Micro-RNAs as Regulators of Senescence and Aging.

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Senescence is the change in the biology of an organism as it ages. It can be caused by different factors, and induced for different reasons. Aging is process, resulting from the accumulation of biological damage. Aging is closely connected to senescence, but has its own regulatory pathways. The p53 and p16 pathway are known to regulate senescence, while the IIS and TOR pathways are the best studied aging pathways. miRNAs have been found to regulate the mechanisms that control senescence and aging, and are also able to influence lifespan after up or down regulation. In this article I will discuss the major pathways that regulate senescence and aging, as well as sirtuins and shelterin. Sirtuins link metabolism to aging, and, acting through the TOR pathway, are another important group of regulators of lifespan. Shelterin is a protective cluster of proteins, safeguarding human telomeres and preventing premature senescence. These pathways and proteins are all involved in senescence and aging, and I will try to link them by a distinct group of cellular regulators; miRNAs.

Introduction

MicroRNAs (miRNA) are an emerging class of posttranscriptional, epigenetic regulators of mRNAs. Since the discovery of the first miRNA, almost two decades ago, the number of miRNAs has exploded, with more than 1000 known today in humans alone. Today, miRNAs have been shown to regulate gene expression in every human cell. In this article, I want to look at the regulatory function of miRNAs in two closely related processes; senescence and aging. The importance of these processes has become more and more appreciated by researchers over the last decades, since they are connected to many diseases. The need to comprehend these processes only increases because humans get older and older nowadays.

Senescence and aging are often used interchangeably, which makes sense because senescence can be regarded as biological aging. In literature although, sometimes a distinction is made between the two, because there are some important differences between the processes. Therefore in this article I want to discuss both processes separately. To underline the differences I will start by defining both senescence and aging. Senescence is the biological change, occurring in an organism as it gets older. Aging is a multifactorial process, resulting from the accumulation of molecular and cellular damage over time (*Smith-Vikos and Slack, 2012).* In this article I will discuss both processes and the underlying mechanisms, with a focus on miRNAs.

Senescence

Cellular senescence was first described in 1965 by Hayflick, who suggested that each cell had a finite doubling potential in culture (*Hayflick, 1965*). After 40-60 doublings, cells become senescent and

lose their ability to proliferate. This does not mean that senescence is just a side effect of cell doubling; it is actually an active biological process, meant to prevent excessive cell proliferation as in cancer. To discern a senescent cell, it is necessary to know the characteristics of a senescent cell. The most important feature of a senescent cell is growth arrest. A senescent cell is unable to go through the cell cycle. Besides growth arrest, senescent cells are resistant to apoptosis, have a unique gene expression and some other markers.

There are a few different categories of senescence which I will discuss shortly (*Campisi et al, 2007*). Telomere dependent senescence, also called replicative cell senescence, is probably the most well-known form of senescence. Telomeres are long, repetitive sequences of DNA, at the end of chromosomes. Because DNA polymerase cannot fully replicate the DNA strand, the telomere end loses 100-200 nucleotides every time a cell divides. After approximately 50 divisions the telomeres will be gone. Because the last part of the DNA strand cannot be fully copied, genetic information would get lost without telomeres. Because of this possible loss, a cell will stop dividing, and becomes senescent. One of the properties of stem cells, is that they are able to divide infinitely (another property is self-renewal). In order to do this, they have high telomerase activity which lengthens the telomeres after every division, preventing chromosomal damage and allowing infinite proliferation.

DNA-damage-induced-senescence is another form of senescence and is telomere independent (*Campisi et al, 2007*). Reactive Oxygen Species (ROS), ionizing radiation and chemotherapy are examples of DNA damage inducing factors. Sometimes oncogenes are regarded as inducers of senescence. But most of these genes only stimulate cells to proliferate, thereby causing telomere dependent senescence. At last there also are extracellular stimuli such as cytokines and growth factors that push a cell towards a senescent state.

Regulatory pathways involved in the development of cellular senescence

The p53 and p16 pathways

To stop a cell from going through the cell cycle, the hallmark of a senescent cell, two different pathways play a major role, the p53 and the p16 pathway (*Figure 1*).

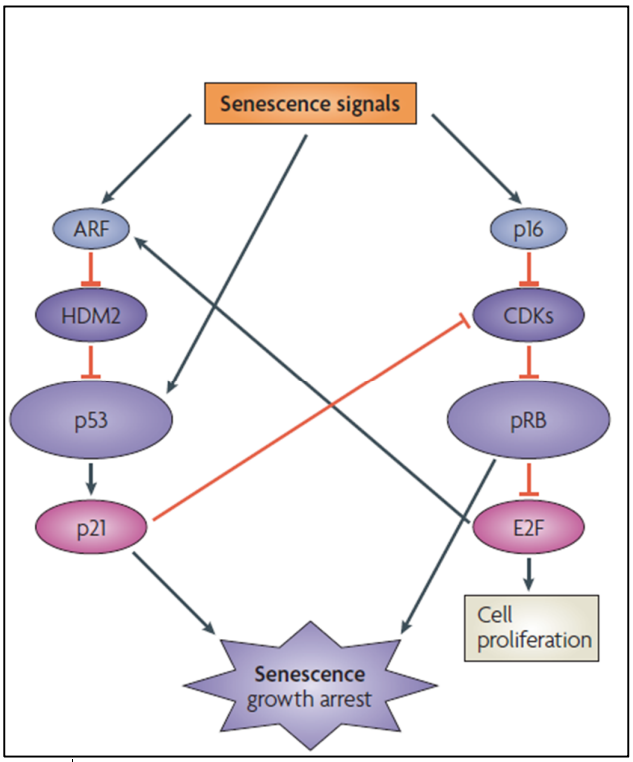

Figure 1. The p53 and p16 pathway, controlling senescence (Campisi et al, 2007). Senescence signals activate the p53 and p16 pathway, which act separately but also interact, and together regulate senescence.

Upon activation, these pathways eventually lead to growth arrest, although not every activation of one of these pathways causes a cell to become senescent. For example, when the activating signal is DNA damage, it is possible for a cell to repair the damage instead of becoming senescent. In both cases the p53 and p16 pathways are activated, but it is unclear why a cell sometimes senesces and at other times is restored.

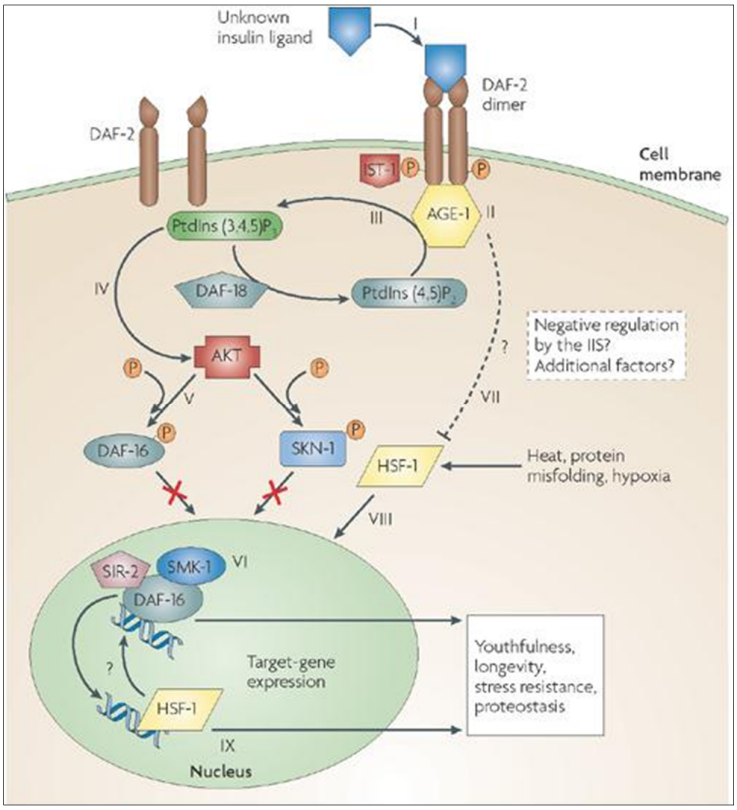

Figure 2. The IIS pathway in C. Elegans (Cohen and Dillin, 2008).DAF-2 is a membrane receptor, that activates the IIS pathway. DAF-2 then recruits the PI3 kinase AGE-1 and IST-1, an insulin receptor substrate. AGE-1 catalyzes the generation of Phosphatidylinositol-3,4,5-triphosphate (PI3) which activates AKT, a kinase. DAF-18 is the opposite of AGE-1, reducing AKT activation. Once AKT is activated, it phosphorylates the Forkhead Box O (FOXO) transcription factor DAF-16 and SKN-1, also a transcription factor. Phosphorylated DAF-16 cannot enter the nucleus. Dephosphorylated DAF-16 goes into the nucleus, where it regulates its target genes, together with cofactors SIR-2 and SMK-1. The target genes are involved in aging and other processes. AGE-1 also inhibits Heat shock Factor 1 (HSF-1), required for longevity and stress resistance.

Aging

Aging, as defined in the introduction, is a multifactorial process (senescence being one of them), which eventually leads to death. Research in the past years, however, has showed that aging, as well as senescence, is a controlled process. Two major pathways, the IIS and the TOR pathway, have been identified to play an important role in aging. That is why I will discuss and show these first. Later on, some regulatory factors of these pathways will be discussed.

Aging pathways

The IIS pathway

The Insulin/ IGF (Insulin-like Growth Factor) signaling (IIS) pathway is a known life span regulatory pathway. The pathway is best known from the model organism C. elegans, but studies in mice and human show that the pathway is identical in these organism and highly conserved (*Smith-Vikos and Slack, 2012*). For details on the pathway, see *figure 2.*

The TOR pathway

One other important pathway involved in aging is the TOR (target of rapamycin) pathway. TOR kinases regulate cell growth and proliferation in response to nutritional cues, and forms the connection between nutrition, metabolism and longevity (*Vellai et al, 2003*). The TOR pathway is highly conserved, and designated mTOR in mammals. As shown in figure 3, mTOR targets S6 and 4EBP.

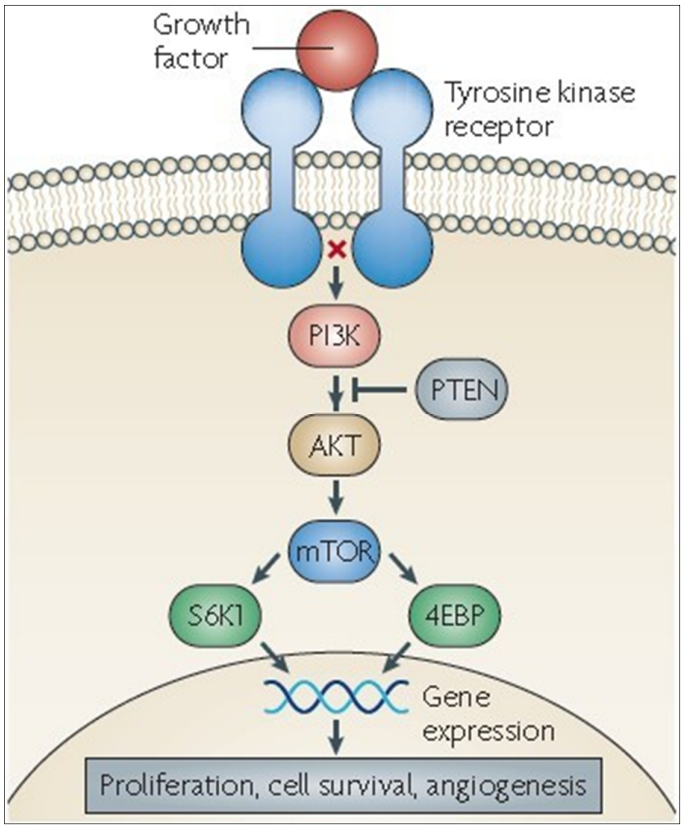

Figure 3. A simplified reproduction of the mTOR pathway (Atkins et al, 2009). The mTOR pathway also is activated through PI3 kinase and AKT. But it is different from IIS, because it acts independently of FOXO and DAF-16. mTOR activates S6, a protein kinase, and 4EBP, the 4E binding protein.

Inhibition of S6, and overexpression of 4EBP have been shown to increase lifespan in several different organisms (*Kenyon, 2010*). Down regulation of TOR activity after chronic dietary restriction in worms, flies and mice is connected with extended lifespan, indicating that TOR indeed is a regulator for aging (*Kenyon, 2010*).

Non-coding RNA's

Only 1,5% of human DNA is translated into proteins. About 10% of the DNA encodes non-translated, regulatory RNA and is therefore called non-coding RNA. Among others, there are small nucleolar RNAs (snoRNAs), small interfering RNAs (siRNAs) and microRNAs (miRNAs)(*Aalto and Pasquinelli, 2012)*. Because miRNAs are the most abundant and best known cellular regulators, I will focus on their function in senescence and aging.

miRNAs

MicroRNA's are small (about 22 nucleotides), non-coding RNAs that regulate gene expression, by posttranscriptional regulation.

MiRNA precursors (pri-miRNAs) are synthesized by RNA polymerase II, or the pri-miRNAs arise from introns and then enter the miRNA processing steps (Berezikov et al, 2007). The processing comprehends the cutting of the hairpin shaped pri-miRNA to the short, single stranded miRNA. This processing acts by a protein complex called RNA Induced Silencing Complex (RISC). Once a miRNA is formed it is able to bind a complementary nucleotide sequence on target mRNAs. Depending on the strength of the match, a miRNA can either induce degradation of mRNA, or inhibit translation (*Figure 4*).

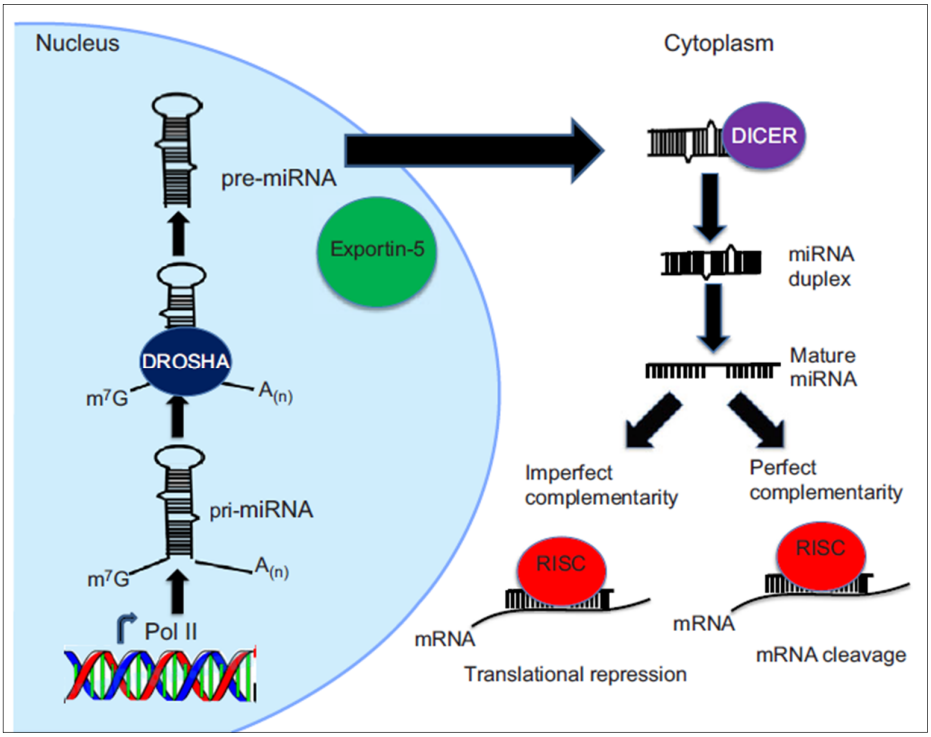

Figure 4. Biogenesis of miRNA (Smith-Vikos and Slack, 2012). miRNA is transcribed by RNA polymerase II, and by the enzyme DROSHA converted into pre-miRNA, which is exported from the nucleus into the cytoplasm. In the cytoplasm, DICER cleaves pre-miRNA into a short double stranded RNA. RISC, together with argonoute takes the double stranded miRNA and processes it into a single stranded miRNA (not shown). The miRNA guides RISC to a complementary mRNA, and represses translation of the mRNA or causes degradation of the mRNA.

The so called seed sequence is an important factor in binding a complementary sequence, since the seed sequence is almost always completely homologous to the target sequence, in contrast to the rest of the miRNA. The seed sequence consists of 6-8 base pairs that are complementary to the target. The imperfect binding of the miRNA to its target enables the miRNA to bind to many different target mRNAs which makes their function very complex. However, their function is studied and elucidated in more and more processes in humans, senescence and aging being two of them.

miRNAs and senescence

A number of studies have shown that miRNAs are key players in the regulation of cellular and organismal longevity. One very interesting study on the importance of miRNA has been done by Mudhanasi et al (*Mudhanasi et al, 2008)*. To better understand miRNAs in regulating cell growth, they created a mouse bearing a conditional *Dicer* allele. This enabled the researchers to turn of Dicer and as you can see in figure 2, dicer is necessary for the generation of miRNA. The loss of miRNA caused an increase in p19 (part of the p53 pathway) and p53 activity, contributing to senescence and also inhibited cell proliferation, increased DNA damage and a premature senescent phenotype *in vivo* and *in vitro*. Deletion of p19 and p53 could prevent premature senescence *in vitro* , indicating a pivotal role for the p53 pathway in senescence.

In 2005 Boehm and Slack showed that a single miRNA, lin-4, had a huge influence on the lifespan of C. Elegans (*Boehm and Slack, 2005*). A lin-4 knock-out had a significantly lower lifespan, compared to wild-type, and overexpression of the lin-4

miRNA led to an extended lifespan. The same study showed that the lin-4 target lin-14 effected the changed life span, by activating the IIS pathway. Lin-4 down regulates lin-14, and lin-14 inhibits DAF 16 by phosphorylation. Phosphorylated DAF 16 remains in the cytoplasm. When the phosphorylation of DAF 16 is inhibited, it moves into the nucleus where it activates and represses several genes involved in processes that, taken together, contribute to longevity (*Smith-Vikos and Slack, 2012)*.

De Lencastre et al showed that miRNA -71, 238, 239 and 246 regulate life span in C. elegans (*De Lencastre et al, 2010)*. Interestingly enough, they showed that both up regulation (miRNA-71 and miRNA-246) and down regulation (miRNA-239) increases longevity. The miRNAs that have been investigated in this study are assumed to target two DNA damage response pathways, specifically CDC25 and CHK1. CDC25 is a phosphatase and GHK1 is a kinase, both of them involved in controlling the cell cycle.

All these studies have been performed on model organisms, and currently there are no studies that investigate the role of miRNAs in human longevity. Some studies, however, show that the tissue-specific expression levels of miRNA in humans alters during aging (*Smith-Vikos and Slack, 2012)*. This indicates that miRNAs might also be involved in human aging. For senescence, the role of miRNAs is better understood. miRNAs are known to target several senescence associated pathways, like the MAPK pathway, the p16 and p53 pathways (shown above) and the IIS pathway (*Smith-Vikos and Slack, 2012)*. Through targeting these pathways miRNAs are able to influence the process of senescence.

Altogether, miRNAs are acknowledged to play an important role in regulating senescence and other essential biological processes, but in many cases the mechanism of regulating is unclear, and the controlling of the regulation remains to be elucidated.

Sirtuins

Now that the influence of miRNA on aging and senescence has been discussed, I want to look at two interesting groups of proteins, sirtuins and shelterin, because of their pivotal role in senescence and aging. First I will describe the proteins and then discuss miRNAs as their possible regulators.

Sirtuins are NAD^+ dependent deacetylases, that are highly conserved across species. Sirtuins in yeast, flies and worms have been shown to regulate lifespan (*Kenyon, 2010)*. It is not entirely clear how sirtuins expand lifespan upon overexpression. For C. elegans, however, the mechanism has been described. The sirtuins sir2 activates DAF-16 (*Figure 2*) by acetylation, and as we have seen, activated DAF-16 targets genes involved in longevity and stress resistance.

Also in mammals sirtuins have been described and suggested to be involved in the regulation of senescence. Besides the sirtuin SIRT1, the homolog for the nematode sir2, there are the sirtuins SIRT1 – SIRT7. Mammalian sirtuins act in different subcellular levels (*Finkel et al, 2009)*. SIRT6 and SIRT7 are nuclear proteins, SIRT3, 4 and 5 mitochondrial and SIRT1 and 2 are active both in the nucleus and cytoplasm. Recent studies show that sirtuins are involved in major biological processes contributing to senescence and aging. The fact that sirtuins depend on NAD^+ indicates that they connects them to

intracellular energy (*Finkel et al, 2009)*. This has led to the idea that sirtuins are a possible link between food intake and lifespan. To date, (a part of) the function of SIRT 1 and SIRT3 is known. Especially SIRT1 has been well studied in relation to senescence and aging (*Houtkooper et al, 2012)*. SIRT1, the most studied mammalian sirtuin, has been shown to target p53 and to control the acetylation of FOXO transcription factors. We have already seen that p53 is a major component in the senescence regulating pathway and FOXO transcription factors play a pivotal role in the IIS pathway. One of the functions of SIRT3 is to protect cells against ROS, a potential cause for senescence (*Houtkooper et al, 2012)*.

A recent study, however, shows that also the overexpression of SIRT6 in male mice increases the lifespan with approximately 80 and 100 days in two groups of mice derived from two different transgenic lines (*Kanfi et al, 2012)*. This research also showed some evidence that the SIRT6 regulates lifespan through the known IIS pathway, shown in figure 2. IGF-1 serum levels were low in male mice overexpressing SIRT 6. IGF-1 is a ligand for the insulin/IGF receptor of the IIS signaling. When the IIS pathway was suspected to be involved, other components of this pathway were examined. Levels of phosphorylated AKT were found to be lower. This also caused a down regulation of FOXO phosphorylation. As you can see in figure 2, this allows the FOXO to enter the nucleus and target genes contributing to longevity.

Although this may seem convincing, scientists are not all together convinced of the crucial role of sirtuins in longevity. It is also possible that sirtuins contribute to health, and that longevity comes as a side effect of improved health (*Lombard and Miller, 2012)*. Also in response to the article of Kanfi et al, discussed above, Lombard and Miller question if an increase of lifespan by overexpression of SIRT6 means that this sirtuin is involved in the process of aging (*Lombard and Miller, 2012)*. According to Lombard and Miller, mutations in the IIS pathway usually have a beneficial effect for female mice, contradictory to the results of Kanfi et al. They also argue for other beneficial functions for SIRT6, like DNA repair, chromosome stability and anti-cancer potential, that might contribute to longevity. Altogether the conclusion that extended lifespan after SIRT6 overexpression means a causal functions for SIRT6 in aging is too simplistic, according to Lombard and Miller. This also means that to get more certainty about the role of sirtuins in aging, especially in mammals, a lot of research is waiting to be done.

miRNAs and Sirtuins

Currently, very few information is available on the role of miRNAs as regulators of sirtuins. Most likely, miRNAs regulate sirtuin levels by blocking translation of sirtuin mRNAs, or by blocking translation of sirtuin inhibitors, thereby up or down regulating sirtuin activity. Several studies indeed prove that this regulation by miRNA occurs, and I will discuss one of them (*Ito et al, 2010)*. This study, by Ito et al., showed that miRNA 34a is up regulated in senescent human endothelial cells (*Ito et al, 2010)*. The SIRT1 mRNA turned out to be a target of the miRNA 34a, resulting in low levels of SIRT1 after overexpression of miRNA 34a. Since SIRT1 was considered a regulator of aging, the researchers concluded that a single

miRNA can have major influence on aging pathways. Unfortunately this study doesn't address the question how levels of miRNA 34a are controlled, since this seems to be regulatory in the process of aging.

Sirtuins are considered pivotal in several pathways connected with senescence and longevity, but many questions remain about their functions and regulatory capacity. One important question to be answered is whether the proteins directly regulate aging, or just regulate biologically beneficial processes, resulting in longevity.

Sheltrins

As said in the beginning of this article, senescence can be induced by different factors. One of them was the shortening of the telomeres. After a certain number of replications, a cell can reach the point where telomeres are too short to undergo replication safely. This is one problem with telomeres. Another one is that telomeres are actually just loose ends of double stranded DNA. How then are they protected against DNA damage repair responses, that are necessary in case of a double strand brake, but also contribute to senescence? This question is known as the end-protection problem. Only recently it has been discovered how telomeres avoid these responses (*De Lange, 2009)*.

Currently four cellular mechanism occurring after DNA damage are known. The first mechanism is by the ATM (Ataxia telangiectasia mutated) kinase pathway, activated directly by DNA ends (*De Lange, 2009)*. Another pathway is the ATR (Ataxia telangiectasia and RAD3 related) kinase pathway that gets activated by a single stranded DNA, which occurs when the 5' end of a double stranded break gets exposed (*De Lange, 2009)*. Besides these two signaling pathways, DNA can undergo Non Homologous End Joining (NHEJ), to protect the exposed ends or Homology Directed Repair (HDR) (*De Lange, 2009)*. These processes cause cell cycle arrest or unwanted fusions of chromosomes, which induces either apoptosis or senescence (*De Lange, 2009)*.

To avoid apoptosis or senescence, telomeres need to be protected against these four mechanisms. In mammals this is done by a protein complex, consisting of six components, called shelterin (*De Lange, 2009)*. Shelterin was first described in 2005, and regarded as a complex that safeguards human telomeres (*De Lange, 2005)*. The components of shelterin are shown in figure 5.

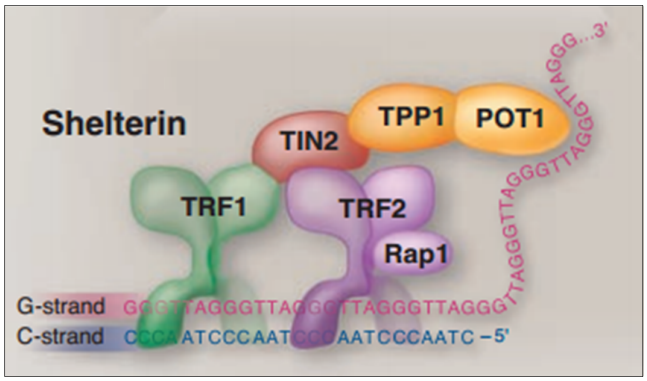

Figure 5. The shelterin complex (De Lange, 2009). The components of the shelterin complex are TRF1, TRF2, TIN2 (, Rap1, TPP1 and POT1. TRF1, TRF2 and POT1 recognize and bind to TTAGGG repeats, typical for telomeres. The other components enable a cell to discern telomeres from double strand brake (De Lange, 2005).

As you can see, TRF1 and TRF2 bind to double stranded parts of the telomere, whereas POT1 binds to the single stranded 3' end. Because these three binding proteins are held together by TIN2 and TPP, only very specific binding is possible

to sequences only occurring at telomeres (*De Lange, 2009)*. The complex as a whole hides the telomeres from DNA damage repair responses, and also regulates the ATM and ATR pathway but the mechanisms for these processes are largely unknown (*De Lange, 2009)*.

To investigate the function of shelterins, several studies have been performed with model organisms, deficient for (parts of) the shelterin complex (*Martinez and Blasco, 2010)*. For example, TRF1 deficient mice show a rapid induction of senescence. Senescence was induced by the pathways shown in figure 1, because abrogation of these pathways prevented the mice from senescing. Bypassing senescence, however, led to chromosomal abnormality and instability (*Martinez and Blasco, 2010)*. This proves once again that senescence is a protective mechanism. Because sheltrins were first described relatively recently, a lot of research needs to be done.

As we have seen, all the mechanisms involved in senescence and aging discussed in this paper, still need further elucidation. This especially holds true for sheltrins. For example, at the moment there is no study available on miRNAs as regulator of shelterin. If you take in mind the enormous interest in miRNAs as regulators, this is highly remarkable. But although they only have been described very recently, their function is quite well known already and seems crucial in telomere protection, which makes them a promising subject for further investigation.

Discussion

As we have seen, many mechanisms controlling aging are partly known. Many factors have been shown to contribute somehow to longevity or senescence. A major issue is the fact that a lot of components act individually, but also interact with other processes. This makes the field of research very complex. Adding to this the many regulatory levels, gives a tantalizing complex system controlling senescence and aging. Because of the complexity, processes are elucidated small steps at a time.

Future research in this field should be focused on how to apply the knowledge of senescence and aging. The aim of studies should be not so much on getting people to live longer, but on preserving biological function as we get older. Many diseases in developed countries arise from failing organs, so a major question is how we can keep our body and organs young, even though we get older.

Everything taken together, one can confidently say that there's a lot of time needed to investigate all the involved mechanisms, and apply this to the human body. But if research in this field will be successful, time won't be a problem anymore.

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