the context of genetic manipulation. Finally, primary cells derived from *N. furzeri* muscle tissue as well as human cardiac fibroblasts (HCFs) were used to validate the findings in fish and human cell culture experiments.

5.1.1 Histone modifications and DNA damage may correlate in the aging *N. furzeri*

The experimental evidence accumulated during the present study showed that in skeletal muscle from old *N. furzeri* histone marks associated with closed chromatin such as H3K9me3, H3K27me3 and H4K20me3 increased with age while those marks associated with open chromatin like H3K4me3, H3K9ac and H4K16ac were significantly lowered. In mammalian model systems different observations have been reported. Global histone methylation decreases with age, accumulating only at certain CpG islands (López-Otín *et al.*, 2013; Weidner and Wagner, 2014). These differences of histone methylation may point towards general discrepancies of epigenetic changes between mammalian and fish aging, which remains to be explored.

In this context, mRNAs of genes related to cell cycle and proliferation were found reduced in the heart and skeletal muscle of *N. furzeri*. Specifically INK4 proteins that are negative regulators of cell cycle significantly decreased in their expression levels, possibly underlying the presence of an aberrant proliferation scenario. Interestingly, it has been recently shown that although INK4 locus expression increases with age it may have a protective effect delaying the aging process and protecting from aging-associated cancer while increasing lifespan in mice (Matheu *et al.*, 2009). The opposite effect has been clearly detected in the *N. furzeri*: INK4 mRNA expression was

downregulated with age and rapid aging could be observed. The mechanism underlying this phenomenon remains to be elucidated and requires further investigations.

Genes associated with DNA damage repair are less expressed at old age in *N. furzeri* skeletal muscle than at young age, hence DNA damage accumulated. DNA damage repair is essential for replication and proliferation as well as tissue regeneration after injury. However, it seemed to be impaired in *N. furzeri* muscle as indicated by the accumulation of the modified γH2AX paralleled by reduced proliferation in muscle. As an important source of aging associated DNA damage, oxidative stress often occurs during aging and cellular senescence as well as during multiple diseases and modulates miRNA expression (Xie and Chen, 2016; Bu *et al*., 2017). It could be observed that with age oxidative stress products accumulated in the heart and in skeletal muscle tissue simultaneously with a reduced number of functional mitochondria, a finding that might be associated with the onset of genomic instability.

It is tempting to speculate about causes for the highly methylated genome and the short lifespan of the *N. furzeri*. At this moment there is no data available regarding DNA damage and DNA damage repair in the aging killifish. However, since DNMTs are also involved in DNA damage repair (Jin and Robertson, 2013), the observation of low expression of *dnmts* with age hints at low DNA repair activity. Moreover, in *N. furzeri* a decrease of mRNA expression from genes associated with DNA synthesis and damage repair could be observed analyzing sequencing data of *N. furzeri* skeletal muscle tissue. Additionally, genes of DNA damage repair have been shown to play essential roles for longevity of other model organisms, suggesting that genome maintenance in short-lived animals is not as evolved as in long-living species (Lidzbarsky *et al*., 2018).

The compaction of the genome by increased heterochromatin formation might also be a protective mechanism. To prevent DNA breakage and further damage from ROS the genome may become hyper-methylated. In HCFs under oxidative stress conditions, in fact, an increase of H3K27me3 could be observed fostering this interpretation. In addition to this, literature also suggests that hyper-methylation is induced in response to DNA damage (Foltánková *et al*., 2013; Li *et al*., 2014; Basenko *et al*., 2015). Several studies have investigated the impact of epigenetic marks on lifespan and the safeguarding role for heterochromatin. In *Drosophila* heterochromatin formation leads to longevity (Larson *et al*., 2012), while a reduction of H4K16ac via deletion of the *sas2* acetylase supports an increased lifespan (Kozak *et al*., 2010). Moreover, it has been reported that accumulation of H3K27me3 leads to increased lifespan and longevity in *C. elegans,* whereas H3K4me3 promotes the aging process (Han and Brunet, 2012; Maures *et al.*, 2011) suggesting that in *N. furzeri* H3K27me3 might have a protective effect. Interestingly, metformin, a drug used for diabetes treatment, has been shown to expand lifespan of animal models (Martin-Montalvo *et al*.,

2013). Yet, a recent study showed that metformin directly targets de-methylase KDM6A and thereby leads to an accumulation of H3K27me3 suggesting a potential mechanism for its beneficial effects (Cuyàs *et al*., 2018) further supporting the hypothesis of a protective role associated with hyper-methylation of histones. However, no study applying metformin in the *N. furzeri* model has been published so far, yet it would be certainly of interest in terms of epigenetic changes and lifespan extension.

In addition the above mentioned experiments, cells derived from young and old *N. furzeri* skeletal muscle tissue were isolated and cultured for subsequent treatments. The cells were used to investigate the effect of inhibitors of histone methylating EZH enzymes, which resulted in a strong reduction of H3K27me3. Interestingly, an increase in DNA damage and reduction of cell proliferation could be observed in treated cells. This evidence supports the idea that histone methylation is a patronizing mechanism in *N. furzeri* muscle that uses H3K27me3 to prevent genomic instability and DNA damage by compaction of the genome. In fact, a recent study has shown that downregulation of EZH2 progressively leads to cellular senescence and upregulation of negative cell cycle regulators as well as pro-inflammatory signaling (Ito *et al*., 2018).

An additional interpretation for the increase in histone methylation and specifically for the accumulation of H3K9me3 in old *N. furzeri* muscle tissue might be the progressive accumulation of senescence markers in muscle tissue occurring with old age. It has been shown, in fact, that the so-called senescence associated heterochromatin foci (SAHFs) are rich in closed chromatin conformation such as H3K9me3 and HP1α (Chandra and Narita, 2012). As shown in this study, an accumulation of cellular senescence markers with increasing age in *N. furzeri* skeletal muscle tissue and cells derived from *N. furzeri* skeletal muscle tissue has been clearly demonstrated.

5.1.2 DNA modifications are differentially distributed in heart and skeletal muscle.

While global DNA methylation is increasing with age in *N. furzeri* skeletal muscle, it is declining in whole hearts. This observation raises some interesting questions that are awaiting to be answered: How is it possible that 5mC accumulates in muscle tissue, despite reduced expression of *DNA methyl transferases*? Is there a particular reason for the difference in DNA methylation level in the heart and skeletal muscle? At present, one can only speculate about the reason for the increase of global methylated DNA despite the evident low expression of the enzymes catalyzing the reaction.

As a hypothesis, it could be argued that expression of *dnmts* is low because the amount of translated protein appears stable and perhaps active and no further transcription might be needed. Future studies should be performed analyzing the expression of the proteins as soon as suitable antibodies

become available. Additionally, a DNMT activity assay could be established to determine methylating properties in freshly isolated fish skeletal muscle and heart samples; unfortunately, this assay could be performed with the frozen material that was available for the present study.

Of interest, taking into account, that miR-29 targets the mRNA of *dnmts* (Morita *et al.*, 2013), low amounts of *dnmts* mRNA could to be a logical consequence of miR-29 relative increase occurring with age in the heart and skeletal muscle. Moreover, miR-29 targets also other members of the DNA methylation/demethylation machinery such as *tets* and *tdg*, which are partly reduced in their expression with age in the two tissues investigated. This finding suggested for a general regulation of the DNA methylation/ demethylation machinery occurring during aging in this animals, ultimately leading to global accumulation of methylated DNA.

5.1.3 microRNAs are expressed in an age-dependent manner

After sequencing of microRNA comparing young, adult and old *N. furzeri* heart, activity performed in collaboration with Dr. Mario Baumgart (FLI, Jena, Germany) and Prof. Dr. Alessandro Cellerino (SNS, Pisa, Italy), an age-specific miRNA expression pattern has been shown of which miR-29 expression and function was analyzed in more detail. The results indicated that miR-29 levels were significantly upregulated in the heart of old *N. furzeri*. This finding was similar in skeletal muscle and in brain, liver and skin, as shown by sequencing analysis of Dr. Mario Baumgart (Heid *et al.*, 2017). The accumulation of miR-29 signal was also evident in HCFs isolated from aged (>65 years) human donors. Of note, it has been reported that miR-29 target genes such as collagens and enzymes of the DNA methylation machinery were reduced in their expression levels as miR-29 levels increase (Heid *et al*., 2017). In collaboration with Dr. Roberto Ripa and Prof. Dr. Alessandro Cellerino, it could be demonstrated that miR-29 could be important for maintenance of a healthy heart preventing cardiac fibrosis, hypertrophy and dysfunction and excess methylation (Heid *et al.*, 2017). Without functional miR-29, indeed, the gene expression in the heart of zebrafish, the animal model that was used for genetic manipulation, changed significantly as the genome became highly methylated giving further indications that excess methylated DNA may be associated with cardiac fibrosis (Tao *et al.*, 2013). Furthermore, in the heart of miR-29 Sponge animals a hypoxic phenotype including cardiac hypertrophy and higher cellular density could be observed with histological staining. The morphology resembled a phenotype very similar to zebrafish exposed to severe hypoxic conditions (Marques *et al*., 2008; Heid *et al*., 2017).

Interestingly, in an independent study on the aging process in the brain of *N. furzeri*, it has be shown that miR-29 family increases and limits iron uptake, thereby possibly limiting oxidative stress and

protecting from neurodegeneration (Ripa *et al.*, 2017). Together, these findings suggest a generally protective role for miR-29 in the aging process of *N. furzeri*.

As miRNA expression might be modified by oxidative stress (Magenta *et al.*, 2013, 2011) and senescence (Weilner *et al.*, 2015), it was tempting to speculate whether miR-29 expression itself could be regulated by oxidative stress. In this context, taking advantage of HCFs as a model system, expression levels of miR-29 significantly increased under oxidative stress conditions realized by supplementing media with hydrogen peroxide. This experiment showed that not only miR-29 expression increased in the presence of excess oxidative stress, but, accordingly, also the target genes of miR-29 were significantly reduced in expression. This effect was prevented by addition of the potent antioxidant N-acetylcysteine to the HCF complete medium supplemented with hydrogen peroxide. By using a different source of oxidative stress, CCCP instead of hydrogen peroxide, similar results were obtained and the findings could be replicated also in cardiomyocytes of rat origin (Heid *et al*., 2017). Taken together, it might be proposed that miR-29 is sensitive to oxidative stress and that this regulatory response is conserved in cells of different species.

Reportedly, miR-29 increases with age but also with cellular senescence (Hu *et al.*, 2014). In muscle tissue from rats, miR-29b leads to cell cycle arrest of muscle progenitor cells and contributes to some types of muscular atrophy (Li *et al.*, 2017). Hence, the global increase of miR-29 in *N. furzeri* tissue might be a response to the accumulation of senescent cells with potential beneficial side effects for the cardiac health e.g. the limitation of fibrosis. More studies are needed to better understand this important aspect.

As described above, in addition to the global upregulation of miR-29a, miR-27d expression is also increased with age in *N. furzeri* brain, liver, skin and heart. Therefore, it might represent another interesting candidate for additional studies, as elevated levels of miR-27 have not been validated yet in *N. furzeri*. In mammals, miR-27 has been shown to play multiple roles: it is a biomarker for atherosclerosis (Chen *et al.*, 2012), regulates IL-10 (Quinn and O'Neill, 2014) and *myostatin* expression (Wang, 2013); it is a regulator of adipogenesis (Chen *et al.*, 2015; Sun and Trajkovski, 2014) and is involved in mitochondrial fission (Tak *et al.*, 2014). An increase of expression might have beneficial as well as detrimental effects depending on tissue type and perhaps on other micro-environmental which impact is to be investigated yet.

5.2 A global aging phenotype is affecting multiple tissues of *N. furzeri*

The evidence that miR-29 and miR-27 increases with age in several tissue types (Heid *et al.*, 2017) paralleled by the accumulation of H3K27me3 in muscle, heart and brain (Baumgart *et al.*, 2014b) suggests about the presence of a global aging-associated epigenetic phenotype in tissue originating from *N. furzeri* with potential consequences on cellular and organismal metabolism and function. In this respect, a comparison of sequencing data from skin, liver and brain (Baumgart *et al.*, 2016) with sequencing data from skeletal muscle revealed the presence of six common KEGG pathways downregulated and two upregulated in young *versus* old animals. Most strikingly, cell cycle was always significantly downregulated in all four tissues while the expression of inflammation-related genes was enhanced globally. This may indicate that not only one particular organ of the *N. furzeri* such as the heart or the brain is influenced by molecular changes occurring during aging, but rather the whole body is affected and that a global change in gene expression is taking place as the fish ages. Thus it seems likely that also in other tissues, not considered in the analysis object of this study, such as kidney or gills a similar change of the epigenetic landscape might occur. Moreover, immune response and inflammatory pathways are induced in a conserved fashion across different tissues from vertebrates: in a recent study heart, liver and brain samples from rodents, human and killifish (in the latter, only liver and brain samples) were analyzed and a common pattern of upregulated mRNA expression was found (Benayoun *et al*., 2018), which confirms also the findings of this study on skeletal muscle of old *N. furzeri*.

Additional comparison of sequencing data performed by using embryonic mRNA (Reichwald *et al.*, 2015) and skeletal muscle tissue has shown that some of the common pathways downregulated during diapause were among those downregulated with age. Consistently, cell cycle is downregulated in both stages. In the wild, during diapause the embryo undergoes severe stress situations e.g. aridity and temperature changes. Therefore, it shuts down all metabolic and cellular processes that are not required during this particular developmental period, which include also proliferation and DNA repair. The adult individual is exposed to additional environmental stressors and the strategy of *N. furzeri* is to produce as many offspring as possible rather than longevity. Little to no effort might be put into maintenance of repair of double strand breaks and mutations hence DNA damage accumulates with age. Overall, it appears that limited resources at both very early and late stages are important factors which might regulate epigenetically gene expression.

This evidence may also provide an interpretation about why the *N. furzeri* does not show an extension of lifespan when kept in a laboratory under ideal conditions with excess food and no dry seasons (Terzibasi *et al.*, 2008). Independent of its environment the *N. furzeri* may not have efficient repair mechanisms, accumulates DNA damage and thus ages rapidly. In addition to that, is the

genome of the *N. furzeri*, rich in tandem repeats (Reichwald *et al.*, 2009), which are an important source for mutations (Fan and Chu, 2007), that could contribute to genetic aberrations and genotoxic stress occurring with age.

From literature it is known that the *N. furzeri* develops hepatic lesions, kidney insufficiency and skin melanomas, which are related to certain cancer types (Di Cicco *et al.*, 2011). Cancer itself develops from mutations leading to proliferation of damaged cells. Moreover, a hallmark of cancer is genomic instability and very often abnormal histone methylation patterns are observed (Hanahan and Weinberg, 2011). In fact, many cancer types are associated with increased histone methylation and increased expression of EZH2 (McCabe and Creasy, 2014; Völkel *et al.*, 2015) and CBX7 (Pallante *et al.*, 2015). Indeed, PcGs play important roles in tumor progression and promote cancer stem cell proliferation (Richly *et al.*, 2011). In agreement, the experimental evidence of this study determined that increased expression of members of PcGs can be found in old skeletal muscle of *N. furzeri* in association with histone methylation and high expression of DNA methylating enzymes. In addition to that, *p53*, shown to delay aging and protect from cancer (Efeyan and Serrano, 2007; Matheu *et al.*, 2007), was found significantly reduced in the aging skeletal muscle.

Inflammation is another important hallmark of cancer (Diakos *et al.*, 2014) and it goes hand in hand with an aberrant DNA and histone methylation pattern (Jung and Pfeifer, 2015). Sequencing data has shown that inflammatory signaling such as the JAK/STAT -dependent pathway is upregulated upon aging in the skeletal muscle of *N. furzeri*. Moreover, it is well known that immunity is reduced with age and that the adaptive immune response becomes less efficient with age (Castelo-Branco and Soveral, 2013; Pawelec, 2014). The ability to fight off pathogens is decreasing not only in humans but also in the closely related *Nothobranchius guentheri* (Liu *et al.*, 2015) which may lead to increased inflammatory signaling. During cellular senescence, cell cycle progression is not only arrested but cells also undergo major metabolic changes leading to altered proteostasis and secretion of inflammatory cytokines, immune modulators and growth factors which is known as the senescence associated secretory phenotype (SASP) (Coppé *et al.*, 2008). Genomic instability has been shown to cause senescence and subsequently to promote SASP (Andriani *et al.*, 2016). It seems likely, that an insufficient DNA damage repair in *N. furzeri* might also contribute to the upregulation of inflammatory signaling pathways in old animals. In many organisms, including humans, inflammation is upregulated with age and chronic, low-grade inflammatory signaling associated with age is also known as “inflammaging” (Franceschi and Campisi, 2014) which is believed to contribute to aging-associated diseases and cancer. It is of note, that non-canonical JAK/STAT signaling is involved in heterochromatin formation and genome stabilization (Shi *et al*., 2008; Yan *et al*., 2011; Silver-Morse

and Li, 2013). If this plays a role in the accumulation of histone methylation of aged *N. furzeri* skeletal muscle and heart tissue remains to be elucidated.

Further, KEGG pathway analysis has shown that pathways associated with glycolysis are upregulated in aged *N. furzeri* skeletal muscle. Glycolysis is a major source for reactive metabolites such as methylglyoxal that leads to formation of advanced glycation end products (AGEs) and triggers oxidative stress (Dhar *et al*., 2014; Allaman *et al*., 2015). AGEs accumulate during aging and disease in many organs, which further support oxidative stress and DNA damage (Ott *et al.*, 2014). Therefore, enhanced glycolysis in *N. furzeri* skeletal muscle could promote ROS mediated senescence and increase DNA damage via oxidative stress. Taking into account that the number of mitochondria decreased in muscle and heart, but also in other tissues (Hartmann *et al.*, 2011) it could be likely that, in order to maintain the necessary energy supply, alternative sources have to be made accessible. Metabolic changes due to deregulated nutrient sensing are a hallmark of aging (López-Otín *et al.*, 2013) but also of cancer. In fact, the so-called Warburg effect describes the phenomenon of glucose uptake being highly increased to meet the energy demand of tumorigenic cells even if mitochondria are still functioning properly (Liberti and Locasale, 2016). It is of note, that metabolic pathways influence epigenetics in various ways. Substrates and intermediates from the Krebs cycle are important for methylating and de-methylating enzymes (Chiacchiera *et al.*, 2013) as mitochondria provide energy but also important substrates for generation and modification of epigenetic marks (Matilainen *et al.*, 2017). Taken together, a large number of processes may lead to the pronounced aging phenotype in *N. furzeri* (Fig.5-1).

Despite all the evidence, it remains unclear whether cancer may develop in the heart or in the skeletal muscle tissue of *N. furzeri*. However, in humans tumor development is very rare in soft tissue like skeletal muscle and heart and often is a secondary form of tumor that stems from metastasis invading the tissue (Loukas, 2014). *N. furzeri* spontaneously develops tumors in liver (Baumgart *et al.*, 2014a; Di Cicco *et al.*, 2011), but until now it has not been investigated whether these tumorigenic lesions spread into skeletal muscle or heart tissue. Hence, the observations that could be reported here must be considered aging related occurring in the whole organism, but it is unclear whether they may lead to cancer only in certain types of tissue. Liver is known to be able to regenerate life-long and therefore proliferates more than other types of tissue which make genomic mutations leading to tumors more probable. Thus, as liver tissue of *N. furzeri* has not been investigated in detail, it appears to be an exciting subject of future studies.

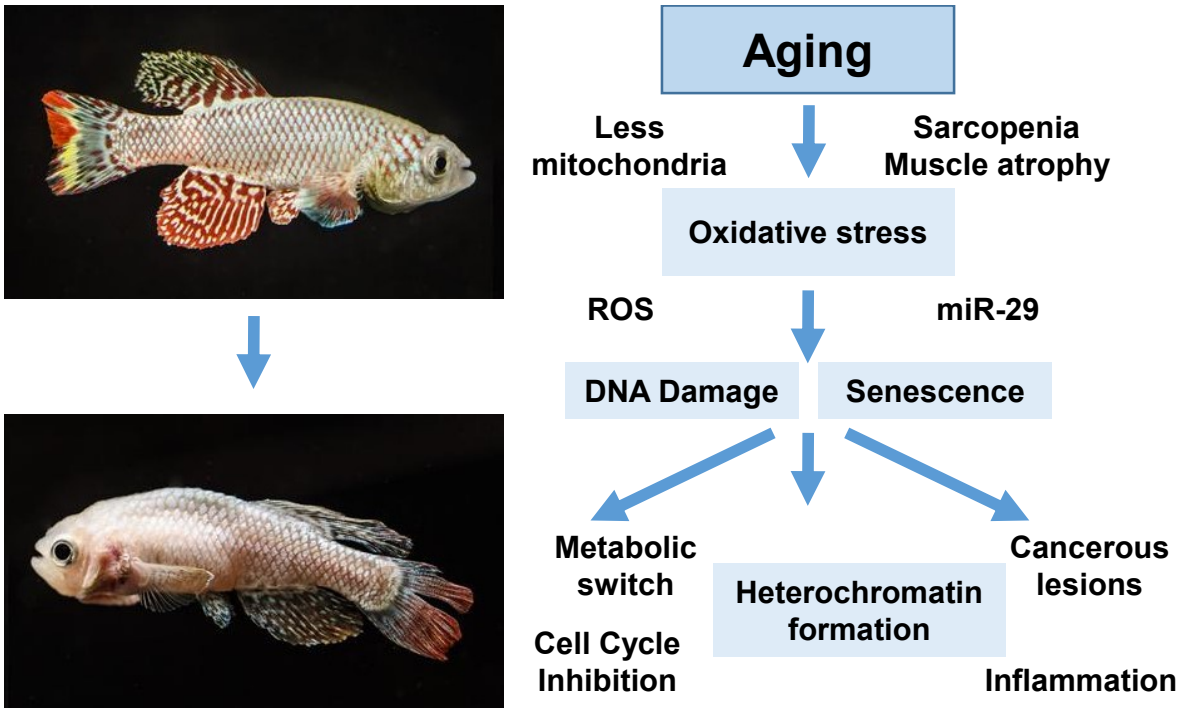

Figure 5-1: Aging leads to global changes in the *N. furzeri* : Upper picture of an adult *N. furzeri*, lower of an old *N. furzeri*, note the overall phenotypical changes (pictures on the left with courtesy of the FLI Jena/Nadine Grimm/Evelyn Kästner). In old *N. furzeri* a series of alterations can be detected that are caused by aging (right side of the panel).

5.3 Advantages of using the *N. furzeri* as a model

Among vertebrates, with a lifespan of 4-12 months the *N. furzeri* has the shortest lifespan known so far (Valdesalici and Cellerino, 2003; Terzibasi *et al*., 2008; Dorn *et al*., 2011). Specifically, it lives much shorter than other animal models such as rodents or zebrafish. Hence, it might represent a very attractive *in vivo* system in which to perform lifespan assays and experiments spanning over multiple generations. As a vertebrate, in *N. furzeri* many features are conserved and similar to mammalian species in terms of gene expression and function (Hu and Brunet, 2018). Its genome has been sequenced recently (Harel *et al.*, 2015; Reichwald *et al.*, 2015) and comprises 19 chromosomes. Sex is determined in a XY fashion, like in humans (Reichwald *et al.*, 2015). Techniques for genetic manipulation (Valenzano *et al.*, 2011) become increasingly advanced and first genetic mutants derived by CRISPR/Cas9 technology have been reported recently as well (Harel *et al.*, 2015; Harel and Brunet, 2015). With the short generation time of the *N. furzeri*, it would be of great interest to introduce mutations that e.g. alter the epigenetic landscape by modifying enzymes or miRNA or the activity thereof and monitor the effect on the mutated animals and their offspring as well as the impact on lifespan.

The aging phenotype of the *N. furzeri* is very pronounced and resembles in many aspects human aging on organismal as well as on molecular level, e.g. spinal curvature, reduced locomotor activity and learning capacity, telomere shortening and emaciation (Cellerino *et al.*, 2016). In addition to the before mentioned phenotypical changes, also neoplasia formation has been observed (Di Cicco *et al*., 2011; Baumgart *et al*., 2014a), which makes the *N. furzeri* a promising model for oncological treatment studies, too. Besides studies with biomedical background, the *N. furzeri* can be used also for studies focusing on evolutionary ecology and eco-toxicity of substances. Because of its short generation time *N. furzeri* is also interesting for genetic screens in multiple consecutive generations and population genomics (Cellerino *et al.*, 2016). Moreover, a recent study could show that transcriptional changes occurring with age are conserved across species and the most pronounced alterations can be detected in tissue from *N. furzeri* (Aramillo Irizar *et al*., 2018). Taken together, the *N. furzeri* represents an interesting and promising animal model not only for further aging research studies.

During the progress of this study cells from young and old *N. furzeri* skeletal muscle tissue were isolated to test i) whether they could be cultured and ii) used for treatment with epigenetic modulators. Since application of drugs in aquatic environments is technically challenging, it was decided to assess the impact of the epigenetic modulators first on cultured cells. These experiments, to test the EZH inhibitors (Ciarapica *et al.*, 2014), were performed as prodromal to future *in vivo* experiments. In the experimental setting, cells that resemble at best the features and constitution of *N. furzeri* muscle are cells directly isolated from the target tissue rather than immortalized or commercially available cells lines. Nevertheless one has to keep in mind that cells might not completely resemble their tissue of origin and may behave differently in a culture system compared to their naïve surroundings. The lack of extracellular matrix and signal molecules may complicate the translation of results and are thereby limiting factors.

Graf *et al.* 2013 described in their work the isolation and cultivation of skin fibroblasts isolated from *N. furzeri*. They observed no senescence in these cells; cells were cultured without reaching the Hayflick limit (Graf *et al.*, 2013). In the cells isolated from skeletal muscle similar observations could be made, but only for cells from young *N. furzeri* muscle tissue – these cells could be passaged over months almost to 100 passages, without showing phenotypically signals of senescence. Here it could be interesting to investigate if the ploidy is affected by the passaging and if DNA damage is manifesting. Interestingly, cells from old tissue could be cultured for only thirty passages before they became senescent and did not proliferate further. A clear difference between young and old cells could be observed: cells derived from old tissue contained more senescent cells which might be one reason for the different numbers of possible passages. Although reported previously the absence of

replicative senescence in cells from young individuals is not clearly explainable (Graf *et al*., 2013). Most likely, cell cycle regulation might be functioning in a different manner and therefore cells continue proliferating. In these aspects, it could be observed only under EZHi treatment cell cycle negative regulators such as products of the INK4 locus were upregulated thus decreasing cellular proliferation. This observation suggests that histone methylation processes, occurring at the level of endogenous cell cycle inhibitors, could be important for cell cycle control in *N. furzeri*.

Hence, one can postulate here that cells isolated from *N. furzeri* tissue might serve as model system to screen for epigenetic drugs relevant to the aging process before setting up *in vivo* studies and lifespan assays. Useful information with respect to properties, dynamics and mode of action can, in fact, be determined. In the present case, EZHi were not tested on living animals for additional reasons. Application of the drug in water could have been difficult as well as the appropriate disposal of large quantities of water used for the experiment. In addition to these obstacles, the legal requirements for animal preclinical experimental tests are very restrictive worldwide and particularly in the location of the collaborators.

Furthermore, it would be of interest to further characterize the cells obtained from *N. furzeri* skeletal muscle. A combination of different cell types grew and proliferated in cell culture. With antibodies for specific marker proteins one could determine the constitution of these cells e.g. to which extent and percentage fibroblasts are part of the mix, if satellite cells were present and if myoblasts were proliferating at the same speed. As a first step one could sort cells via flow cytometry using specific markers or simply by size. An initial staining against myosin heavy chain was performed and some cells were positively stained (data not shown). However, the lack of marker antibodies specifically for *N. furzeri* was an obstacle that emerged. Antibodies for zebrafish tissue could be used as an alternative if the sequence of the epitope is similar between the two species. Antibodies against PCNA and γH2AX could be successfully applied on that account.

5.4 Limitations of the study

The killifish aging model has been established only recently and data about the biology of aging in this animal are still limited. In addition, methods and techniques for genetic manipulations have been described only recently (Valenzano *et al*., 2011; Hartmann and Englert, 2012; Allard *et al*., 2013; Harel and Brunet, 2015), as knowledge about the genome has been published three years ago (Harel *et al.*, 2015; Reichwald *et al.*, 2015). Hence, for this thesis, genetic manipulation was realized in the more established zebrafish model and data could be only indirectly extrapolated to *N. furzeri*. Moreover, the laboratory in which the work for this thesis was performed had no direct access to the

N. furzeri facility and all tissue samples were provided by the collaborators in Jena, Pisa and Cologne. Unfortunately, it was not possible to generate a genetically modified killifish during the present study. For future investigations, however, the technology to genetically manipulate this fish has been more established now and, taking advantage from the initial observations provided here, it might represent a great opportunity to investigate the *in vivo* mechanisms of epigenetic changes occurring with age in *N. furzeri*. With more than 30,000 publications in worldwide literature, the zebrafish is, so far, one of the most widely used model organism for genetic manipulations. It represents a well-established model when it comes to genetic engineering, and its genomic sequence is available since 2001 provided to the scientific community by the genome sequencing project by the Sanger Institute, UK. However, with a maximum lifespan of five years, the zebrafish model is not a first choice for aging studies. The lifespan of the *Nothobranchius* species is exceptionally short and it represents a very convenient model for aging studies in vertebrates. Having access to both model organisms, advantage from the main assets of each animal has been taken for the present study. However, translation of the results into mammalians has to be very carefully considered. There are, in fact, distinct differences in morphology of the heart between fish and higher organisms. While mammalians have a four chambered heart with two ventricles and two atria, fishes have only one of each, representing a somewhat simplified version of the cardiac system. It is of note, that whole hearts from fish were used for expression analysis, Western blotting and DNA methylation, therefore, one can only speculate whether fibroblasts or rather myocytes are responsible for the effects of miR-29 expression that were observed. It would be interesting, however, to investigate whether different cell types express miR-29 differently during aging and respond in different or similar manner to oxidative stress. One could assess the expression profile of the different cell types via single cell sequencing investigating their contribution to the overall phenotype.

Furthermore, it has to be acknowledged that lifestyle, environmental surroundings and habitat of mammals and fish are completely different, which has to be kept in mind when investigating the effects of aging. This simple consideration, however, may explain many of the differences between the mammalian and the teleost epigenetic landscape as environmental factors have a determining impact on epigenetics. While it is indeed well established, that heterochromatin marks decrease with age in mammalian genome the contrary could be observed in muscle and heart of the *N. furzeri*. It would be of great interest to investigate tissue from other fish species such as zebrafish or Medaka and to assess epigenetic changes occurring with age in these species: observations like these might clarify whether epigenetic modifications found in *N. furzeri* tissue are specific for only this species or are a phenomenon that might occur generally in teleost fish. Nevertheless, it could be shown that molecular principles and pathways such as the induction of miR-29 expression via oxidative stress could have been observed also in a mammalian *in-vitro* setting and appear to be conserved across

species. Transfer of the results of studies performed in the *N. furzeri* into mammalian systems would be of great interest potentially unravelling the role of epigenetic mechanisms. It could shed light on the processes controlled by miR-29 and H3K27me3, which are active during heart and skeletal muscle aging and elucidate the effect of heterochromatin in the context of genomic instability.

6 Outlook

To gain insight on the methylated genome, one could perform chromatin immune precipitation (ChIP) by using antibodies raised towards histone modifications including H3K27me3, H3K9me3 and H4K20me3 to precipitate stretches of chromatin enriched in these signals and sequence them. In a first attempt with collaborators from the Fritz-Lippmann-Institute in Jena, Germany no enrichment has been detected at specific sites for H3K27me3 on the whole chromatin of *N. furzeri* skeletal muscle, but rather the presence of signal dispersed across the genome. In this regard, it is important noting, that taking into account the novelty of the *Nothobranchius* as a model for aging research, very little information regarding techniques and antibodies are available. In addition to that, sequencing itself has been shown to be somewhat difficult as the genome of the *N. furzeri*, similar to that of other fish including zebrafish, is rich in repetitive sequences, which might be an obstacle for mapping short sequences. Furthermore, there are at the moment two reference genomes with varying quality available from different sources hampering analyses. Hopefully with the advancement of the knowledge about this model, better experiments performed by using ChIP can be realized in the near future.

It is of note that the focus of this study was on heterochromatin and markers of thereof. Nevertheless, it would be certainly of interest to study in greater detail open chromatin conformation changes and their underlying mechanisms upon aging in tissue of the *N. furzeri*.

To further investigate epigenetic changes on a global level without sequencing technology, one could also assess the degree of genome condensation of young and old *N. furzeri* by performing a karyogram. This method uses a staining, e.g. Giemsa, for heterochromatic regions so they can be distinguished from less condensed regions. It seems likely, that the banding pattern will change with age as the global histone methylation increases in *N. furzeri* skeletal muscle and heart.

It has been demonstrated, that certain variants accumulate with age influencing the epigenetic landscape (Tvardovskiy *et al*., 2017) and may induce inflammatory gene expression (Contrepois *et al*., 2017). Moreover, sequencing data of *N. furzeri* skeletal muscle tissue revealed that mRNA expression of core histones changes, hence, it could be of interest to study these interactions in detail.

To further analyze the impact of epigenetics in this model one could test more epigenetic drugs that modify the epigenetic landscape and evaluate their impact on lifespan. An example of this approach has been provided applying EZHi to reduce H3K27me3 enrichment in cells of *N. furzeri*. Although difficult for the required authorization and experimental long term, similar drugs could be tested in living animals. As an initial experiment, it would be particularly interesting to compare global histone methylation levels in the short-lived GRZ and the longer-living MZM strains at old age as well as EZH2 protein and activity levels. Most likely, the large differences in aging phenotype and lifespan (Terzibasi *et al*., 2008) are the result of different epigenetic landscape and not due to genetic differences. Therefore, one could explore whether the genome becomes hyper-methylated in the GRZ strain as in the MZM strain. With drugs or genome editing one could modulate the methylation status and assess changes in lifespan. Using the GRZ strain, one could also test whether hyper-methylation has a protective, lifespan extending effect.

Moreover, it would be interesting to examine the DNA methylation and hydroxy-methylation pattern in more detail. The 5mC and 5hmC enrichment could be assessed genome-wide via bisulfite-sequencing or in the promotor region of selected genes of interest with an enzymatic approach followed by qRT-PCR analysis. Using either approach one could shed more light on the epigenetic regulation of gene expression in tissue from young and old *N. furzeri*.

In addition to the previously mentioned experiments, it would be of great interest to investigate the mechanisms of DNA repair, establishing at which extent this mechanism is active and sufficient to maintain genomic stability. As an initial experiment the accumulation of 8-oxoguanine, a marker for DNA and RNA lesions (Radak and Boldogh, 2010), could be investigated to further support the evidence of DNA damage aggregation. Interestingly, the mRNA expression level of *8-oxo-guanine DNA glycosylase* (*ogg1*), member of DNA damage repair pathway, was reduced according to sequencing results in the aging *N. furzeri*. Therefore it could be of interest to validate this finding via qRT-PCR and evaluate protein levels and activity. One could also attempt enhancing DNA damage repair in the killifish by modulation of expression of genes involved in DNA repair by CRISPR/Cas9 or other genome editing techniques to evaluate the impact on killifish lifespan.

A recent study has shown that, similar to other species, mitochondria are the main source of oxidative stress in killifish (Baumgart *et al.*, 2016). By limiting the oxidative damage the authors could increase the lifespan of *N. furzeri*. Moreover, it has been shown that lower water temperatures limit oxidative stress in the *N. furzeri* and prevent accumulation of lipofuscin and lead to longer lifespan (Valenzano *et al.*, 2006). However, no assessment of global histone modifications including methylation was performed in these studies. Therefore, it would be very interesting to evaluate the effect of oxidative stress and that of the interventions aimed at limiting on epigenetic changes

especially of H3K27me3 and the expression of miR-29 in selected tissues of the animal. In addition to that, by applying relatively simple methods, e.g. via Western blot for γH2AX or ELISA assays one could assess the status of DNA damage.

Regarding microRNA expression and changes occurring thereof one could perform further experiments aimed at modulating expression of specific microRNAs. While it has been looked at the effect of a miR-29 Sponge construct that impeded the effect of endogenous miR-29, also investigation of the effect of overexpression or inactivation of miR-29 could be of interest. A genetic mutant killifish overexpressing or lacking certain microRNAs e.g. miR-29, in fact, could provide more insights on their impact on lifespan and cardiac health in *N. furzeri.* The induction of miR-29 expression in desired tissues or the targeted delivery of exogenous miRNAs or mimics could be of interest as well to treat accumulation of fibrosis in the heart (Gourdie *et al.*, 2016). Furthermore, the role of miR-27 in the aging *N. furzeri* could be investigated in greater detail, as this miRNA is increased with age in many tissue types. Since miR-27 has been reported to play diverse roles in atherosclerosis, inflammation and lipid metabolism as well as mitochondrial function it would be interesting to understand at what extent it might contribute to the aging phenotype of the *N. furzeri*.

With increasing age an organism may face many different obstacles that lead overall to an aging phenotype marked by impairment of molecular and cellular mechanisms leading to reduced general function. For instance, regenerative capacity declines with age: in the fin of *N. furzeri* this has been well demonstrated (Wendler *et al.*, 2015), however no skeletal muscle or heart regeneration was investigated yet. However, one could speculate that it declines as well. In addition to that, less mobility with age has been observed in *N. furzeri* (Valenzano *et al.*, 2006) and this ultimately leads to a decrease in muscle mass and sarcopenia. This is associated with disturbance of mitochondrial function and ultimately leads to oxidative stress and DNA damage. Accumulation of damaged DNA results in cell cycle arrest and genomic instability which is commonly associated with cancer. In this regard, it has been demonstrated that locomotor activity is greatly impaired in aged *N. furzeri*. Old *N. furzeri* showed less swimming activity and mobility hinting that sarcopenia occurs with age (Valenzano *et al.*, 2006). From literature it is known that sarcopenia is associated with increased oxidative stress and decreased muscle function (Powers *et al.*, 2012). Therefore, it would be interesting to investigate whether there are certain individuals that are more active at old age or to perform exercise with old animals, assessing oxidative stress level and DNA damage of these fish. Additionally, their histone methylation and rate of neoplasia formation could be assessed comparing the results to other, old individuals. Furthermore it could be of interest to explore the composition of muscle fibers and their properties upon aging. With a staining that distinguishes between red and

white muscle mass one could check whether the ratio of the two fiber types changes or remains consistent as the fish ages.

Taken together, the *N. furzeri* represents an exciting new animal model in which many aspects of aging process could be molecularly investigated and yet to be uncovered. Moreover, the reversible nature of epigenetic modulation represents a promising approach for a more healthy and youthful state by modifying gene expression without introducing changes to the DNA sequence.

7 Summary

This dissertation aimed to shed light on changes of the epigenetic landscape in heart and skeletal muscle tissue of the turquoise Killifish *N. furzeri*, a novel, short-lived animal model for aging research. The following results could be obtained:

1. A global trend towards closed chromatin conformation could be observed; histone markers for H3K27me3, H3K9me3 and H4K20me3 accumulated in skeletal muscle tissue from old *N. furzeri*. Markers for open chromatin conformation such as H3K4me3, H3K9ac and H4K16ac decreased in old skeletal muscle tissue. In old hearts from *N. furzeri* an accumulation of H3K27me3 could be detected while H3K9ac was found to increase with age as well. mRNA expression levels of methylating enzymes were higher in skeletal muscle tissue from old *N. furzeri* when compared to expression levels in skeletal muscle tissue from young *N. furzeri.*
2. The shift of epigenetic pattern was accompanied by a change of gene expression. Via mRNA sequencing in collaboration with the MPI, Bad Nauheim it could be shown that genes associated with cell cycle and DNA repair were lower expressed in skeletal muscle tissue from old *N. furzeri* than in tissue from young *N. furzeri.* Genes, associated with inflammatory signaling and glycolysis, displayed increased mRNA levels in skeletal muscle tissue from old *N. furzeri.* These results could be confirmed by Western blot and qRT-PCR analyses.
3. Markers for DNA damage and senescence increased in skeletal muscle tissue from old *N. furzeri*.
4. Cells derived from young and old *N. furzeri* skeletal muscle could be isolated and cultured for many passages. These cells were a mix of different cell types with properties and features of the native tissue. They could be used for treatment with drugs and/small compounds modulating the epigenetic landscape via specific interference with methylating enzymes.
5. DNA methylation and hydroxy-methylation were found to go in different directions in skeletal muscle and heart tissue from *N. furzeri*: while increasing in skeletal muscle tissue, a both DNA modifications declined in heart tissue with age.
6. In the heart of *N. furzeri* microRNA expression changes with age were assed with sequencing in collaboration with the FLI, Jena. It could be demonstrated that miRNA expression is age-dependent. Particular focus was on miR-29 and its target genes: miR-29 was highly upregulated in heart and skeletal muscle tissue, while target genes such as *collagens* and *dnmts* were reduced with age in the heart of *N. furzeri*.
7. Cardiac function remained stable with age and no accumulation of collagens could be found when comparing hearts of young and old *N. furzeri* despite the increase of markers for oxidative stress.

8. Cell culture experiments with human cardiac fibroblasts revealed that miR-29 is upregulated with increasing age of the donor. In addition to that, it could be shown that miR-29 is positively regulated by oxidative stress.
9. A zebrafish mutant with modified expression of miR-29 that was created in collaboration with the SNS, Pisa, presented a severe hypoxic phenotype and an altered mRNA expression profile compared to Wild Type control zebrafish. Cardiac dysfunction and hypertrophy were observed as well as an increase in DNA methylation and collagens.

Taken together, it could be shown that the aging process in skeletal muscle and heart tissue from *N. furzeri* leads to a series of changes on epigenetic levels. It remains to be elucidated whether these changes are result or cause for further changes of mRNA expression, protein levels and pathophysiology, yet the *N. furzeri* represents a promising research model for further aging studies.

8 German summary – deutsche Zusammenfassung

Mit zunehmenden Alter steigt das individuelle Risiko für Krankheiten, die vor allem ältere Menschen betreffen: Diabetes, Herz-Kreislauf-Erkrankungen, Krebs und Demenz treten häufig erst in der zweiten Lebenshälfte auf. Da die Bevölkerung zunehmend älter wird und die Lebensspanne steigt, steigt auch die Zahl jener Menschen, die von altersbedingten Krankheiten betroffen sind. Neue Methoden und Forschung zur Vermeidung bzw. Behandlung dieser altersbedingten Krankheiten sind deshalb gefragt. Dabei steht die Aufklärung der molekularen Mechanismen von Krankheiten in der Grundlagenforschung im Vordergrund. Lopez-Otin *et al.* Haben die molekularen Merkmale des Alterns beschrieben und neun Kennzeichen des Alterns identifiziert (López-Otín *et al.*, 2013). Hierzu zählen Instabilität des Genoms, Verkürzung von Telomeren, epigenetische Veränderungen, Verlust der Proteostase, deregulierte Nährstoffwahrnehmung, mitochondriale Dysfunktion, zelluläre Seneszenz, Erschöpfung der Stammzellen und veränderte interzelluläre Kommunikation. Für die vorliegende Dissertation wurde insbesondere der Aspekt der epigenetischen Veränderung in einem neuen Modelorganismus für Altersstudien näher beleuchtet.

Die Epigenetik beschreibt die Regulation der Genexpression ohne Veränderung der DNA Sequenz selbst, dabei unterscheidet man zwischen verschiedenen Mechanismen der Regulierung: zum einen können Histone, zum anderen aber auch DNA selbst, modifiziert werden. Darüber hinausgehend gibt es auch nicht codierende RNAs (ncRNA), die Einfluss auf Transkription und Translation von Genen haben, im weiteren Verlauf wird insbesondere auf die Expression von microRNA (miR) eingegangen. In welchem Zustand ein betreffender DNA-Abschnitt vorliegt wird unter anderem durch Histonmodifikationen bestimmt. Die DNA engverpackt und für Polymerasen unzugänglich, liegt sogenanntes Heterochromatin vor, die DNA ist um Histone gewickelt. Als Euchromatin hingegen werden zugängliche DNA-Abschnitte bezeichnet. Die Veränderung der Histonmodifikationen variiert mit dem Alter und nimmt damit Einfluss auf die Genexpression. Dadurch können andere Gene abgelesen werden als es im jungen Organismus der Fall ist. Modifikationen der DNA stellen eine weitere Möglichkeit der Expressionsregulation dar. Beispielsweise wird durch Anhängen von Methylresten zur Kompaktierung des Genoms beigetragen. Die microRNAs stellen eine negative Regulationsmöglichkeit der Genexpression dar: durch Bindung an mRNA wird der Abbau des RNA-Duplex eingeleitet und somit eine Translation des entsprechenden Gens verhindert.

Für die Erforschung altersbedingter Krankheiten gibt es eine ganze Reihe verschiedener Modellorganismen, die aus unterschiedlichen Gründen interessant für die jeweiligen Fragestellungen sind. Die Bandbreite reicht dabei von einfachen Pilzen wie *Podospora anserina* oder *Saccharomyces* über *Drosophila* bis zu Wirbeltieren. Allen voran sind hierbei Nagetiere wie Mäuse und Ratten zu nennen, aber auch Nacktmulle werden aufgrund ihrer außergewöhnlich langen Lebensspanne

untersucht. Die Forschung an Menschenaffen liefert oftmals interessante Einblicke in Prozesse des Alterns, allerdings sind diese Versuche mit hohem finanziellem und technischem Aufwand verbunden. Weiterhin gilt es bei allen Tieren die zu erwartende maximale Lebensdauer zu berücksichtigen. Während *Podospora* zwischen 3-6 Wochen alt werden, können *Drosophila* bis zu vier Monate alt werden. Mäuse haben eine Lebenserwartung von etwa drei Jahren und Zebrafische können bis zu fünf Jahre alt werden.

Der türkisfarbige Killifisch *Nothobranchius furzeri* ist ein neuer Modellorganismus für die Alternsforschung. Gekennzeichnet durch seine sehr kurze Lebenserwartung von 4-12 Monaten ist er das Wirbeltier mit der derzeit kürzesten bekannten Lebensdauer. Das Herz ähnelt in seiner Funktion dem der Säugetiere, ist jedoch einfacher aufgebaut. Muskelaufbau und –funktion sind ebenfalls denen der Säugetiere ähnlich.

In der vorliegenden Arbeit wurde die Veränderung epigenetischer Marker mit zunehmendem Altern in dem neuen Modellorganismus *Nothobranchius furzeri* (*N. furzeri*) untersucht. Hierbei wurden Herz- und Muskelgewebe von jungen, adulten und alten Fischen auf verschiedene Modifikationen hin untersucht sowie deren mögliche Ursachen ermittelt.

Mittels Western Blot und Immunfluoreszenzmikroskopie konnte ein Anstieg von Histonmarkern, die mit einer Kompaktierung des Genoms in Verbindung stehen, nachgewiesen werden. Mit zunehmendem Alter akkumulieren H3K27me3, H3K9me3 und H4K20me3 in Muskelgewebe von *N. furzeri*. Auch im Herzgewebe konnte ein Anstieg an H3K27me3 mit dem Alter festgestellt werden. Histonmarker, die mit Euchromatin assoziiert werden, nehmen im alternden Muskel hingegen ab. Zudem werden Gene, die für die Trimethylierung von H3K27 verantwortlich sind, in Gewebe von alten Fischen stärker exprimiert als in Muskel von jungen Fischen. Anhand von Sequenzierungsdaten konnte eine Verringerung von DNA Reparaturmechanismen und Zellzyklusgenen mit dem Alter festgestellt werden. Dies konnte sowohl in Muskel- als auch Herzgewebe von *N. furzeri* durch Western Blot bestätigt werden. Gene, die mit Glykolyse und Inflammation assoziiert sind, sind hingegen in Muskel von alten Fischen in ihrer Expression hochreguliert, was auch mittels qRT-PCR für die mRNA einzelner Gene nachgewiesen werden konnte. In Muskelgewebe von altem Fisch konnte zudem eine Akkumulation von Seneszenzmarkern festgestellt werden.

Während die DNA-Methylierung in Muskelgewebe des *N. furzeri* mit dem Alter zunimmt, wird diese im Herzen mit der Zeit verringert. Die Genexpression der dafür zuständigen Enzyme wird reduziert.

Um die Veränderung der miRNA Expression in Herzen von *N. furzeri* zu ermitteln, wurden zusammen mit Dr. Mario Baumgart (FLI, Jena) die microRNA von jungen, adulten sowie alten Killifischherzen sequenziert. Dabei konnte gezeigt werden, dass die miRNA Expression altersabhängig ist. Von

besonderem Interesse war der signifikante Anstieg der Expression von miR-29a und miR-29b, die nicht nur in *N. furzeri* Herzen und Muskelgewebe nachgewiesen werden konnte, sondern auch in Kulturen humaner Kardiofibroblasten (HCFs). Gleichzeitig wurde im Herzen ein Anstieg von Markern für oxidativen Stress verzeichnet. Mittels *in-vitro* Versuchen mit HCFs konnte gezeigt werden, dass oxidativer Stress einen Anstieg von miR-29 Niveaus induziert. Die Expression von Zielgenen von miR-29 (unter anderem Kollagene) war dabei vermindert. Im Herzen von *N. furzeri* kam es mit zunehmendem Alter zu einer Verringerung der Expression von Kollagenen. Eine Akkumulation von Kollagen-artigen Proteinen konnte mittels histologischer Färbung nicht nachgewiesen werden. Eine vergleichende Untersuchung von jungen und alten Fischen ergab, dass die Herzen auch im fortgeschrittenen Stadium kontraktil und damit funktional blieben. Des Weiteren kann durch erhöhte Expression von miR-29 einer Hypermethylierung der DNA und damit verbundener Expressionsveränderung, sowie einem hypoxischen Phänotypen entgegengewirkt werden.

Um diese Entdeckung weiter zu untersuchen, wurde in Kollaboration mit Dr. Roberto Ripa und Prof. Dr. Alessandro Cellerino (SNS, Pisa) ein transgenes Zebrafischmodell generiert. Diese Fische exprimieren keine funktionale miR-29 (miR-29 Sponge). Es konnte gezeigt werden, dass diese Fische Kollagene in ihren Herzen ansammeln und das Herz dadurch weniger kontraktil ist. Zudem war die DNA-Methylierung stark erhöht im Vergleich zu Herzen der Kontrollgruppe. Dies führte zu einer Veränderung der Genexpression in Herzen der miR-29 Sponge Tiere. Gene, die mit Hypoxie in Verbindung stehen, werden stärker exprimiert als in Herzen von Kontrolltieren, des Weiteren kam es zu einer Stabilisierung von Hypoxie-induzierbarem Faktor 1alpha (HIF1α).

Die epigenetische Landschaft verändert sich im türkisfarbigen Killifisch nicht nur auf der Ebene der microRNAs, wie Analysen von Histonmodifikationen und DNA Methylierung ergeben haben. Im Gegensatz zu Säugetieren, bei denen eine Abnahme von H3K27me3 festgestellt werden konnte, akkumulierte H3K27me3 in Muskel- und Herzgewebe und auch im Gehirn von *N. furzeri* (vergl. Baumgart *et al.*, 2014b). Gleichzeitig wurden DNA Schäden nicht ausreichend repariert und Doppelstrangbrüche nahmen zu. Es kann angenommen werden, dass eine reduzierte Anzahl an Mitochondrien zu oxidativem Stress im Gewebe führt und reaktive oxidative Spezies (ROS) DNA Schäden verursachen. Im Zuge dieser Dissertation konnte gezeigt werden, dass oxidativer Stress zu einer Erhöhung des Expressionslevels von miR-29 führt und miR-29 in verschiedenen Gewebearten mit dem Alter zunimmt (Heid *et al.*, 2017).

Bislang ist nichts über die Aktivität von DNA-Reparaturmechanismen in *N. furzeri* bekannt, aufgrund seiner kurzen Lebensdauer und auch der Umweltbedingungen in seinem ursprünglichen Habitat ist jedoch davon auszugehen, dass diese nur begrenzt aktiv sind. Um eine Weitergabe etwaiger Schäden an andere Zellen zu verhindern kommt es zu einer Verringerung der Proliferation im Gewebe; zudem

sind Wachstumsprozesse abgeschlossen und eine erhöhte Zellteilung ist nicht weiter notwendig. Dies führt dazu, dass sich im Muskelgewebe Marker für zelluläre Seneszenz anreichern und die Proliferation weitestgehend zum Erliegen kommt.

Es ist anzunehmen, dass es aufgrund der unzureichenden Reparatur von DNA-Schäden zu einer Kompaktierung des Genoms kommt, weitere Schäden werden damit verhindert. Durch vermehrte Trimethylierung von Histonmarkern kommt es zur Bildung von Heterochromatin, um das Genom zu stabilisieren. Von *in-vitro* Versuchen ist ein solches Phänomen der Zunahme von geschlossener Chromatinkonformation unter oxidativen Bedingungen bekannt (Basenko *et al.*, 2015; Foltánková *et al.*, 2013; Li *et al.*, 2014). Zudem treten mit zunehmendem Alter auch sogenannte Seneszenz-assoziierte Heterochromatin Foci (SAHF) auf, welche mit H3K9me3 und HP1α angereichert sind (Chandra and Narita, 2012), die Kompaktierung des Genoms könnte also auch durch einen Anstieg seneszenter Zellen verursacht sein. Gleichzeitig wurde durch zahlreiche Studien gezeigt, dass verschiedenste Krebsarten zu einer Veränderung der Genexpression durch veränderte Epigenetik führen. Oftmals kommt es zu einem Anstieg der mRNA Expression von methylierenden Enzymen, die Zellzyklus-Kontrollgene mittels Hypermethylierung in ihrer Expression einschränken und damit unkontrolliertes Zellwachstum fördern. Dabei kann es zur vermehrten Bildung von Entzündungsfaktoren und einer Veränderung des Metabolismus. Der sogenannte Warburg-Effekt beschreibt eine metabolische Verschiebung von oxidativer Respiration hin zu vermehrter Glykolyse, auch wenn funktionale Mitochondrien vorliegen. Anhand der mRNA Expressionsanalyse konnte gezeigt werden, dass ein Anstieg von Genen, die mit Entzündungsreaktionen und mit Glykolyse assoziiert sind, mit dem Alter in *N. furzeri* Muskelgewebe zunehmen. Darüber hinaus sind kanzerogene Läsionen in Nieren und Leber bekannt (Di Cicco *et al.*, 2011) sowie die Melanomabildung in alten *N. furzeri* (Cellerino *et al.*, 2016).

Durch eine vergleichende Analyse der mRNA Expression mit Datensätzen verschiedener Studien konnte gezeigt werden, dass sich die Genexpression nicht nur in einem bestimmten Gewebe verändert, sondern dass sich die Expression im ganzen Fisch mit dem Alter in eine ähnliche Richtung verändert. So konnte eine Expressionsabnahme von Zellzyklus-assoziierten und DNA-Reparaturgenen in Muskel, Gehirn, Leber und Haut nachgewiesen werden, während es in allen Geweben zu vermehrter Expression von Entzündungs-assoziierten Genen kommt. Diese Ergebnisse wie auch die Tatsache, dass bestimmte epigenetische Veränderungen in mehreren unterschiedlichen Geweben sich ähneln, lassen darauf schließen, dass der ganze Organismus des Killifisch altert und global von den Auswirkungen des Alterns betroffen ist. Nichtsdestotrotz konnten auch Unterschiede in der epigenetischen Veränderung mit dem Alter festgestellt werden. Hierbei handelt es sich

wahrscheinlich um gewebetypische Veränderungen, die von der unterschiedlichen Konstitution und Funktion des jeweiligen Gewebes beeinflusst werden.

Für weitere Analysen dieser epigenetischen Landschaft wäre es interessant herauszufinden, welche Genomabschnitte von einer Hypermethylierung mit zunehmenden Altern betroffen wären. Dafür könnten mittels Chromatin-Immun-Präzipitation (ChIP) unter Verwendung eines geeigneten Antikörpers entsprechende Sequenzen identifiziert werden. Erste Versuche in diese Richtung der Arbeitsgruppe Gaetano haben leider bisher zu keinem Ergebnis geführt.

Zudem wäre es von Interesse, DNA-Reparaturmechanismen im Killifisch genauer zu untersuchen. Gezielte Manipulation oder Verbesserung der DNA-Reparatur mittels Medikamentengabe oder genetischer Editierung (z.B.: CRISPR) könnte möglicherweise die Lebensspanne des *N. furzeri* verändern. Des Weiteren könnte der Zusammenhang von mitochondrialen Stoffwechselprodukten und epigenetischen Veränderungen näher betrachtet werden. Eine Analyse der Entzündungs-assoziierten Reaktionen und ihren Anteil an Alterungsprozessen in diesem Modellorganismus könnte obendrein für künftige Forschung von Belang sein.

Insgesamt bleibt festzuhalten, dass es sich bei dem türkisen Killifisch *Nothobranchius furzeri* um einen interessanten und vielversprechenden Modellorganismus handelt, der sich durch seine kurze Lebensdauer sehr gut für generationsübergreifende Versuche und Fragestellungen eignet. Da *N. furzeri* erst dabei ist, weitere Verbreitung zu erfahren und ein etablierter Modellorganismus zu werden, sind derzeit noch viele Details und Mechanismen zu klären. Erste Untersuchungen hinsichtlich der neun Merkmale des Alterns und deren Manipulation konnten bereits unternommen werden (Harel *et al*., 2015).

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Web links for teleost fish physiology:

http://esi.stanford.edu/exercise/exercise6.htm

https://esi.stanford.edu/circulation/circulation5.htm

https://esi.stanford.edu/circulation/circulation6.htm

9.2 Abbreviations

Table 9-1: List of utilized abbreviations in the text.

Abbreviation	Full Name
2-HG	2-hydroxyglutaric acid
5hmC	5-hydroxymethyl cytosine
5mC	5-methyl cytosine
A	Atrium
AGE	advanced glycation end product
AGO2	Argonaute 2 protein
APS	ammonium persulfate
ATP	adenosine tri-phosphate
B	*Bulbus arteriosus*
BCA	Bicinchoninic acid assay
BER	base excision repair
cbx	*chromobox*
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
cd40	cd40 molecule
cepbp	*CCAAT/enhancer binding protein beta*
cDNA	complementary DNA
-CH3	methyl residue
ChIP	Chromatin immune precipitation
col	*collagen*
CS	Cockayne Syndrome
CT	cycle threshold
CVD	cardiovascular disease
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dnmts	*DNA methyl transferases*
Dre	*Danio rerio*
DSB	double strand break
ECM	extra cellular matrix
EDA	end-diastolic area
EED	embryonic ectoderm development
ELISA	enzyme-linked immunosorbent assay
ESA	end-systolic area
EtOH	ethanol
ezh	*enhancer of zeste*
EZHi1/2	inhibitor molecules 1and 2 of EZH proteins, described in Ciarapica *et al*. (2014)
FAC	fractional area change
FBS	fetal bovine serum
FDA	Food and Drug Administration, US authority
Fig.	figure
FLI	Fritz-Lippmann-Institute; Jena, Germany
gadd45γ	*growth arrest and DNA damage inducible 45γ*
gapdh	*glyceraldehyde-3-phosphate dehydrogenase*

GO	gene ontology
h	hour
H_2O_2	hydrogen peroxide
H3	histone 3
H3K27me3	histone 3, lysine 27, tri-methylation
H3K4me3	histone 3, lysine 4, tri-methylation
H3K9ac	histone 3, lysine 9, acetylation
H3K9me3	histone 3, lysine 9, tri-methylation
H4K16ac	histone 4, lysine 16, acetylation
H4K20me3	histone 4, lysine 20, tri-methylation
hats	*histone acetyl transferases*
HCFs	human cardiac fibroblasts
hdacs	*histone de-acetylases*
HGPS	Hutchinson-Gilford progeria syndrome
HRR	homologous recombination repair
HP1α	heterochromatin protein 1α
Hsa	*Homo sapiens*
HSP	heat shock proteins
IF	immunofluorescence
jak3a	*janus kinase 3*
jmjc	*jumonjic*
K	lysine
kdms	*lysine de-methylases*
KEGG	Kyoto Encyclopedia of Genes and Genomes
MI	myocardial infarction
min	minutes
miR	microRNA
miR-29 Sponge	transgenic Zebrafish with non-functional miR-29 expression
mmp	*matrix-metalloprotease*
mRNA	messenger ribonucleic acic
myog	*myogenin*
N	number of replicates
NAC	N-acetylcysteine
NAD^+	Nicotinamide adenine dinucleotide
NER	nucleotide excision repair
Nfu	*Nothobranchius furzeri*
NHEJ	non-homologous end joining
p0	*ribosomal protein subunit p0*
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline Tween-20
PCA	principal component analysis
pcna	*proliferating antigen*
PFA	paraformaldehyde
polγ	*polymerase gamma*
PRC	polycomb repressive complex
PTM	post-translational modifications
ptx3a	*pentraxin 3a*
qRT-PCR	quantative real-time polymerase chain reaction

RISC	RNA-induced silencing complex
ROS	reactive oxygen species
RT	room temperature
s	seconds
SAHF	senescence-associated heterochromatin foci
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate
SFB	„Sonderforschungsbereich“; special research area
SIRT1	Sirtuin 1
SNS	Schuola Normale Superiore; Pisa, Italy
ssbp	*mitochondrial single-stranded dna binding protein*
stat1a	*signal transducer and activator of transcription 1a*
suz	*polycomb subunit suz12*
TCA	tricarboxylic acid
tdg	*thymine dna glycosylase*
TEMED	Tetramethylethylenediamine
tet	*ten-elven translocase*
tfam	*mitochondrial transcription factor*
tgfβ	*transforming growth factor β*
tnfaip	*tnf alpha induced protein 2*
V	Ventricle
vs.	*versus*
WB	Western blot
WHO	world health organization
wks	weeks
WS	Werner syndrome
αTub	α Tubulin
γH2AX	histone 2 AX, with phosphorylated serine 139

9.3 List of figures

9.4 List of tables

10 Appendix

10.1 Contributions from collaborators

Table 10-1: List of collaborators and their contribution to the present thesis.

Name	Contribution
Dr. Mario Baumgart, FLI, Jena	animal sacrifice, tissue isolation; partial RNA isolation and cDNA preparation from muscle and heart tissue; miRNA sequencing
Prof. Dr. Thomas Braun, MPI, Bad Nauheim	mRNA sequencing of killifish muscle and zebrafish hearts
Prof. Alessandro Cellerino SNS, Pisa/ FLI, Jena	tissue donation: paraffin-embedded muscle and liver samples
Dr. Stefan Günther, MPI, Bad Nauheim	mRNA sequencing of killifish muscle and zebrafish hearts
Dr. Carsten Künne, MPI, Bad Nauheim	mRNA sequencing of killifish muscle and zebrafish hearts
Dr. Giuseppina Milano, IRCCS, Milan	Echocardiography of killifish and zebrafish hearts
Prof. Dr. Guilio Pompilio, IRCCS, Milan	Echocardiography of killifish and zebrafish hearts
Roberto Ripa, SNS, Pisa / MPI, Cologne	generation of a transgenic zebrafish (miR-29 Sponge), zebrafish husbandry
Dr. Alessandro Scopece, IRCCS, Milan	Echocardiography of killifish and zebrafish hearts
Dr. Francesco Spalotta	Confocal imaging

10.2 German affidavit - Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbständig und nur unter Zuhilfenahme der hier angegebenen Quellen und Hilfsmittel verfasst habe.

Die Dissertation wurde bisher keiner anderen Fakultät vorgelegt.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass keine Aberkennung eines bereits erworbenen Doktorgrades vorliegt.

Frankfurt, den 26.06.2018

Johanna Heid